Impaired Polyglutamation of Methotrexate as a Cause of Resistance in CCRF-CEM Cells after Short-Term, High-Dose Treatment with This Drug


ABSTRACT

Two methotrexate-resistant sublines, CCRF-CEM R3/7 and CCRF-CEM R30/6, were selected from the human leukemia T-lymphoblast cell line, CCRF-CEM, after repeated exposures (7 and 6 times, respectively) for 24 h to constant concentrations (3 and 30 μM) of the drug. Analysis of the mechanism of resistance revealed no differences in levels of dihydrofolate reductase activity, its binding affinity for methotrexate, or in methotrexate transport between the CCRF-CEM parent and methotrexate-resistant cell lines. The development of resistance to methotrexate was associated with a marked decrease in the intracellular level of methotrexate polyglutamates. Although the resistant sublines were able to form substantial amounts of folate polyglutamates when measured with [14C]folic acid, the level of polyglutamates formed was decreased to about 50% of that formed by the parent cell line. No qualitative differences in folate polyglutamates formed were noted between the parental and resistant sublines.

This is the first example of a cell line which displays resistance which is solely attributable to defective methotrexate polyglutamate synthesis.

INTRODUCTION

MTX is a useful drug for the treatment of several human malignancies such as choriocarcinoma, acute lymphocytic leukemia, osteogenic sarcoma, diffuse lymphomas, head and neck cancer, and breast cancer (1).

The mechanism of MTX action is related to its potent inhibition of the enzyme DHFR, with consequent depletion of reduced folate cofactors and block of the de novo purine and thymidylate pathways as well as the synthesis of certain amino acids. Intracellular MTX polyglutamate formation is a recently discovered event (2-16) that appears to be a major determinant of tumor cell responsiveness to MTX (17-20). MTX polyglutamates are as potent inhibitors of DHFR as the parent drug (7, 20-22) and are selectively retained by cells after removal of extracellular drug (23-27). MTX polyglutamates are also more potent than MTX as inhibitors of folate requiring enzymes in de novo purine synthesis (glycinamide ribonucleotide and aminoimidazole carboxamide ribonucleotide transformylases) and de novo thymidylate synthesis (thymidylic synthase) than MTX itself (28-30).

Previous studies have established that resistance to MTX may occur in mammalian experimental tumor cells by one or more of five mechanisms (reviewed in Ref. 31): (a) an increase in the levels of the target enzyme, DHFR, generally as a consequence of gene amplification; (b) an alteration in DHFR, leading to an enzyme with a decreased binding affinity for MTX; (c) a decrease in the uptake of MTX into cells; (d) decreased thymidylate synthase activity (32); and (e) impaired ability to form MTX polyglutamates (17, 18, 20).

The majority of previous in vitro studies of MTX resistance have used selection procedures whose clinical relevance is unclear. These methods are based on continuous exposure of long-term tumor cell cultures to increasing concentrations of the drug (33-36) or single exposure to very high concentrations of MTX to select highly resistant mutants (37).

The human leukemia CCRF-CEM cell lines resistant to MTX were developed by means of a more clinically relevant model. This was accomplished by repeated 24-h exposures of cultures to constant drug concentrations in the range of those that are achievable in the clinic with the use of moderate- to high-dose MTX regimens (e.g., 3, 30 μM). Using the time of recovery to achieve exponential growth as a measure of drug resistance, it was demonstrated that cell populations which emerged within 6 treatment cycles were nearly maximally resistant. The mechanisms of resistance have been investigated, and the results showed that the CCRF-CEM R3/7 and R30/6 cell lines did not exhibit resistance by virtue of the first three of the aforementioned mechanisms of resistance, but did show impaired polyglutamation of both MTX and of folic acid.

