Relative Frequency and Kinetic Properties of Transport-defective Phenotypes among Methotrexate-resistant L1210 Clonal Cell Lines Derived in Vivo

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ABSTRACT

Information was sought on the relative extent to which transport-defective, methotrexate-resistant phenotypes emerge among the total subpopulation of resistant phenotypes during therapeutic challenge of leukemic cells in vivo. A number of monoclonal methotrexate-resistant sublines of the L1210 leukemia were derived during methotrexate therapy of leukemic mice and biochemically characterized. Of the total number of 14 sublines derived, five exhibited altered $[^3]$Hmethotrexate transport alone, five exhibited increased dihydrofolate reductase content alone (2- to 18-fold), and four showed alterations in both of these properties. Methotrexate binding and substrate turnover rate for dihydrofolate reductase appeared to be unchanged in any of the resistant sublines. The relative resistance of each subline was accounted for by the biochemical alterations observed. Among the transport-defective sublines, one subcategory showed a 3- to 4-fold reduction in apparent influx $V_{max}$ for $[^3]$Hmethotrexate, a second category showed both a 5-fold reduction in influx $V_{max}$ and a 3-fold increase in the apparent influx $K_m$, and one subline showed only a 2-fold increase in $K_m$. Otherwise, Michaelis-Menten saturation kinetics for influx was observed in each case and in the case of the parental line and the other resistant sublines. None of the resistant sublines exhibited altered efflux of $[^3]$Hmethotrexate. Steady-state levels measured for intracellular exchangeable (osmotically active) fractions of drug accurately reflected the values for specific kinetic parameters determined for each sensitive and resistant cell line. These studies show that transport-defective phenotypes represent a major category of methotrexate-resistant cell types which emerge initially from leukemic cell populations under therapy in mice. Based on considerations discussed here, it is reasonable to assume that a similar relative occurrence of this phenotype would result during methotrexate therapy of leukemia patients.

INTRODUCTION

The rapid development of acquired resistance to methotrexate remains a major limitation to its effective clinical utility. Consequently, studies begun a number of years earlier (1, 5-7, 9, 10, 18, 23, 25, 27, 29, 30, 38, 39) on the mechanisms of resistance to this agent continue (2, 11-15, 17, 19, 21, 22, 25). Although these studies have been useful in identifying a number of resistant phenotypes, the relative frequency of occurrence of each during initial therapeutic challenge in vivo, i.e., within the nutritional and pharmacological limitations of the host, remains an open question. A number of methotrexate-resistant sublines of both experimental and human tumors have been derived (1, 5, 9-18, 20-22, 26) in cell culture by a stepwise serial transfer to extremely high drug concentrations. These studies have provided some basis for concluding that the same major categories of resistant phenotypes emerge in both animal and human tumor cells, namely, impaired transport of drug and elevation of the target enzyme, dihydrofolate reductase. However, the relevance of data on phenotypic frequency observed after multiple selection steps under these in vitro conditions to the corresponding in vivo situation has not been established.

In our view, information from meaningful studies on the relative occurrence of specific resistant phenotypes at a pharmacological level is of importance in providing direction for biochemical research aimed at circumventing this problem at a clinical level. Toward this goal, we now describe studies carried out with the L1210 leukemia. In these studies, a number of monoclonal, resistant sublines were derived which emerged early during therapy of leukemic mice with methotrexate. Phenotypes which either were transport defective or had elevated levels of dihydrofolate reductase were found to occur in approximately equal frequency. Moreover, detailed analysis of methotrexate transport in these sublines revealed 3 subcategories of transport-defective phenotypes which differed in kinetic properties. Overall, the results suggest that the transport-defective category of methotrexate-resistant phenotypes occurs with relatively high frequency following selection at a pharmacological level.

