Evolution of Methotrexate Resistance of Human Acute Lymphoblastic Leukemia Cells in Vitro

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ABSTRACT

A human acute lymphoblastic T-cell line, MOLT-3, was fed with Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal bovine serum and antibiotics which contained increasing concentrations of methotrexate (MTX). The development of drug resistance was associated initially with progressive decrease in MTX transport. When the cells became 200-fold resistant, a rise in the dihydrofolate reductase was noted which was short-lived in the absence of the drug. A 10,000-fold increase in MTX resistance was accompanied, in addition to further decrease in MTX transport, by a 10-fold increase in the dihydrofolate reductase activity. While the purely transport-related resistant cell lines had a collateral sensitivity to lipid-soluble antifols, the sublines which had both transport- and enzyme-related MTX resistance contained a subpopulation highly resistant to these antifols. Chromosome analysis of the subline with increased dihydrofolate reductase activity showed an expanded abnormally banded region in chromosome 5.

INTRODUCTION

Mechanisms of resistance to MTX have been studied by developing resistant cell lines both in vivo, using transplantable animal tumor cells, and in vitro, using animal and human tumor cell lines. Many of these resistant lines developed in vitro were shown to have impaired membrane transport of the drug (15, 26, 41, 42), elevated levels of target enzyme DHFR (1, 5-8, 12, 14, 21, 23, 32, 34, 40), or both (16, 17, 19, 20, 24, 36, 39). A decreased affinity of the enzyme for MTX was also recognized in some cell lines (1, 5, 15). MTX-resistant cells developed in vitro without changes in membrane transport are rare, and only one such instance has been recorded (12). These observations suggest that the initial step of MTX resistance in vitro is often impaired MTX transport, which is then followed in a second step by changes in DHFR either an increase in the activity or an altered affinity to MTX, or both. We have identified steps involved in the development of MTX resistance in vitro using a human acute lymphoblastic leukemia cell line, MOLT-3.

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4 The abbreviations used are: MTX, methotrexate; DHFR, dihydrofolate reductase; TMQ, 2,4-diamino-5-methyl-6(3',4',5'-trimethoxyanilino) methylquinazoline; BW301U, 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-d]pyrimidine; LV, leucovorin; GIBCO, Grand Island Biological Co.; 5-methyl-THF, 5-methyltetrahydrofolate; HSR, homogeneously stained region.

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MATERIALS AND METHODS

The cell line used, MOLT-3, was of human acute lymphoblastic leukemia origin, which retained T-cell characteristics (37). An earlier communication detailed the development of a transport-resistant subline, MOLT-3/MTXo (41). This subline was approximately 30-fold resistant as compared to the parent line, MOLT-3. To reflect expression of the degree of resistance rather than the mechanism of resistance, we redesignated this subline MOLT-3/MTXa. This subline was further cultured in the presence of increasing concentrations of MTX in RPMI-Medium 1640 (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (GIBCO) and antibiotics (41).

The degree of MTX resistance was determined by a cell growth inhibition assay. For this assay, cells were removed from the MTX-containing medium and allowed to grow for more than 2 weeks prior to initiating experiments to quantitate resistance. Ten-ml suspensions of the parent cell line and MTX-resistant sublines were prepared in individual culture tubes (No. 3033, Falcon, Oxnard, CA), each at an initial cell density of 1.5 x 10⁶/ml, to which 0.1 ml of drug solutions at graded concentrations was added. The cells were incubated at 37°. Three days later, the viable cell number was counted by the trypan blue dye exclusion method, and dose-response curves were obtained by calculating the percentage of viable cells in drug-treated tubes as compared to those in control tubes without drugs. The degree of resistance was expressed with 50% inhibitory concentration on Day 3. Effects of other antifols were similarly determined. TMQ (obtained courtesy of the National Cancer Institute, Bethesda, MD) (11, 41) and BW301U (courtesy of Dr. Charles Nichol) (13, 22) used in the experiments were lipid-soluble antifols and did not use the classical reduced folate transport system for entry into the cell. To determine the reversal effects of LV against folate antagonists, equimolar concentrations of LV (0.1 ml) and each drug (0.1 ml) at graded concentrations were added successively to 10 ml of cell suspensions. After 3 days of incubation at 37°, the protective effect of LV was determined by comparing dose-response curves with and without LV. All experiments were carried out in triplicate and repeated at least twice.