MATERIALS AND METHODS

Chemicals. MTX was obtained from the National Cancer Institute, Bethesda, MD. For biochemical studies, MTX was dissolved in 1 N NaOH, and the pH was adjusted to 7.0. In challenging the cells, clinical MTX (Lederle) was dissolved in saline. Trimetrexate glucuronate was obtained from Warner-Lambert/Parke-Davis, Ann Arbor, MI, and was dissolved in sterile water. The concentration of both drugs was determined by measurement of absorbance, using a mm extinction coefficient at 370 nm (pH 13) for MTX of 8.1, and of 41.1 at 242 nm (pH 1) for TMQ. 7-Fluoromethotrexate was a gift from Dr. J. K. Coward, University of Michigan, Ann Arbor, MI. Dihydrofolic acid was synthesized by the method of Blakley (38). 2-5-H-deoxyuridine, [3',5',7',9-H]-methotrexate, and [3',5',7',9-H]-folic acid were purchased from Moravek Biochemicals, Brea, CA. Purity of [3',5',7-H]MTX was determined by HPLC using a reverse-phase column (Ultraphase-ODS 25 cm x 4.6 mm inner diameter, 5 μM; Altex, Berkeley, CA). The mobile phase was ammonium acetate (100 mM, pH 5.5) containing 7% (v/v) acetonitrile (J. T. Baker, Phillipsburg, NJ); under these conditions MTX had a retention time of 21 min, well-separated from any breakdown products which elute earlier (chromatogram not shown). If the purity was less than 95%, the labeled compound was purified, 100 μCi per injection, using these same HPLC conditions. The fractions containing the purified MTX were pooled and lyophilized (Virris freeze-dryer; Gardner, NY), and, after reconstitution in a minimal volume of water, an aliquot was reinked to determine final purity.

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The purity of [3H]folic acid was determined using the same HPLC conditions except that the concentration of acetonitrile was 4%. The retention time for folic acid was 15 min. If purification was required, multiple injections were made; fractions were pooled and lyophilized; and an aliquot was reinjected to determine final purity.

All other chemicals were purchased from Sigma Chemical Company, St. Louis, MO. All other chemicals were of the highest purity available.

Cell Lines. A cloned subline of the human T-lymphoblast cell line CCRF-CEM (40) was used. The MTX-resistant subline CCRF-CEM R1, characterized by an increased DHFR activity (41), and CCRF-CEM R2, which has normal DHFR levels but impaired with MTX transport (42), were used as controls. All cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO2 atmosphere.

Selection Procedure. Exponentially growing CCRF-CEM cells at a concentration of 4 to 6 x 10^5 cells/ml were divided into two identical populations and were exposed to MTX (either 3 or 30 μM) for 24 h. After treatment, cells were harvested by centrifugation (1000 x g, 5 min, 37°C), and washed twice with drug-free medium, and the cell pellet containing approximately 10^6 cells was resuspended in drug-free medium at a density of about 2 x 10^4 viable cells/ml. This time was considered “time zero” for the calculation of the recovery time period (see below). Cells were counted every 24 h using a Model D Coulter Counter (Counter Electronics, Ltd., Luton, Bedfordshire, England), and the viability was assessed by the trypan blue exclusion test.

Cells were passaged either when cell numbers exceeded 10^6/ml or, on a weekly basis when cell densities fell below 5 x 10^4/ml, the cells were concentrated 2- to 4-fold on passage. At the earliest convenient time for the CCRF-CEM, parent R3/7, and R30/6 was 21 h for all cell lines.

Enzyme Assays. DHFR activity was determined spectrophotometrically as described (43). The decrease in absorbance at 340 nm, which occurs when NADPH and dihydrofolate are converted to NADP and tetrahydrofolate, respectively, at 37°C, was measured using a Gilford Model 2000 recorder (Gilford Instruments, Oberlin, OH) attached to a Beckman DU spectrophotometer (Beckman Instruments, Fullerton, CA).

IgG values for MTX with DHFR were obtained by inhibition assay in which the drug was added at various concentrations to the assay mixture which contained enzyme (activity A_290 nm, 0.040/min at 37°C). Protein concentrations were determined by the semimicrobiuret method.

2'-[3H]Deoxyuridine Tritium Release Assay. For the detection and activation of MTX resistance, the method of Rodenius was used (44). In this assay, the inhibition of thymidylate biosynthesis caused by antifolates is estimated in situ using 2'-[3H]deoxyuridine. After cellular uptake of 2'-[3H]deoxyuridine and conversion to 5'-[3H]dUMP, tritium is released as H2O into the medium when thymidylate synthase replaces the 5'-H by a methyl group (45).

