Proliferation-dependent Cytotoxicity of Methotrexate in Murine L5178Y Leukemia

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ABSTRACT

The basis for the proliferation-dependent cytotoxicity of methotrexate has been investigated in mice bearing the L5178Y ascites leukemia. Methotrexate at 60 mg/kg i.p. reduced the viability of logarithmically growing ascites cells (55% active S phase cells) to 28% of control, whereas the viability of the slowly growing cells (18% active S phase) was decreased to only 59% of control. Log phase tumor cells accumulated 8-fold higher levels of methotrexate polyglutamates compared to cells that had approached the stationary phase. However, no differences between log phase and slowly growing tumor cells were observed in the cellular levels of unmetabolized methotrexate. Intestinal mucosa and bone marrow from non-tumor-bearing mice resembled slowly growing tumor cells and had markedly lower levels of methotrexate polyglutamates than logarithmically growing cells. The greater accumulation of methotrexate polyglutamates in the logarithmically growing tumor cells was consistent with an increased synthesis of methotrexate polyglutamates in these cells. The enhanced methotrexate polyglutamylation in log phase versus slowly growing cells was not related to changes in the rates of either cellular methotrexate transport, transmembrane efflux of methotrexate, or hydrolysis of methotrexate polyglutamates. Thymidylate synthase activity measured in situ and in extracts from log phase cells was 4- and 2-fold higher, respectively, than in the more slowly growing cells. Methotrexate produced a 2.4-fold greater depletion of polygamma-glutamyl derivatives of 5,10-methylenetetrahydrofolatepolyglutamate in log phase cells compared to slowly growing cells, and this was a function of both the increased methotrexate polyglutamate accumulation and thymidylate synthase activity in the rapidly proliferating cells. These results provide further evidence that the selectivity of methotrexate for tumors with a high growth fraction is a consequence of the rapid rates of both cellular methotrexate polyglutamate synthesis and oxidation of 5,10-methylenetetrahydrofolatepolyglutamates by thymidylate synthase.

INTRODUCTION

Early laboratory studies of MTX activity against murine leukemias indicated that the drug was more effective in prolonging the survival of mice bearing a low tumor burden compared to those with advanced disease (1). This correlation of drug effect with proliferative activity was subsequently related to differences in the rates of DNA synthesis (2) and MTX transport (3) between rapidly and slowly growing tumors. MTX is currently a component of curative regimens for gestational trophoblastic disease (4), acute lymphocytic leukemia in childhood (5), diffuse histiocytic lymphoma (6, 7), Burkitt’s lymphoma (8), and adjuvant therapy of premenopausal breast cancer (9, 10). This agent also has significant palliative activity in head and neck cancer (11). It is interesting to speculate that the one feature shared by the MTX-responsive tumors is a higher proliferative activity compared to those tumors against which MTX is less effective.

This study was carried out in order to further define the biochemical basis for the proliferation-dependent activity of MTX in mice bearing the L5178Y ascites leukemia. A primary goal was to identify those biochemical parameters that would likely be of importance to MTX scheduling in the clinic. Various cellular processes including dTMP synthase activity (12), MTX transport (3) and polyglutamylation (13), and MTX-Glu4, hydrolysis (14) have been reported to be proliferation linked and important to MTX efficacy. The present study represents an attempt to evaluate these and other biochemical parameters within a single tumor system, and to relate the results obtained to the proliferation-dependent cytotoxicity of MTX.

MATERIALS AND METHODS

Male AKR/J X DBA/2J F1 mice, weighing 20–22 g were used in all experiments. Tumor cell lines were maintained by weekly i.p. passage of cells in male DBA/2 mice. All mice were obtained from The Jackson Laboratory, Bar Harbor, ME. The murine L5178Y tumor was obtained from Dr. Joseph G. Mayo of the National Cancer Institute. MTX was purchased from Lederle Laboratories, Pearl River, NY. RNase (specific activity of 70 Kunitz units/mg of protein) and propidium iodide were obtained from Sigma Chemical Co., St. Louis, MO. [3', 5', 7-3H]MTX (specific activity of 120 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. [5-3H]dUrd and [6-3H]-FdUMP (specific activities of 20 and 23 Ci/mmol, respectively) were obtained from Moravek Biochemicals, Brea, CA. Authentic standards of MTX-Glu4 to MTX-Glu5 were prepared by Dr. Charles Baugh of the Department of South Alabama and were obtained from the National Cancer Institute. [methyl-3H]Thymidine (specific activity of 20 Ci/ mmol) was purchased from New England Nuclear, Boston, MA. The IU-4 monoclonal antibody directed against BrdUrd was a gift of Dr. Joseph L. Livornese of the Medical University of South Carolina. Noble agar was purchased from Difco Laboratories, Detroit, MI.