MATERIALS AND METHODS

General. L1210 leukemic cells (L1210V and L1210V/C) were passaged by i.p. implantation of 10⁶ cells in C57BL/6 x DBA/2 F₁, mice. L1210V/C is a clonal subline of L1210/V established in culture. When transplanted in mice, it is essentially indistinguishable from the parental L1210V in all of the transport and enzymic parameters evaluated in the present study. Cells harvested from the peritoneal cavity 2 to 3 days prior to death of the animals were used for all of the biochemical and transport studies. Specific details of the harvesting procedure are described in previous reports (32-39) from this laboratory. Methods of extracting drug following transport experiments were also described in detail (32-39). $[^3]$Hmethotrexate obtained from Moravek Biochemicals, City of Industry, Calif., was purified just prior to use by paper chromatography (34). Samples of unlabeled methotrexate were provided by the Drug Procurement and Synthesis Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Both radioactive and unlabeled samples of drugs were found spectrophotometrically (24) to be >98% pure.

Derivation of Resistant Sublines. Each parental cell line (L1210V and L1210V/C) was transplanted into groups of 5 mice each. Beginning 24 hr later, each group was treated with 12 mg (maximum
tolerated dose) of methotrexate on a schedule of every 2 days for 5 doses. Treatment resulted in an ILS2 of +150% (average survival time of 17.3 ± 2 (S.E.) days compared to 6.9 ± 0.5 days for controls). One or 2 days prior to death of the treated groups, cells were removed from one mouse in each treated group and transplanted into 5 more mice, and therapy was initiated again 24 hr later. This process was repeated for a total of 5 transplant generations. However, cell suspension from transplant generation 3 or 4 was used for clonal isolation described below. Beginning with the second transplant generation, there was a reduction in the ILS (see Chart 1). In some cases, this reduction was gradual, and the line was stabilized at an ILS of 50 ± 10% at transplant generation 4. In a smaller number of cases, the reduction in ILS was more rapid, the subline was stabilized at an ILS of 12 ± 5% by transplant generation 3. This difference was associated with no known variable during transplantation and therapy. Three other sublines were derived during therapy at 3 mg methotrexate per kg. The initial ILS for these sublines was 110 ± 10%. All 3 sublines stabilized at transplant generation 3 with an ILS of 40 ± 10%.

Cloning of each resistant L1210V subline was carried out by transplanting serially diluted ascites cell suspensions taken from one mouse in each of the groups into a total of 5 mice for each dilution. No therapy was given to these mice. A single ascitic animal was then selected from the group of mice receiving the highest dilution which resulted in ascites development. This process was repeated for a total of 3 times. Cloning of resistant L1210V/C sublines was carried out by plating diluted suspensions of cells in the absence of drug in screw-capped tubes containing Roswell Park Memorial Institute Tissue Culture Medium 1640 plus folic acid (1 µg/ml), 15% calf serum, and 0.3% agar (Difco Laboratories, Inc., Detroit, Mich.). After 7 days of incubation at 37°, a single colony was picked and grown for 2 to 3 days at 37° in the same medium without agar. The process was repeated twice more. All of the clonal lines were maintained by transplantation in mice while treating with a dose of methotrexate (1 to 3 mg/kg) which had no effect on the average life span. Ascites cell suspensions of each subline used for biochemical and transport studies were obtained from untreated mice.

**Determination of Relative Resistance Level.** Logarithmic-phase cells (10⁶ cells/ml) of each L1210V/C subline in Roswell Park Memorial Institute Tissue Culture Medium 1640 plus folic acid (1 µg/ml) and 15% calf serum were dispensed into 16 × 25-mm culture tubes (final volume, 5 ml) containing various concentrations of methotrexate. Cell growth in tubes with or without drug was monitored after 72 hr with a Model ZBI Coulter Counter. In 2 or 3 determinations by each of 2 methods which agreed within 10%, the time course after 15 sec to the vertical axis. Values obtained by either method are the same and equal to values obtained after subtracting the exchangeable intracellular drug (32) and the nonexchangeable (dihydrofolate reductase bound) intracellular drug from the total. Values for intracellular water were similar for all lines. Measurements Related to Dihydrofolate Reductase Activity. Three separate methods were used for determination of the dihydrofolate reductase content of cell-free extract from the various methotrexate-resistant sublines. (a) Measurements were made by titration inhibition of enzyme activity with methotrexate (40). The details of the procedure actually used are described in a previous report (32) from our laboratory. This procedure also allows for the identification of any resistant phenotypes (7, 31) which synthesize dihydrofolate reductase with reduced affinity for methotrexate. The data generated by this method are used to devise a value of K (19) and relative turnover rate. In the latter case, the change in absorbance obtained in the absence of drug is converted to the amount of dihydrofolate reduced (2.62 µmol = 0.01 at 340 nm/min at 37°). This value is divided by the titration inhibition value expressed in drug-binding equivalents of dihydrofolate reductase which assumes one binding site per molecule of enzyme. (b) Values for dihydrofolate reductase content of cell-free extract are derived from measurements of the slowly dissociable fraction of radioactivity during dialysis at pH 6.2 of an aliquot of cell-free extract treated with [³H]methotrexate. This method requires only small amounts of cell extract and avoids possible errors following the large dilutions necessary in this test. Methods described previously. (c) Dihydrofolate reductase content is determined from measurements of nonexchangeable levels of radioactivity following efflux from cells preloaded with [³H]methotrexate. This method requires a measurement of the time course for efflux and an accurate value for cell surface adsorption. At least 2 of the 3 methods were used for determining values for dihydrofolate reductase content in each cell line. The values shown are an average of at least 2 determinations by each of 2 methods which agreed within 10%.