MTX Uptake. Exponentially growing cells (CCRF-CEM, CCRF-CEM R3/7, and CCRF-CEM R30/6) were harvested by centrifugation and suspended at a density of 1 to 3 x 10^6 cells/ml in complete medium in the presence of [3H]MTX and unlabeled MTX to a concentration of 1 μM (230 cpm/pmol). One hundred-μl aliquots were taken, in duplicate, at 5, 10, 15, 30, and 60 min and added to 200 μl of 0% sucrose and 700 μl of 0.9% saline solution in microfuge tubes, centrifuged 2 min at 16,000 x g, and washed twice with 1 ml of ice-cold 0.9% saline solution. The pellets were resuspended in 500 μl of 5% perchloric acid, boiled 15 min, and centrifuged 2 min at 16,000 x g, and the radioactivity in the supernatant was quantitated using a liquid scintillation counter.

Analysis of MTX Polyglutamates. Exponentially growing cells were harvested by centrifugation (500 x g, 5 min, ambient temperature). Cells were resuspended at 0.4 to 1.0 x 10^6 cells/ml in fresh medium in spinner flasks, and [3H]MTX and unlabeled MTX were added to yield the appropriate final concentration. In experiments in which efflux was followed, the cells were collected at the time indicated, centrifuged, washed twice in prewarmed complete medium, and resuspended at the same cell density in fresh complete medium. After the cells were incubated with [3H]MTX, 40- to 45-μl aliquots were transferred to glass tubes in ice, centrifuged (4°C), and washed twice with ice-cold 0.9% saline solution. The cell pellet was suspended in 1 ml of boiling 50 mM sodium phosphate, pH 5.5, and boiled for 5 min. Cellular debris was removed by centrifugation at 2000 x g for 10 min at room temperature (20). The supernatant was stored at -20°C until analysis by HPLC.

HPLC analysis of cell extracts was performed using a modification of the method of McGuire et al. (46). An anion exchange column, Whatman Partisol SAX:10 μm, was eluted with a linear gradient from 50 to 450 mM sodium phosphate, pH 3.3, at 1.0 ml/min in 24 min. Sufficient chemically synthesized MTX polyglutamate was added to radiolabeled samples to provide an internal standard.

Determination of Intracellular Folate Polyglutamates. Seventy-five μCi of [3H]folic acid were added to 80 ml of complete medium containing 3 x 10^5 cells/ml (271 cpm/pmol). After incubation for 24 h (37°C), the cells were harvested by centrifugation (500 x g, 5 min), and the pellet was washed twice with ice-cold 0.9% saline solution. Folate derivatives were extracted by the addition of 1 ml of boiling 25 mM sodium phosphate, pH 6.0, containing 200 mM 2-mercaptoethanol and boiled for 10 min. Cellular debris was removed by centrifugation at 3000 x g for 15 min at 4°C. The supernatant was stored at -20°C until analysis by HPLC.

A reversed phase ion-pair chromatography system was used to separate the folyl-polyglutamates. Briefly, the column was an Ultrasphere, C18, 25-cm x 4.6-mm inner diameter, 5 μm (Rainin, Woburn, MA). Solvent A was methanol:acetonitrile (2:1, v/v), 10% in 100 mM ammonium phosphate with 1 M Tris base, pH 6.5, and Solvent B was methanol:acetonitrile (2:1, v/v), 25% in 100 mM ammonium phosphate.
RESULTS

Development of Resistance to MTX. CCRF-CEM cell lines resistant to MTX resulted when the cells were exposed repetitively for 24 h at drug concentrations of 3 or 30 \( \mu \)M. The growth curves in drug-free medium after each exposure are shown in Fig. 1, A and B. The time of recovery of these leukemic cell lines treated with MTX decreased as a function of the number of drug treatments. The development of MTX-resistant cell lines occurred during the first six cycles and was apparent even after two cycles at the higher dose. The recovery times for the sixth cycle were 92.5 and 95 h, respectively, for the cells treated with 3 and 30 \( \mu \)M MTX. These values were 46% and 27% of the recovery time for cells treated only once (200 and 345 h).

In the case of the eighth and last treatment, the time of recovery was 70 and 75 h for CEM R3 and CEM R30, or 35 and 21%, respectively, of the time of recovery from the first challenge. Thereafter, the time of recovery did not shorten significantly. Under these culture conditions the untreated parental cell lines required 40 to 55 h for quadruplicating their number.