For the determination of DHFR, the cells were washed twice in Dulbecco's phosphate-buffered saline [formulation is as follows (in g/liter): KCl, 0.20; KH₂PO₄, 0.20; NaCl, 8.00; and Na₂HPO₄·7H₂O, 2.16] (GIBCO). The cell pellets were frozen and thawed 3 times in 0.05 M Tris buffer (pH 7.5) containing albumin (1 mg/ml) and 0.15 M KC1. The 20,000 x g supernatant was subjected to enzyme assay by 2 methods as has been described previously (41). The first method was the labeled dihydrofolate method (25). The incubation mixture contained 50 μmol phosphate buffer (pH 6.0), 0.1 mm NADPH, 0.04 mm [³H]dihydrofolate (American-Searle, Arlington Heights, IL), 35,000 to 45,000 cpm, 0.2 mm KC1, and approximately 8 to 64 ng of protein in a final volume of 200 μl. The reaction was initiated by the addition of NADPH at 37° and terminated in a 0° bath by the addition of 25 μl of 0.13 M folate. The folate and dihydrofolate were precipitated by the addition of 25 μl of 0.17 M zinc sulfate in 25% acetic acid. The appropriate blanks contained either all components except NADPH or all components except enzyme, in which case enzyme was added immediately prior to terminating the reaction. Blank values were subtracted from the values obtained from complete assay mixtures to yield the data presented in this paper. The reaction...
mixtures were centrifuged at 17,000 x g for 10 min. Aliquots of 100 μl of the supernatant were added to 5 ml of ACS (Amersham) and analyzed for radioactivity.

The second method was that of MTX binding capacity assay (29). For this study, [3H]MTX (6 to 12 Ci/mmol; Amersham) was purified on DEAE-cellulose as needed. The reaction was carried out at 4°C in a total volume of 1.2 ml. To 0.9 ml of 50 mM phosphate buffer (pH 6.0), containing 1 mg albumin and 0.1 μmol NADPH/ml, were added 0.1 ml of standard MTX solution containing 2.2 to 90.0 pmol/ml and 0.1 μl [3H]MTX containing about 1.5 pmol of MTX. The reactants were mixed, and the incubation was started by the addition of 0.1 ml of the supernatant with serial dilutions (binding capacity of 1 to 1.2 pmol MTX, enough to bind 70 to 80% of the [3H]MTX). After gently shaking to ensure mixing, the reaction mixture was incubated for 10 min at 4°C in the dark. The incubation was terminated by the addition of charcoal coated with dextran.

For the study of MTX and methyl-THF uptake, the parent and resistant cells were washed twice and resuspended in Earl’s balanced salt solution (GIBCO) at a cell concentration of 2 x 10^6/ml. After preincubation of the cells for 15 min at 37°C, [3H]MTX or [5-14C]-methyl-THF (58 μCi/mmol; GIBCO) at a cell concentration of 2 x 10^7/ml. After preincubation of the cells for 15 min at 37°C, the reaction mixture was incubated for 10 min at 4°C in the dark. The samples were counted in a Beckman model 250 liquid scintillation counter at 30% counting efficiency. The results were calculated as a net uptake as measured by the total uptake minus the diffusion value at 0°C. Efflux was similarly measured after diluting an aliquot of the cells into 20-fold excess drug-free medium.

For chromosome analyses, the cells were exposed to Colcemid (0.2 μg/ml medium) for 10 min. The cells were washed by centrifugation, resuspended in 0.38% KCI for 12 min, and fixed in cold Carnoy’s solution (methanol: glacial acetic acid, 3:1). Slides for microscopic examination were prepared by dropping the fixed cell suspension on iced methanol-treated slides and staining with routine orcein Quinacrine mustard (9) or trypsin-Giemsa (46). Screening for double minutes was done on 100 sequential unbanded metaphase plates.