**RESULTS AND DISCUSSION**

A total of 14 monoclonal resistant cell lines was derived during the course of the present study. The properties of these cell lines are summarized in Table 1. All of the lines exhibited...
Methotrexate-resistant L1210 Clonal Cell Line Phenotypes

Table 1
Properties of methotrexate-sensitive and -resistant sublines of the L1210 leukemia

Values were derived from separate determinations made in parallel on the parental sensitive and resistant cell lines. Additional details are given in the text and legends of the charts.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dihydrofolate reductase level (nmol/g, dry wt)</th>
<th>Influx</th>
<th>Resistance level (resistant/sensitive)</th>
<th>Pheno-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210V</td>
<td>3.64 ± 0.3c</td>
<td>3.92 ± 0.5c</td>
<td>7.34 ± 1.9c</td>
<td>12-fold Td</td>
</tr>
<tr>
<td>L1210V-R1</td>
<td>3.59 ± 0.4</td>
<td>10.70 ± 0.9</td>
<td>1.57 ± 0.5</td>
<td>10-fold T</td>
</tr>
<tr>
<td>L1210V-R2</td>
<td>4.04 ± 0.2</td>
<td>10.30 ± 1.4</td>
<td>1.34 ± 0.3</td>
<td>10-fold T</td>
</tr>
<tr>
<td>L1210V-R3</td>
<td>54.50 ± 6.1</td>
<td>4.85 ± 0.6</td>
<td>8.41 ± 2.1</td>
<td>16-fold E</td>
</tr>
<tr>
<td>L1210V-R4</td>
<td>67.60 ± 5.3</td>
<td>3.51 ± 0.2</td>
<td>6.54 ± 1.6</td>
<td>20-fold E</td>
</tr>
<tr>
<td>L1210V-R5</td>
<td>6.55 ± 7.1</td>
<td>4.08 ± 0.6</td>
<td>3.02 ± 0.6</td>
<td>5-fold TE</td>
</tr>
<tr>
<td>L1210V-R6</td>
<td>8.23 ± 9.2</td>
<td>8.21 ± 1.3</td>
<td>7.82 ± 1.1</td>
<td>3-fold TE</td>
</tr>
<tr>
<td>L1210V-R7</td>
<td>4.15 ± 3.1</td>
<td>4.08 ± 0.5</td>
<td>3.64 ± 0.7</td>
<td>2-fold T</td>
</tr>
<tr>
<td>L1210V/C</td>
<td>3.52 ± 4.6</td>
<td>3.63 ± 0.4</td>
<td>9.29 ± 2.3</td>
<td>2-fold T</td>
</tr>
<tr>
<td>L1210V-R1</td>
<td>3.98 ± 2.9</td>
<td>4.40 ± 0.2</td>
<td>2.91 ± 0.8</td>
<td>3-fold T</td>
</tr>
<tr>
<td>L1210V-R1</td>
<td>17.73 ± 6.6</td>
<td>4.08 ± 0.9</td>
<td>2.63 ± 0.6</td>
<td>10-fold TE</td>
</tr>
<tr>
<td>L1210V-R19</td>
<td>36.30 ± 5.3</td>
<td>4.61 ± 0.6</td>
<td>4.09 ± 0.7</td>
<td>20-fold TE</td>
</tr>
<tr>
<td>L1210V-R20</td>
<td>3.87 ± 4.2</td>
<td>3.52 ± 0.7</td>
<td>3.12 ± 0.3</td>
<td>2-fold T</td>
</tr>
<tr>
<td>L1210V-R21</td>
<td>16.10 ± 2.1</td>
<td>3.82 ± 0.4</td>
<td>7.11 ± 1.3</td>
<td>4-fold E</td>
</tr>
<tr>
<td>L1210V-R22</td>
<td>27.10 ± 3.2</td>
<td>4.34 ± 0.9</td>
<td>9.24 ± 1.1</td>
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</tr>
<tr>
<td>L1210V-R23</td>
<td>40.80 ± 5.9</td>
<td>4.55 ± 0.8</td>
<td>8.61 ± 0.9</td>
<td>12-fold E</td>
</tr>
</tbody>
</table>