Following the sixth and the seventh MTX treatment cycle, the R30 and R3 resistant cell lines were exposed to MTX continuously for 72 h. The \( ED_{50} \) values for the cells resistant to 24-h exposures to 3 and 30 \( \mu \)M MTX (R3/7 and R30/6) were only slightly different than the \( ED_{50} \) of the parent CCRF-CEM cell line (i.e., 15, 18, and 10 nM, respectively) (Fig. 2). The \( ED_{50} \) for the CCRF-CEM R1 cell line, resistant to MTX because of increased levels of DHFR (41), and for the CCRF-CEM R2 cell line, resistant to MTX because of impaired drug transport (42), were higher (i.e., 580 and 2300 nM).

The MTX-resistant cell lines R3/7 and R30/6 and the parent line were equally sensitive to the nonclassical antifolate, trimetrexate, which differs from MTX in transport and intracellular metabolism (\( ED_{50} \) values for continuous exposure of 2.3, 3.5 nM and 1.6 nM, respectively).

The resistance in CCRF-CEM R3/7 and R30/6 changed slightly during a 4-mo period of growth in drug-free medium (data not shown).

Detection and Classification of MTX Resistance. As a screening system to detect the mechanism of resistance to MTX (44), in the R3/7 and R30/6 cell lines, the effects of three antifolates, MTX, TMQ, and FMTX, on thymidylate biosynthesis were studied in resistant and parent cells (Table 1). At the end of a 3-h drug exposure period, thymidylate synthase activity in the parent line was inhibited by all three drugs; after a 6-h efflux period, thymidylate synthase activity was inhibited by MTX and TMQ, but not by FMTX. In contrast, the two resistant cell lines showed a different pattern of inhibition: after a 3-h drug exposure period, marked inhibition was produced by all three drugs; after the 6-h efflux period, however, inhibition was maintained only in TMQ-treated cells, but not in MTX- or FMTX-treated cells.

These data suggested that impaired polyglutamation of MTX was the probable cause of resistance to the high doses of MTX (44). In order to provide definitive evidence for decreased polyglutamation of the drug as the sole mechanism of resistance, additional studies were carried out as described below.

DHFR Activity. DHFR specific activity was similar in the parent line and in the two resistant lines, CCRF-CEM R3/7 and CCRF-CEM R30/6 (0.29, 0.36, and 0.34 \( \mu \)mol/h/mg protein, respectively). Also, \( IC_{50} \) values for MTX showed no evidence for a decreased affinity of MTX for DHFR in the resistant lines, 8.7 and 6.6 nM for CCRF-CEM R3/7 and R30/6, respectively, compared to 7.4 nM for the parental CCRF-CEM cell line.

Uptake of \(^3\text{H}\)MTX. The results of the uptake studies of \(^3\text{H}\)MTX (1 \( \mu \)M) are presented in Fig. 3. Under these conditions no significant differences in the level of intracellular drug concentrations were observed among the parent and the two resistant lines, R3/7 and R30/6.

MTX Polyglutamate Formation. The three cell lines were exposed for different periods of time (4 and 24 h) to different concentrations of \(^3\text{H}\)MTX (1 and 10 \( \mu \)M). For each time period, the MTX content was analyzed using an HPLC method (Table 2). The MTX polyglutamate content increased with the number of treatment cycles. The data show a 3- to 4-fold increase in MTX polyglutamate levels in the two resistant lines compared to the parent cell line.

Table 1. \( ED_{50} \) Values for Continuous Exposure of MTX-resistant Cell Lines

<table>
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<tr>
<th>Cell Line</th>
<th>( ED_{50} ) (nM)</th>
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<tbody>
<tr>
<td>Parent</td>
<td>7.4</td>
</tr>
<tr>
<td>R3/7</td>
<td>8.7</td>
</tr>
<tr>
<td>R30/6</td>
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Table 2. \( IC_{50} \) Values for MTX and TMQ

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<th>Antifolate</th>
<th>( IC_{50} ) (nM)</th>
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<tr>
<td>Parent</td>
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<tr>
<td>R3/7</td>
<td>8.7</td>
</tr>
<tr>
<td>R30/6</td>
<td>6.6</td>
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</tbody>
</table>

Fig. 2. Inhibitory effects of MTX on the growth of CCRF-CEM parent and MTX-resistant cell lines. Cells (2 \( \times \) 10\(^6\)/ml) were incubated in the presence of MTX at various concentrations. The number of cells was counted after 72 h of incubation. The cell lines are: CCRF-CEM (○); CCRF-CEM R3/7 (□); CCRF-CEM R30/6 (△); CCRF-CEM R1 (△); and CCRF-CEM R2 (■).