RESULTS

The initial concentration of MTX in the feeding medium was 5 x 10^-9 M. At the end of 3½ years with the increasing concentrations of MTX in the medium, the cells were able to grow avidly in the presence of 5 x 10^-5 M MTX.

The chronological changes in MTX resistance are illustrated in Chart 1. Ten, 32, and 41 months‘ exposure of cells to increasing concentrations of MTX in the medium resulted in the development of sublines of 30-, 200- and 10,000-fold, respectively, resistant to the drug. MOLT-3/MTX30, MOLT-3/MTX200, and MOLT-3/MTX10,000. These sublines were released from the MTX-containing medium and were subjected to characterization.

The MTX-resistant sublines grew slower than did the parent line. Thus, the doubling time of MOLT-3/MTX200 is approximately 32 h, and MOLT-3/MTX10,000 is approximately 36 h, as compared to 24 h for the parent MOLT-3 line.

The initial uptake of radioactive MTX into the parent line and 3 MTX-resistant sublines is shown in Chart 3. There was progressive impairment of MTX uptake with increasing MTX resistance. Chart 3 also shows the efflux of MTX (open circles), which appears similarly impaired. Since the resistant cells have altered uptake and/or possibly amplified intracellular binding capacity, the defective efflux is more likely due to its inability to achieve intracellular free drug. The initial uptake of MTX in the 4 cell lines is replotted in the Lineweaver-Burk plot shown in Chart 4A. There were progressive decreases in the velocity of the initial uptake with increasing MTX resistance. The Km values were similar, however, for the 4 cell lines, with a concentration of approximately 6.6 μM. The Lineweaver-Burk plot of 5-methyl-THF uptake showed changes similar to those seen in MTX (Chart 4B).

MOLT-3/MTX30, the least MTX-resistant line, had the resistance of impaired transport, and the observations made with this subline were communicated earlier (41). MOLT-3/MTX200 was initially shown to have a high level of DHFR (Table 1). In followup studies, however, the DHFR activity was found to be unstable and returned to the original level. This cell line continues to be 200-fold resistant, however. MOLT-3/MTX10,000 was developed in the medium which contained MTX at a concentration of 5 x 10^-5 M. This cell line was shown to have both decreased MTX transport and increased DHFR activity. The increase in DHFR activity has been stable in this cell line.

The inhibitory effects of TMQ on the MTX-resistant cell lines are shown in Chart 5. The sublines with purely transport-related MTX resistance, MOLT-3/MTX30 and MOLT-3/MTX200, are sensitive to TMQ (Chart 5A). The addition of equimolar LV reversed the inhibitory effects of TMQ on the parent cells (shown by broken lines) but not on MTX-resistant sublines. This observation can be contrasted with nonspecific reversal by LV of MTX effects on parent lines and resistant sublines (Chart 2). The dose-response curves of TMQ for MOLT-3/MTX10,000 were, however, entirely different. Thus, although there are populations of cells
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Chart 2. Dose-response curves of the parent and resistant sublines to MTX by growth inhibition assay. See "Materials and Methods" for the experimental procedure used. Protective effects of equimolar concentrations of LV on MTX-induced growth inhibition are shown in broken lines. Each data point represents a mean value of 2 or 3 experiments, each with triplicate determinations. Bars, SD

Chart 3. Uptake (•) and efflux (○) of [3H]MTX (10 μM) from the parent line and the MTX-resistant sublines. The data shown are combined from 3 separate experiments; each time point represents an average from 3 separate experiments carried out in duplicate.

Chart 4. Uptake kinetics of [3H]MTX (A) and [5-14C]methyl-THF (B) from the parent cell line and the MTX-resistant sublines. V is expressed as nmol of MTX or 5-methyl-THF uptake/2.5 min/10⁷ cells. Each time point represents an average from 3 separate experiments carried out in duplicate.

sensitive to this lipid-soluble antifol, approximately 10% of the cells were shown to be extremely resistant to the antifol, and these resistant cells were insensitive to LV (Chart 5B). Patterns of collateral sensitivity and resistance of these cell lines to BW301U were essentially identical to those of TMQ (data not shown).