Flow Cytometry. On days 4 and 7 after i.p. inoculation of 104 tumor cells, groups of 3 mice each received an i.p. injection of 100 mg/kg BrdUrd. Twenty min later the ascites cells were harvested. The incorporation of BrdUrd into DNA and the cellular DNA content were determined by flow cytometry (15). Nuclei from active S-phase cells, which incorporated BrdUrd into DNA, were stained green via an indirect antibody technique using an IU-4 monoclonal antibody directed against BrdUrd. The nuclei were then counterstained red with propidium iodide and analyzed on a Becton Dickinson FACS analyzer system. The cytokinetic values were obtained by computer analysis of the histogram by using the FSC program (16).

Rates of DNA and Protein Synthesis. Mice were given injections i.p. of 106 tumor cells. On days 4 to 8 after tumor inoculation the ascites cells from 5 mice were harvested, pooled, and washed once with ice-cold 0.9% NaCl solution and then resuspended at a density of 106 cells/ml in Fischer’s medium containing 10% horse serum. Following a 30-min preincubation at 37°C, 1 µCi of [3H]thymidine or [3H]leucine was added to each sample, and the cultures were incubated for 1 h. After this time, the incorporation of [3H]thymidine or [3H]leucine was measured. DNA and protein were isolated from the cells, and the incorporation of the radioactivity into DNA and protein was determined by liquid scintillation counting. Statistical significance was determined by Student’s t-test.
added to each flask. The incorporation of radioisotopes into DNA and protein was measured as previously described (17) and were linear over the time course of the experiment. The rates were calculated from linear regression analysis of the data and were expressed as percentages of the rates obtained with day 4 tumor cells (control). Each experiment was repeated two additional times with similar results.

**MTX Transport.** The procedure followed was a modification of that which we previously described (18). Briefly, groups of 5 mice each were sacrificed on days 4 and 7 after i.p. inoculation of 10⁵ L5178Y cells. The tumor cells were washed once with ice-cold 0.9% NaCl solution, and then resuspended at a density of 5 x 10⁸ cells/ml in Eagle’s minimal essential medium containing 40 mM 3-[N-morpholino]propanesulfonic acid buffer, pH 7.4. The cell suspensions were preincubated for 15 min at 37°C, and transport was initiated by the addition of [3H]MTX to yield a final concentration of 0.1 μM (final specific activity of 17.8 Ci/ mmol). Aliquots of the cell suspensions (150 μl) were removed at 1- min intervals over an 11-min time period and immediately layered over 100 μl of silicone oil. [3H]MTX influx was terminated by centrifugation of the samples at 12,000 x g in a microcentrifuge. The [3H]MTX in the supernatants and pellets were counted. [3H]Glucose and [3H]2O₂ spaces were determined similarly, and the actual intracellular concentrations of [3H]MTX were calculated, following correction for [3H]-MTX in the extracellular space (18).

**Measurement of Intracellular MTX and MTX-Glu.** Mice were inoculated with 10⁵ tumor cells i.p. On days 4 or 7 after tumor transplantation, the mice received an i.p. injection of [3H]MTX of either 2.5 mg/0.5 mCi/kg or 60 mg/1.6 mCi/kg. The cells from 5–6 mice per time point were pooled and washed twice with ice-cold 0.9% NaCl solution, and the cell pellets were resuspended in 400 μl of water and immediately placed in a 100°C water bath for 10 min. The samples were then centrifuged at 10,000 x g and 4°C. The supernatants were used for HPLC analysis and protein determination. MTX and MTX-Glu were measured in the small intestine of non-tumor-bearing mice in order to avoid the possibility of contamination of these normal tissues with infiltrated tumor cells. These mice were given injections of [3H]MTX as described above, and 4 h later the animals were sacrificed. Approximately 3 cm of small intestine were surgically removed from 12–18 mice per group. The intestines were opened longitudinally, rinsed in 0.9% NaCl solution with a syringe and needle. Marrow from 18 mice per group was pooled, Cells were treated at 4°C, washed 3 times, and resus in the pellet were removed by hypotonic lysis in 0.2% NaCl solution. Immediately after isolation, the intestinal mucosa and the bone marrow were placed in 400 μl of boiling water and the MTX-Glu was extracted as described above.