Fig. 1. Time course of recovery in CCRF-CEM cell lines after repeated exposure for 24 h to constant concentrations of MTX. Logarithmically growing CCRF-CEM cells (4 to 6 \( \times \) 10\(^7\) cells/ml) were repetitively exposed for 24 h to a constant concentration of MTX (3 or 30 \( \mu \)M), as described in "Materials and Methods." The bar refers to the duration of exposure of the cells to MTX (24 h). n refers to the starting cell concentration; 4n is 4 times the initial concentration.
Cells (4 × 10^7/ml) were suspended in complete medium. Five 400-µl aliquots were incubated for 3 h in the presence of: no drug; 1 µM MTX; 0.1 µM MTX; 1 µM TMQ; or 5 µM FMTX. [3H]Doxycycline was then added, and samples were taken at 0, 15, 30, and 45 min and processed as described in "Materials and Methods." To determine efflux, four 400-µl aliquots were incubated for 3 h in the presence of: no drug; 1 µM MTX; 1 µM TMQ; or 5 µM FMTX. The cells were then washed 3 times in complete medium, suspended in drug-free medium, and incubated for another 6 h. The tritium release assay was then carried out as described for the 3-h incubation. The results are expressed as the percentage of the slope (H release versus incubation time) of untreated control cells.

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**DISCUSSION**

The technique commonly used to develop MTX-resistant sublines *in vitro* is based on continuous exposure of cells to low concentrations of MTX, with subsequent small stepwise escalation of drug concentration. This procedure results in development of resistance to MTX in a stepwise fashion, usually corresponding to elevated levels of DHFR as a function of amplification of the DHFR gene (36, 41). In the clinic, most patients are now treated with pulse doses of MTX at moderate to high dosage levels at weekly or biweekly intervals. It was therefore of interest to derive sublines of tumor cells resistant to MTX as a consequence of high concentration, short-term intermittent high-dose treatment after 7 and 6 cycles, respectively. No significant change in the pattern of the polyglutamates was observed after efflux.

After 24 h of incubation with 10 µM MTX the main metabolite in the parental cell line was the triglutamate (142 pmol/10^7 cells), but MTX, di-, tetra-, and pentaglutamates were also present in high concentration (72.9, 46.2, 28.1, 15.4 pmol/10^7 cells, respectively). The efflux showed the same level of tri- (138.1 pmol), slightly higher tetra- and pentaglutamate (45.4 and 22.0 pmol), and loss of MTX (12.8 pmol) and diglutamate (13.9 pmol/10^7 cells). The resistant cell line CCRF-CEM R3/7 had a high amount of MTX (78.3 pmol) and a considerable amount of diglutamate (11.4 pmol/10^7 cells). After efflux the level of triglutamate increased about 2.5-fold (from 3.6 to 9.5 pmol/10^7 cells). The CCRF-CEM R30/6 showed only a high concentration of MTX (73.4 pmol) with low di- (3.8 pmol) and triglutamates (2.4 pmol). After efflux there was no change in the pattern, except for a decrease in the MTX concentration to 41.9 pmol/10^7 cells.

**Folate-Polyglutamate Formation.** Since the data presented above were consistent with impaired MTX polyglutamate formation as a basis for the resistance to pulse exposures to high concentrations of MTX, it was also of interest to measure the ability of these resistant cells to form folate polyglutamates. After incubation with [3H]folic acid for 24 h, the parent line had a 2-fold higher concentration of total intracellular folates (19 pmol/10^7 cells) than did either of the resistant lines (9 pmol/10^7 cells) for both R3/7 and R30/6. Only 1 to 3% of the total folates was folic acid; the predominant forms for all three cell lines were in the region of the tetra-, penta-, and hexaglutamates (Fig. 4).