The parent and MTX-resistant MOLT-3 cell lines showed a modal chromosome number of 89. The original report on this line soon after its establishment described a chromosomal mode of 47 which has evolved to hypertetraploid within 11 months (28). Our counts ranged from 81 to 93 in 29 cells, and most of the increases were trisomies or tetrasonies of unaltered human chromosomes. Two Y-chromosomes in each metaphase plate were identified by quinacrine fluorescence. A few marker chromosomes which were common to parental and drug-resistant sublines (see Fig. 1) appeared to have originated from breakage or recombination at or near centromers. The highly resistant subline MOLT-3/MTX10,000 was similar to the parent line in chromosome number. The parent line was typically trisomie for chromosomes 4 and 5. In the resistant sublines, 1 or 2 long acrocentric marker chromosomes were present in every cell. A typical karyotype is shown in Fig. 2. The more compact metaphase spreads contained a non-banded region or HSR involving most of the long (q) arms but, in less contracted metaphase spreads, this region could be resolved into a repetitiously banded pattern or abnormally banded region (Figs. 1 and 2). The region of homology between the banded region of the HSR-bearing chromosome and its normal counterpart was short, consisting mainly of the short (p) arm. In most of the cells examined, this most nearly resembled the 5p. No double minute chromosomal fragments were identified in any of cells examined. The altered
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Table 1
DHFR activity of human lymphoblastic leukemia cell line MOLT-3 and MTX-resistant sublines

<table>
<thead>
<tr>
<th>MTX concentration in the culture medium (m)</th>
<th>[3H]MTX assay (pmol MTX bound/10^6 cells)</th>
<th>[3H]Dihydrofolate assay (nmol dihydrofolate reduced/min/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOLT-3</td>
<td>0</td>
<td>1979 September 1981/16/18/13/10/22/ND</td>
</tr>
<tr>
<td>MOLT-3/MTX&lt;sub&gt;50&lt;/sub&gt;</td>
<td>10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>1979 September 1981/16/18/20/20/22/ND</td>
</tr>
<tr>
<td>MOLT-3/MTX&lt;sub&gt;500&lt;/sub&gt;</td>
<td>5 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>1979 September 1981/NA/200/14/ND</td>
</tr>
<tr>
<td>MOLT-3/MTX&lt;sub&gt;10,000&lt;/sub&gt;</td>
<td>5 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>1979 September 1981/NA/85/113</td>
</tr>
</tbody>
</table>

ND, not done; NA, cells were not available.

DISCUSSION

The present study establishes the evolution of MTX resistance in human acute lymphoblastic cells in vitro, in that decreased membrane transport of MTX appeared first, followed by increased DHFR activity, which was accompanied by the development of a HSR or an abnormally banded region.

Although a large number of MTX-resistant cell lines have been reported, the development of different mechanisms for MTX resistance has not been clearly described, and such a report was heretofore unavailable for human tumor cells. The only similar data were reported by Flintoff et al. (16) and Flintoff and Essani (17), who described decreased transport in Chinese hamster ovary cells as the first step in MTX resistance, followed by an increase in DHFR as a second step selection.

For the survival of cells in the presence of increasing MTX concentration in vitro, changes in membrane properties appear to be the initial and easiest step in adaptation to the new environment. As MTX concentrations reach beyond a critical level, decreases in membrane transport alone may not be able to keep the drug out of the cell, and the resultant cellular folate deficiency could have triggered the development of gene amplification. In a human lymphoblastic cell line, CEM, 2 distinct routes for MTX entry have been shown, an energy-dependent low-dose route and an energy-independent high-dose route, the latter being observed only at MTX concentrations higher than 2 x 10<sup>-5</sup> M (48). Our observation that increases in DHFR activities were seen in cells exposed to 5 x 10<sup>-5</sup> M of MTX but not in those exposed to 5 x 10<sup>-6</sup> M is consistent with this report.
We found that, for the highly MTX-resistant sublines MOLT-3/MTX200 and MOLT-3/MTX10,000, the addition of LV resulted in increased cell growth (see Chart 2). The reason for this observation is unclear; it may be related to somewhat slower cell growth than is MTX, and/or the growth-reversal effects of LV are more efficacious than the inhibitory effects of equimolar MTX. Slow cell growth has been observed in other MTX-resistant cell lines as well (12).