MTX and MTX-Glu were quantitated by HPLC as described previously (19). Authentic standards of MTX and MTX-Glu were co-injected with each sample and monitored by absorbance at 254 nm. Protein concentrations in the cell extracts were estimated by the Coomassie blue staining method (20).

**Measurement of MTX Retention and Efflux.** At 4 and 7 days after i.p. implantation of 10⁵ L5178Y cells, groups of 5 mice each were given a single i.p. injection of [3H]MTX (2.5 mg/0.5 mCi/kg). Four h later the tumor cells were harvested, pooled, and washed once with ice-cold 0.9% NaCl solution, and then resuspended at a density of 2 x 10⁶ cells/ml in 150 ml of Fischer’s medium containing 10% horse serum. The cell suspension was incubated at 37°C in a shaking water bath and at 0-, 1-, 3-, and 5-h intervals 40-ml aliquots were removed for determination of protein concentration and HPLC analysis of MTX retention as described above. In another series of experiments groups of 5 mice each bearing either day 4 or 7 tumors were given injections i.p. of [3H]-MTX (2.5 mg/0.5 mCi/kg) and sacrificed 15 min later. The tumor cells were pooled, washed once at 4°C with 0.9% NaCl solution, and resuspended in drug-free medium at 37°C. The intracellular levels of MTX and MTX-Glu were measured at 0-, 15-, 30-, and 45-min intervals as described above.

Hydrolysis of MTX-Glu. Tumor cells were harvested and pooled from duplicate groups of 3 mice each on days 4 and 7 after i.p. tumor transplantation. The cells were washed once with ice-cold 0.9% NaCl solution, and then resuspended in either 50 mM Tris-HCl, pH 7.2, containing 1 mM EDTA, or 50 mM sodium acetate, pH 4.5, plus 1 mM EDTA. The cell suspensions were then sonicated with three 15-s bursts of 15 W with a Branson 200 sonifier equipped with a microtip. The suspensions were centrifuged at 12,000 x g for 10 min at 4°C and the supernatants, which had protein concentrations between 0.1 and 0.5 mg/ml were removed and placed in a water bath at 37°C. Authentic MTX-Glu was added to each sample to yield a final concentration of 25 μM. At 0, 30, 60, and 180 min after the addition of MTX-Glu, 200- to 400-μl aliquots were removed for the determination of MTX and MTX-Glu levels by HPLC as described above. The hydrolysis of MTX-Glu was determined by monitoring the decrease in absorbance at 254 nm of the MTX-Glu peak as a function of time. The rate of hydrolysis of MTX-Glu was linear for at least 180 min.

dTMP Synthase Activity in situ and in Cell Extracts. The activity of this enzyme was measured in intact cells by the method of Yalowich and Kalman (21) with the following modifications. Mice were inoculated with 10⁴ tumor cells on day 0, and the tumor cells were harvested from the mice on either day 4 or 7. The cells were preincubated for 2 min at a density of 10⁶ cells/ml in Fischer’s medium maintained at 37°C. [5-3H]dUrd was added to duplicate flasks per group to achieve a final concentration of 22 nM (final specific activity of 23 Ci/mmole). At 0, 5, 10, 15, and 20 min after the addition of [3H]dUrd, 200-μl aliquots were removed and pipeted into microcentrifuge tubes. The reactions were terminated by immediately adding 1 ml of ice-cold 5% (w/v) activated charcoal suspended in 0.15 N trichloroacetic acid. The mixtures were centrifuged at 10,000 x g for 5 min, and the radioactivity in 800 μl of the supernatant was counted. When the experiment was repeated similar results were obtained. Enzyme activity measured in the in situ assay was sensitive to inhibitors of dTMP biosynthesis, since incubation of tumor cells with either 1 μM MTX or 1 μM 5-fluoro-2'-deoxyuridine for 30 min decreased dTMP synthase activity in situ by 98% and 95%, respectively, compared to control cells.

dTMP synthase activity was assayed in extracts from day 4 and day 7 tumor cells for 30 min by the tritium displacement method of Lomax and Greenberg (22) as modified by Dolinick and Cheng (23) in a final volume of 40 μl. Tritium release was linear with respect to time and protein concentration under the above assay conditions. One enzyme unit is defined as the quantity of enzyme required to form 1 nmol of product per min. Statistical analysis was carried out by using the two-tailed t test.

