Folate Requirements of Methotrexate-Resistant Human Acute Lymphoblastic Leukemia Cell Lines

By Yasuhiko Kano, Takao Ohnuma, and James F. Holland

We studied the folate requirements of a human acute lymphoblastic leukemia cell line, MOLT-3, and methotrexate (MTX)-resistant sublines established in vitro. The requirement of pteroylglutamate (PGA) for optimal cell growth was different for each cell line. With increasing MTX resistance, there was progressive increase in PGA requirements, moving the PGA concentration--cell growth curve (dose-response curve) 1 log order of magnitude to the right. The increases in the requirement of 5-methyltetrahydrofolate (5-methyl-THF) by the resistant sublines were more pronounced than PGA requirement, moving the dose-response curve nearly 3 log orders in magnitude to the right. The concentrations in vitro of 5-methyl-THF required for optimal growth of the MTX-resistant sublines far exceeded the normal serum 5-methyl-THF concentrations known in humans. These observations show that MTX-resistant cells established in vitro in culture media containing PGA instead of 5-methyl-THF, a physiological folate, cannot be expected to grow in vivo. The collateral sensitivity of transport-impaired MTX-resistant sublines to 2,4-diamino-5-methyl-6-[(3',4',5'-trimethoxyanilino) methyl] quinazoline (trimetrexate, TMQ) was negated in the absence of PGA. With the addition of 5-methyl-THF, the parent cells became more resistant than the transport-impaired sublines to TMQ. These data indicate that the collateral sensitivity of MTX-resistant cells to the substituted 2,4-diaminoquinazoline is due to functional folate deficiency by virtue of the impaired transport of folate.

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\textbf{METHOTREXATE (MTX)} is one of the most widely used agents for the treatment of leukemia, lymphoma, and other malignant diseases. Frequently, tumors that originally appeared to be responsive to MTX became increasingly resistant to this compound. The mechanism of acquired resistance to MTX has been studied in detail in a number of experimental systems including transplantable animal tumors in vivo and tumor cell lines in vitro. These studies revealed three major mechanisms for the development of MTX resistance: (a) an increase in dihydrofolate reductase activity; (b) a decrease in the affinity of dihydrofolate reductase to MTX; and (c) a decrease in the transport of the drug to the cell. As a fourth mechanism of MTX-resistance, the inability of cells to convert the drug to its polyglutamate derivatives has recently been suggested. Among them, the increase in dihydrofolate reductase and the decrease in membrane transport have been considered as the major mechanisms of MTX-resistance, since a majority of MTX-resistant cells established, mainly in vitro, had either of them or both. When MTX-resistant cells were established in vivo, however, in only a few isolated cases has impaired-transport been recognized as the mechanism of resistance. At the clinical level, no reports have indicated impaired transport of MTX as the mechanism of an acquired resistance. In cases of acquired resistance, an elevated dihydrofolate reductase or a decreased affinity of the enzyme to MTX was demonstrated.

The in vitro culture condition is different from the in vivo condition in that commercial media used for maintaining cells or establishing MTX-resistant cells contain pteroylglutamate (PGA), an oxidized form of folic acid, instead of 5-methyltetrahydrofolate (5-methyl-THF), a physiological folate. It is known that MTX shares the common carrier-mediated process with reduced folates, 5-methyl-THF, and 5-formyltetrahydrofolate (5-formyl-THF), to enter cells, and the transport of these reduced folates is decreased in transport-impaired MTX-resistant cell lines. Although the nature of the PGA transport system is not precisely understood, a major portion of PGA was shown not to use a reduced-olate--MTX-transport system to enter cells. Thus, it is possible that although transport-related MTX-resistant cells grow avidly in vitro, they may not be able to take up sufficient quantities of 5-methyl-THF for optimal cell growth in vivo.