The uptake Vmax decreased progressively for both MTX and 5-methyl-THF as MTX resistance increased. However, the degrees of changes were not identical (compare Chart 4, A and B). Indeed, a 10,000-fold increase in MTX resistance resulted in a 7-fold decrease in the Vmax for MTX uptake but only a 1.8-fold decrease in that for 5-methyl-THF. Both MTX and 5-methyl-THF are known to enter the cell using a similar transport system. The differences in the degrees of Vmax changes between MTX and 5-methyl-THF include possible differences in the affinity of MTX versus 5-methyl-THF to transport protein. Reasons for the discrepancy between uptake and cell growth inhibition have been discussed (41). The nature of the transient rise in DHFR activity observed in MOLT-3/MTX200 is unclear. An unstable rise in DHFR was associated with the appearance of double minute chromosomes in murine tumor cells (31). We did not analyze the chromosomes during the period when the cell line had a transient rise in DHFR.

The development of HSRs in MTX-resistant human cells has been reported in several laboratories, with different karyotypic localizations, including 10q26 (49) and 5q, 6q, and 19q (4, 47). The 5q+ in the latter, which was found in 60% of the cells examined, appears morphologically similar to that reported herein. The localization of the human DHFR gene by chromosomal segregation in interspecies hybrid cells has now been reported on chromosome 5 (35). However, convincing evidence has been presented that the HSR representing an amplified gene may appear in a chromosomal site apparently unrelated to the native gene localization (30). Thus, while the altered chromosomal morphology correlates with DHFR gene amplification, the localization to 5q in the MOLT-3 cell line could be completely random.

The observation that LV specifically reverses the effects of TMQ in MTX-resistant human tumor cells by virtue of impaired transport may play a role in the treatment of human cancer, since TMQ plus LV will be specifically toxic to MTX-resistant neoplasms with such mechanisms (27, 41).

One cannot generalize about the problem of development of MTX resistance from a single human cell line. Nevertheless, we find that MTX-resistant cells developed in vitro have, in general, impaired in membrane transport, whereas those developed in vivo often have elevated DHFR activity (18, 38, 43). If the transport was impaired in the latter case, it was only to a mild degree (44, 45). Although a correlation between impaired MTX transport and natural resistance to MTX has been described (33), impaired MTX transport as a mechanism of acquired MTX resistance in clinical samples has not been recorded (2, 3, 10, 21). Thus, whether cells with acquired MTX resistance developed in vitro can serve as model for acquired MTX resistance in vivo is yet to be determined. This in vitro/in vivo relationship in the development of MTX resistance is currently under investigation in our laboratory. Only after this relationship is clarified will the role of lipid-soluble antifols, TMQ or BW301U, plus LV as a specific therapeutic approach to the treatment of human cancer be further defined.

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REFERENCES


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Fig. 1. Karyotype prepared from a MOLT-3/MTX\textsubscript{10,000} cell. Two long acrocentric markers (M) are shown in the upper right, near chromosomes 4 and 5. Other markers typical of these lines, a t(3p;6p) and 2 copies of t(7q;10q), are present in the center of the lowest line of chromosomes. The sex chromosomes are located at the right end of the line. The remaining members of the karyotype are in the traditional locations.
Fig. 2. Representative No. 4, No. 5, and marker (M) chromosomes from 6 cells of MOLT-3/MTX are shown. The short arm (p) of the marker (M) bears a close resemblance to 5p in most of the cells examined. The altered region (enclosed by a dotted line) on the long arm of M (Mq) appears homogeneous (HSR) in some less well-banded cells (middle and lower markers, right column) and is distinguished by a repeated abnormal banding pattern in more extended, better-banded cells (left column and upper marker, right column) (also see Figure 1). At least one such chromosome was found in every cell. A dark band is present at approximately three-fourths of the distance from the centromere to Mqter, interrupting the homogeneous or repeat regions.
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