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![Graph showing [3H]MTX uptake by CCRF-CEM parent and MTX-resistant cell lines.](image-url)
Table 2 Intracellular concentration of MTX and MTX polyglutamates in CCRF-CEM parent and resistant cell lines

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<th>Glu 4</th>
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<th>Glu 6</th>
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</table>

* 4-NH₂-10-CH₃ PteGlu₅, methotrexate polyglutamates; Glu 1, monoglutamate (others defined similarly).

Fig. 4. Folylpolyglutamates in CCRF-CEM parent and CCRF-CEM R30/6 cells. Cells were incubated with [3H]folic acid for 24 h, harvested, boiled, and centrifuged as described in "Materials and Methods." An aliquot was injected onto the HPLC column; 1-μl fractions were collected and counted for radioactivity. The hatched area represents radioactivity; the solid line, absorbance at 280 nm; n, elution position of standards, according to the number of glutamyl moieties. A, CCRF-CEM parent, 10⁷ cells; B, CCRF-CEM R30/6, 10⁷ cells.

(i.e., CCRF-CEM R3/7 and CCRF-CEM R30/6) without an increased ability to survive long-term (72 h) low-dose treatment. Using the recently published method for detecting and classifying MTX resistance (44), the short-term 2'-[5-³H]deoxyuridine tritium release assay system, it was shown that thymidylate synthesis in resistant cell sublines was inhibited only in the presence of extracellular MTX, while recovery occurred rapidly once the external drug supply was removed. Similar results were obtained by exposing cells to FMTX. The latter compound was used as a positive control, since FMTX (47) is capable of inhibiting DHFR with a potency equivalent to that of the parent drug and has transport properties essentially identical to that of MTX. However, the presence of the electronegative fluorine substitution adjacent to the potential amide forming carboxyl group in the molecule of the analogue greatly diminishes its ability to form polyglutamate derivatives. The finding that TMQ sensitivity was not affected in the resistant sublines indicated that increased levels of DHFR or DHFR with altered affinity for 2,4-diamino inhibitors (48) were not the basis for MTX resistance since the latter compound enters the cell through a pathway independent from that of MTX (49) and does not undergo intracellular conversion to polyglutamate derivatives (50), but would be affected by qualitative or quantitative changes in DHFR. This assumption was confirmed by measurement of the levels of DHFR in both R3/7 and R30/6 sublines and measurement of the apparent affinity of MTX for the DHFR in each cell line.

These data, together with the cytotoxicity results, suggested that the biochemical alteration that was involved in the MTX resistance in CCRF-CEM cells was diminished synthesis of MTX polyglutamates. The total intracellular concentration of MTX by 4 and 24 h was lower in resistant cells following incubation with two concentrations of the drug (1 and 10 μM MTX). This difference is attributed to the inability of MTX-resistant cells to convert intracellular MTX to polyglutamate forms which are preferentially retained, rather than impaired uptake of MTX (Fig. 3). The finding that the resistant cells can form folypolyglutamates to a lesser extent than the parent line after exposure to [3H]folic acid makes it likely that the FPGS enzyme may be altered in its affinity for MTX and folates.
Other possible causes for a decreased level of polyglutamates of MTX in resistant cells are: an increased catabolism of the polyglutamates; increased efflux of short-chain polyglutamates (27); decreased intracellular glutamates; or altered intracellular regulation of FPPGs, i.e., decreased affinity of MgATP or glutamate for the enzyme as recently suggested by Cook (51). This cell line represents the first example of a cell line which displays resistance which is solely attributable to defective MTX polyglutamate synthesis. Previous reports (17, 18) have described cell lines with defective MTX polyglutamylation associated with a decreased MTX transport, and in one case, a reduced glutamate synthesis. Previous reports (17, 18) have described cell line represents the first example of a cell line which displays resistance which is solely attributable to defective MTX polyglutamate synthesis. Since our findings may have important clinical implications, we are examining the basis for the decreased polyglutamate forms of MTX observed in these cell lines and plan to examine tumor cells from patients before and after treatment with MTX to establish the generality of this observation.

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IMPAIRED POLYGLUTAMYLATION OF MTX


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