We examined the folate requirements of MTX-resistant sublines of a human acute lymphoblastic leukemia cell line, MOLT-3, established in vitro. We also compared the effect of 5-methyl-THF on the sensitivity of these cells against MTX and 2,4-diamino-5-methyl-6-[(3',4',5'-trimethoxyanilino) methyl] quinazoline (trimetrexate, TMQ).

\textbf{MATERIALS AND METHODS}

\textbf{Cell lines.} The cell lines used were human acute lymphoblastic leukemia cell line MOLT-3\textsuperscript{29} and 3 MTX-resistant sublines. The sublines were established in our laboratory and were maintained as a suspension in culture flasks containing RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO) and antibiotics (penicillin G 100 U/mL and streptomycin 100 \mu g/mL).

Sublines MOLT-3/MTX\textsubscript{30}, MOLT-3/MTX\textsubscript{300}, and MOLT-3/MTX\textsubscript{3000} were 30, 200, and 10,000 times more resistant to MTX, respectively, as compared with the parent MOLT-3 cells when tested in RPMI 1640 medium plus 10% FBS or in McCoy's 5a medium devoid of PGA and vitamin B\textsubscript{12} (Nissui Seiyaku, Tokyo) that was supplemented with 20% FBS plus 2.3 \times 10\textsuperscript{-4} PGA (Sigma, St Louis) plus 2 mg/L vitamin B\textsubscript{12} (Sigma). MOLT-3/MTX\textsubscript{30} and
MOLT-3/MTX200 have impaired transport of MTX and 5-methyl-THF and no significant elevation of dihydrofolate reductase activity. MOLT-3/MTX10,000, derived from MOLT-3/MTX200, has both impaired transport of MTX and elevated dihydrofolate reductase activity. Data regarding the doubling time, MTX and methyl-THF transport, and dihydrofolate reductase activity of these cells are summarized in Table 1.

**Cell maintenance and preparation.** All cell lines originally maintained in RPMI 1640 medium plus 10% FBS plus antibiotics were transferred to the special McCoy's 5a medium (devoid of PGA and vitamin B12) supplemented with 20% FBS, vitamin B12, and PGA. PGA concentrations used were 2.3 x 10^-4 mol/L for MOLT-3 and MOLT-3/MTX200 and 1 x 10^-5 mol/L for MOLT-3/MTX10,000. A larger concentration of PGA was required for the optimal cell growth of MOLT-3/MTX200 and MOLT-3/MTX10,000 (Table 1).

**Folate requirement studies.** Logarithmically growing cells were harvested, washed three times in the special McCoy's 5a medium devoid of PGA and vitamin B12, and resuspended to a final concentration of 1 x 10^6 cells/10 mL of the medium containing 20% dialyzed FBS and vitamin B12 with or without PGA. Fifteen milliliters of FBS were dialyzed three times at 4°C for 24 hours against 1 L of a solution containing 8 g of NaCl, 0.4 g of KCl, 1 g of glucose, and 0.35 g of NaHCO3 and used as dialyzed FBS.

Initially, in order to determine the time period required to exhaust intracellular folate pools, cell growth in the media with or without PGA was studied for seven days. Ten-milliliter aliquots (10^6 cells) of the cell suspensions with or without PGA were placed in a tissue culture flask (Falcon, Oxnard, Calif, No. 3033) and incubated in 5% CO2 in humidified air for four days, and the viable cell number was counted with an improved Neubauer hemocytometer. Cell viability was defined as the ability of cells to exclude trypan blue dye. PGA concentrations required for optimal cell growth were determined. Values are mean ± SD. PGA concentrations in commercial culture media are shown in Table 1.