a All but 3 (R5, R6, and R7 isolated at 3 mg/kg) were isolated during therapy at 12 mg methotrexate per kg.

b Average ± S.E. (n = 4).
c Derived by inference from data in Table 2.
d T, transport deficient; E, elevated dihydrofolate reductase.

Table 1: Transport

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<td>3.51 ± 0.2</td>
<td>6.54 ± 1.6</td>
<td>20-fold E</td>
</tr>
<tr>
<td>L1210V-R5</td>
<td>6.55 ± 7.1</td>
<td>4.08 ± 0.6</td>
<td>3.02 ± 0.6</td>
<td>5-fold TE</td>
</tr>
<tr>
<td>L1210V-R6</td>
<td>8.23 ± 9.2</td>
<td>8.21 ± 1.3</td>
<td>7.82 ± 1.1</td>
<td>3-fold TE</td>
</tr>
<tr>
<td>L1210V-R7</td>
<td>4.15 ± 3.1</td>
<td>4.08 ± 0.5</td>
<td>3.64 ± 0.7</td>
<td>2-fold T</td>
</tr>
<tr>
<td>L1210V/C</td>
<td>3.52 ± 4.6</td>
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<tr>
<td>L1210V-R19</td>
<td>36.30 ± 5.3</td>
<td>4.61 ± 0.6</td>
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</tr>
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<td>L1210V-R20</td>
<td>3.87 ± 4.2</td>
<td>3.52 ± 0.7</td>
<td>3.12 ± 0.3</td>
<td>2-fold T</td>
</tr>
<tr>
<td>L1210V-R21</td>
<td>16.10 ± 2.1</td>
<td>3.82 ± 0.4</td>
<td>7.11 ± 1.3</td>
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<td>L1210V-R22</td>
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a All but 3 (R5, R6, and R7 isolated at 3 mg/kg) were isolated during therapy at 12 mg methotrexate per kg.

b Average ± S.E. (n = 4).
c Derived by inference from data in Table 2.
d T, transport deficient; E, elevated dihydrofolate reductase.

Relatively low levels (3- to 20-fold greater than the 2 parental sensitive cell lines) of resistance to methotrexate. Five of the sublines (R1, R2, R7, R17, and R20) exhibited alterations in the transport of [3H]methotrexate while 5 of the other sublines (R3, R4, R21, R22, and R23) exhibited increased dihydrofolate reductase levels. The remaining 4 sublines showed alterations in both of these properties. In every case, the biochemical alterations observed appeared to account for the relative level of resistant to methotrexate exhibited by each line.