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**Table 1. Characteristics of Human Acute Lymphoblastic Cell Line, MOLT-3, and Its MTX-Resistant Sublines**

<table>
<thead>
<tr>
<th></th>
<th>Doubling Time (h)</th>
<th>Influx (Vmax)</th>
<th>MTX-Dihydrofolate Reductase Activity</th>
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<tr>
<td></td>
<td>in Culture Medium</td>
<td>of MTX</td>
<td>Assay† Assay‡</td>
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<td></td>
<td>2.3 x 10^-4</td>
<td>1.0 x 10^-4</td>
<td>MTX THF</td>
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<tr>
<td>MOLT-3</td>
<td>24</td>
<td>24</td>
<td>7.7 6.7 18 15</td>
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<tr>
<td>MOLT-3/MTX200</td>
<td>25</td>
<td>24</td>
<td>1.7 5.7 20 22</td>
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<tr>
<td>MOLT-3/MTX10,000</td>
<td>32</td>
<td>27</td>
<td>1.4 3.7 20 14</td>
</tr>
<tr>
<td>MOLT-3/MTX10,000</td>
<td>36</td>
<td>29</td>
<td>1.1 3.3 210 85</td>
</tr>
</tbody>
</table>

*nmol/2.5 minute per 10^7 cells. Km is constant at ~6.6 µmol/L for both MTX and 5-methyl-THF.
†µmol of MTX bound per 10^6 cells.
‡µmol of dihydrofolate reduced per minute per 10^6 cells.

Data on doubling time represent the mean of at least three measurements. Data on influx represent a mean of three separate experiments carried out in duplicate (modified from Ref. 17). Data on dihydrofolate reductase activity are representative values from longitudinal studies. Results are expressed as a mean of an assay done in triplicate. Range of the values in triplicate determination was ±10% of the mean. 17

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**RESULTS**

**Folate requirements.** As expected, all of the cell lines grew well in the medium containing PGA. In the McCoy's 5a medium with 20% dialyzed FBS and devoid of folate, however, the cells grew avidly for the first three days and then cell growth gradually slowed. Therefore, cells incubated without PGA for three days were used for the study of PGA, 5-methyl-THF, and 5-formyl-THF requirements for cell growth. The effects of various concentrations of PGA and 5-methyl-THF on the cell growth of MOLT-3 and MTX-resistant sublines are illustrated in Figs 1 and 2.

PGA concentrations required for optimal cell growth were...
different for each cell line (Fig 1). With increasing MTX resistance PGA requirements progressively increased, moving the drug concentration–cell growth curve (dose–response curve) to the right. The maximal move of the dose–response curve was ~1 log order in magnitude. The most MTX-resistant cell line, MOLT-3/MTX10000, required 1.1 x 10^-6 mol/L of PGA for 50% optimal cell growth, and the PGA concentration for optimal cell growth was ~1 x 10^-5 mol/L. These values can be contrasted with PGA concentrations in commercially available media [eg, 2.3 x 10^-6 mol/L and 2.3 x 10^-5 mol/L for RPMI 1640 medium (GIBCO) and McCoy’s 5a medium (GIBCO), respectively]. These data indicate that PGA concentrations in these media are well above the concentration required for the optimal growth of the parent MOLT-3 cells. MOLT-3/MTX10000 and MOLT-3/MTX10000 cells must have been maintained at threshold or suboptimal concentrations of PGA for cell growth.

Changes in the 5-methyl-THF requirements for the optimal cell growth of the resistant sublines were more pronounced, moving the dose–response curve nearly 3 log orders in magnitude to the right (Fig 2). The changes in the 5-methyl-THF requirements appeared to be parallel to the degree of acquired resistance to MTX for the purely transport-related sublines. Thus, the folate concentration required for 50% optimal cell growth increased 19-fold, 219-fold, and 407-fold of the parent cells for MOLT-3/MTX30, MOLT-3/MTX300, and MOLT-3/MTX10000, respectively. The normal serum folate concentration in humans is reported to be in a range from 5 x 10^-8 to 3 x 10^-8 mol/L, with most folate in the form of 5-methyl-THF. The concentrations in vitro of 5-methyl-THF required for optimal cell growth in the MTX-resistant sublines (3 x 10^-7 to 1 x 10^-6 mol/L) far exceeded the normal serum 5-methyl-THF levels in humans. These observations indicate that highly transport-impaired MTX-resistant cells cannot be expected to grow in vivo.

A similar move in the dose–response curve (~3 log orders in magnitude) was also seen as the requirement of 5-formyl-THF with increasing MTX resistance (data not shown). To produce identical biologic effects, 5-formyl-THF requirements were, on a molar basis, threefold to fourfold less than that of 5-methyl-THF.

**TMQ sensitivity.** Comparative inhibitory effects of TMQ on the parent and MTX-resistant sublines maintained in RPMI 1640 medium containing 10% FBS are shown in Fig 3. Transport-impaired MTX-resistant sublines were found to be more sensitive to TMQ than the parent line was. Thus, the TMQ concentration required for 50% inhibition of cell growth on day 3 (IC50) was 1.8 x 10^-8 mol/L for the parent cell lines and 5.0 x 10^-8 and 4.0 x 10^-9 mol/L for MOLT-3/MTX30 and MOLT-3/MTX10000 cell lines, respectively. MOLT-3/MTX10000 was found to be as sensitive as the parent line to TMQ to the point of IC50; however, the dose–response curve approached a plateau at higher TMQ concentrations, indicating the presence of highly TMQ-resistant cell populations. Their TMQ sensitivities in McCoy’s 5a medium plus 20% dialyzed FBS containing various concentrations of 5-methyl-THF are illustrated in Fig 4. Without 5-methyl-THF, the TMQ sensitivity was virtually identical for MOLT-3, MOLT-3/MTX30, and MOLT-3/MTX10000. In the absence of 5-methyl-THF, however, MOLT-3/MTX10000 was found to be more resistant than the other cell lines. Progressive increases in 5-methyl-THF concentrations resulted in a marked shift of the drug concentration–inhibition curve (dose–response curve) of the parent cells. In contrast, the shift of the dose–response curve to TMQ was progressively less influenced with increasing MTX-resistance.

**DISCUSSION**

The major aim of the present study was to evaluate whether the mechanism of MTX-resistance and collateral
sensitivity of MTX-resistant cells to TMQ, both demonstrated in vitro, could be operational in vivo.

MOLT-3/MTX, MOLT-3/MTX30, and MOLT-3/MTX10,000 cells required much higher levels of 5-methyl-THF, 5-formyl-THF, and PGA than did the parent MOLT-3 cells. For optimal growth, they required at least $3.0 \times 10^{-7}$ mol/L, $3.0 \times 10^{-8}$ mol/L, and $1.0 \times 10^{-5}$ mol/L 5-methyl-THF, respectively. A similar increase was observed for 5-formyl-THF requirements. At physiological levels of 5-methyl-THF, MOLT-3/MTX30 may grow at a substantially reduced rate; however, these concentrations of reduced folate were unable to support cell growth for the highly MTX-resistant sublines. These data indicate that highly transport-impaired MTX-resistant MOLT-3 cells cannot be expected to grow in vivo.

Although 5-methyl-THF and 5-formyl-THF are considered to use the same transport system to enter the cells, we found that 5-methyl-THF requirements were three to four times higher than those of 5-formyl-THF. The reasons for this discrepancy include the instability of 5-methyl-THF during four days of incubation and the difference in the entry site of these two reduced folates in the metabolic pathway. Our observations are consistent with reports that 5-formyl-THF corrected folate deficiency in hematopoietic cells more effectively than did 5-methyl-THF in short-term culture.23,24 The commercial media contained nonphysiological and stable PGA instead of physiological and unstable folate, 5-methyl-THF. The increased requirements of PGA were observed in MTX-resistant MOLT-3 sublines; however, the increases were only 5-fold to 11-fold. Therefore, even if MOLT-3 cells lost their capacity to transport reduced folates into the cell during the process of acquiring MTX-resistance, these cells can still grow in a medium containing PGA.