None of the sublines exhibited a dihydrofolate reductase which was altered in terms of affinity for methotrexate, a rare property. Among the resistant sublines that are defective for [3H]methotrexate transport, 3 kinetically distinguishable phenotypes were discernible (Table 1). One category (R5, R7, R17 to R20) showed a 3- to 4-fold reduction in the value for the apparent influx V_max. One subline (R6) showed a 2-fold increase in apparent influx K_m. A third category (R1 and R2) showed both a 5-fold reduction in the value for influx V_max and a 3-fold increase in the value for apparent influx K_m. The increase in K_m would appear to indicate a reduction in the affinity of some component (carrier?) of the system for [3H]methotrexate. Data from a time course experiment for 2 representative sublines showing transport alterations are shown in Chart 2, bottom.
Data derived during a kinetic analysis of initial influx of [3H]-methotrexate (Chart 1, top) by these 2 sublines are given in Chart 3. In each case, initial influx exhibited Michaelis-Menten saturation kinetics, and the derived values for each kinetic parameter were clearly different. None of the resistant sublines showed an alteration in influx of [3H]-methotrexate. In the case of 9 of the sublines (R1 to R7, R17, and R20), this was determined by a direct measurement of influx as shown in Chart 4. In each case, as with the parental L1210V and the R1 subline for which the data are shown, influx exhibited first-order kinetics in agreement with all of our prior published reports (8, 32-39). With the remaining 5 sublines (R18, R19, R21 to R23), this similarity to parental-type influx of drug was inferred from the agreement between expected and measured values for steady-state level of intracellular exchangeable [3H]-methotrexate. The relationship between exchangeable (osmotically active) and nonexchangeable (drug bound to dihydrofolate reductase) fractions of intracellular [3H]-methotrexate for the accumulation phase is shown in Charts 2 and 5 for the L1210V parental cells, and the R1 and R17 sublines, and the R3 subline which has a 20-fold-higher dihydrofolate reductase level. In the latter case (Chart 5), accumulation in the resistant cells initially shows a very long linear phase in view of the high level of dihydrofolate reductase. However, the exchangeable fraction of intracellular [3H]-methotrexate delineated is the same as in the parental L1210V cells. Values for steady-state level of intracellular exchangeable drug are given in Table 2 for all of the resistant sublines. In every case, the value based on experimental measurements was in close agreement to levels expected based on a calculation (Ref. 8; legend of Table 2) using measured values for each kinetic parameter for influx, the measured efflux rate constant (R1 to R7, R17, and R20), or the assumption that the efflux rate constant was the same (R18, R19, R21 to R23) as that measured in the parental line (L1210V or L1210V/C).

Since the relative increase in resistance observed in the various sublines can be fully accounted for by the biochemical data given in Tables 1 and 2, the occurrence of additional alterations in these sublines can probably be ruled out. Although other enzymic effects involving dihydrofolate reductase or other folate-related enzymes have been observed previously (1, 7, 17, 21, 30), in these earlier studies, stepwise selection of cell lines exhibiting extremely high levels of resistance was used. In view of the data presented here, it would seem that resistant sublines bearing these other alterations emerge only rarely during the initial selection process at least under pharmacological conditions. It is of interest to note that only 4 of the 14 sublines exhibited alterations of both transport and dihydrofolate reductase. Earlier studies in vivo from our own laboratory (39) and earlier in vitro studies (11, 14, 22, 26) suggested that such doubly altered sublines emerge sequentially and that selection for the enzymic alteration eventually occurs within a transport-defective resistant population. In view of this, it is reasonable to conclude that, under the conditions used here, the majority of the resistant sublines which emerge initially are of the transport-impaired type.

The 3 categories of transport-defective phenotypes identified in the present study bring the total number of kinetic varieties already identified to 3. Prior studies from our laboratory (38, 39) and elsewhere (22, 26) have identified phenotypes with an alteration of either the $K_m$ or $V_{max}$ for influx of methotrexate. The current study identified phenotypes with both a reduced $V_{max}$ and increased $K_m$ for influx. Unfortunately, there is no basis in the current study for concluding that the latter category
Transporting [3H]methotrexate is necessary for survival of these coenzyme substrate (5-methyltetrahydrofolate) by the system of phenotype arose by a single genetic event or stepwise as a resistant sublines. In contrast, isolation of this phenotype in cell laboratory, Ref. 32), it is somewhat surprising that the trans sublines in a host (see discussion in a previous report from our result of 2 distinct genetic events. Since transport of the natural selected number of these transport-defective resistant L1210 high. Because of this, additional biochemical studies of a patients during methotrexate therapy would also be relatively usually used as a folate source. This folate appears to serve as port-defective phenotype represents a major category of re in human tumor cell lines, it is reasonable to assume that the basis for the alterations observed.

References

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