An increase in the doubling time of the resistant sublines in RPMI 1640 medium plus 10% FBS and the reversal by increasing PGA concentrations (Table I) indicate that the PGA concentration in the commercial RPMI 1640 medium plus 10% FBS is not adequate for their optimal growth.

The nature of the concomitant shift in the requirements of PGA and reduced folates in MTX-resistant sublines is not clear. PGA has been considered not to use a reduced folate-MTX transport system.29,26,28 The changes in the membrane structure or the transport protein for the entry of reduced folate-MTX may have influenced the entry of other nutrients including PGA. It is difficult, however, to rule out the possibility of a much closer tie between the transport systems of PGA and MTX.

We observed that in the absence of folate in the medium, the degree of TMQ sensitivity was virtually identical among parent and transport-impaired MTX-resistant sublines. The presence of 5-methyl-THF produced a lesser influence on the shift of the TMQ dose–response curves of sublines with higher MTX-resistance, resulting in apparent higher sensitivity to TMQ for the resistant sublines as compared with the parent cells at identical 5-methyl-THF concentrations. These data indicate that the collateral sensitivity of transport-impaired MTX-resistant sublines to TMQ is related to their impaired folate transport. Our observation confirms the statement by Sirotnak et al that collateral sensitivity to lipophilic antifolates in MTX-resistant human cells is probably attributable to relatively low levels of intracellular folate by virtue of impaired transport of folate.15

We find that MOLT-3/MTX10,000 cells, which have both impaired transport of MTX and an elevated dihydrofolate reductase, are more resistant to TMQ in the absence of folic acid (Fig 4). This finding is in accord with the dose–response curve of MOLT-3/MTX10,000 seen in Fig 3. It may be concluded, therefore, that MTX-resistant cells with elevated dihydrofolate reductase have cross-resistance with lipophilic antifolates. This observation is consistent with a report that lipophilic antifolates are not active on MTX-resistant cells with elevated dihydrofolate reductase.15,17

Although transport-resistant cells thus obtained in vitro may serve as a useful tool in the study of membrane transport of nutrients, our data cast strong doubts as to the development of a high degree of transport resistance as the primary cause of acquired MTX resistance in the clinical setting. Bertino et al reported that dihydrofolate reductase activity was found to be elevated in leukemic cells from patients treated with MTX.4,7 Some MTX-resistant–leukemic patients also had a low affinity of MTX binding to dihydrofolate reductase.19 More recently, Curt et al established a
MTX-resistant tumor cell line derived from a patient with small cell lung cancer who had been treated with MTX and had become resistant to MTX. This cell line displayed an elevation of dihydropyrimidine reductase accompanied by amplification of the gene coding for the enzyme. In addition, Hoffbrand et al. reported that there was no difference in MTX transport between MTX-sensitive and MTX-resistant leukemic cells obtained from patients with acute myelogenous leukemia (AML). Therefore, no clinical case has demonstrated impaired MTX transport as the primary cause of acquired MTX resistance. This is in sharp contrast to the report of Kessel et al. demonstrating that MTX transport is a critical determinant of clinical responses to MTX in human leukemias. Their work indicates that decreased MTX-transport is a major cause of natural resistance to MTX. Although Sirotnak et al. established MTX-resistant L1210 cells by virtue of impaired transport of MTX in transplantable animals, the resistance was only about tenfold as compared with that of the parent cells. We believe that in transplanted animals, or even in clinical cases, a mild degree of MTX-resistance may occur by virtue of mildly impaired MTX transport alone. In such cases, the impairment in reduced folate transport is to such a degree that sufficient quantities of reduced folate for optimal cell growth can still be incorporated into the cells. In highly MTX-resistant cells, the elevation in dihydrofolate reductase appears a more likely mechanism of acquired MTX resistance in vivo.

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