**Measurement of 5,10-CH₂H₂PteGlu.** The total cellular pools 5,10-CH₂H₂PteGlu were determined by measuring the ability of cell extracts to stimulate tight ternary complex formation with [3H]FdUMP and dTMP synthase (24). Tumor cells were harvested from mice on either day 4 or 7 after inoculation of 10⁶ cells. Between 2 and 3 x 10⁶ tumor cells were placed in 50 mM Tris-HCl, 50 mM ascorbate, 1 mM EDTA, and 250 mM sucrose, pH 7.4, and boiled for 10 min. The suspensions were then centrifuged and the supernatants were used for determination of 5,10-CH₂H₂PteGlu and protein. The reaction mixture contained 7 x 10⁴ units of dTMP synthase, 250 nM [3H]FdUMP, and tumor cell extract in 200 μl of the above buffer. Following a 20-min incubation at 25°C, 20 μl of 11% (w/v) sodium dodecyl sulfate was added and the samples were boiled for 30 min to disrupt noncovalent ternary complexes. Complexed [3H]FdUMP was separated from free [3H]FdUMP by Sephadex G-25 chromatography as previously described (24).

**RESULTS**

Effects of MTX against Logarithmically and Slowly Growing L5178Y Cells. The viability of tumor cells was determined following a 4-h treatment of mice with MTX on days 4 and 7 after tumor transplantation. When given on day 4 after tumor transplantation, a single i.p. injection of MTX (60 mg/kg) reduced the cloning efficiency of the ascites cells to 28% of control (Table 1). On the other hand, the same dose of MTX decreased tumor cell viability to only 59% of control when administered on day 7. This differential cytotoxicity of MTX...
Table 1 Effect of MTX on the viability of logarithmically and slowly growing L5178Y cells

At 4 and 7 days after tumor inoculation, groups of 4 mice each were given injections i.p. of either 0.9% NaCl solution or MTX (60 mg/kg). Tumor cells from each mouse were harvested aseptically 4 h later, washed once with Fischer's medium, and then cloned in soft agar as previously described (39). Results are the means of 4 determinations ± SD.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>% of control</th>
<th>% of cloning efficiency</th>
</tr>
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<tbody>
<tr>
<td>Day 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>29 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>MTX</td>
<td>8 ± 2</td>
<td>28</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>22 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>MTX</td>
<td>13 ± 2</td>
<td>59</td>
</tr>
</tbody>
</table>

* P < 0.01 compared to the percentage of control cloning efficiency of tumor cells treated with MTX on day 4 (two-tailed t test).

Table 2 Cytokinetic analysis of logarithmically growing and stationary phase L5178Y cells

At days 4 and 7 after i.p. tumor inoculation the ascites cells were harvested, and the total cellular DNA content and uptake of BrdUrd into DNA were measured by flow cytometry as described in “Materials and Methods.” Results are the mean values obtained from 2 separate experiments with range in parentheses.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>S</th>
<th>% of cells in each phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BrdUrd positive</td>
</tr>
<tr>
<td>L5178Y</td>
<td>Day 4</td>
<td>55 (49-61)</td>
</tr>
<tr>
<td>Day 7</td>
<td>18 (15-21)</td>
<td>9 (8-10)</td>
</tr>
</tbody>
</table>

was directly related to the growth rate of the tumor. On day 4 the L5178Y tumor was in the logarithmic phase of cell growth (Fig. 1), and 55% of these cells were in active S phase as defined by DNA content and the ability to incorporate BrdUrd into DNA (Table 2). By day 7 the tumor had approached stationary phase and contained only 18% active S-phase cells. In addition, the rates of [3H]thymidine and [3H]leucine incorporation into DNA and protein on day 7 had declined to 23 ± 5% (SD) and 19 ± 2% (SD), respectively, of the corresponding rates measured in logarithmically growing cells (day 4). These results are in agreement with those of others who demonstrated that MTX is a relatively S-phase-specific agent (2, 25) and is generally more active against rapidly growing tumors compared to tumors with a low growth fraction (1, 2). It was not feasible to use completely stationary tumor cells in our studies because of the extensive contamination of the advanced ascites tumors with erythrocytes and the obviously moribund state of the mice after day 7.

Accumulation and Retention of MTX. It was of interest to determine if the proliferation-dependent cytotoxicity of MTX shown in Table 1 was related to differences between log and near stationary phase cells in their ability to accumulate MTX and MTX-Glu4. In all studies MTX-Glu4 accumulation was measured at 4 h after MTX administration, since preliminary experiments revealed that maximal levels of MTX-Glu4 were achieved within 4 h in both day 4 and day 7 tumor cells (data not shown). Table 3 indicates that the total intracellular MTX level after a 4-h MTX treatment was 2.2-, 3.1-, and 52-fold greater in logarithmically growing tumor cells than in slowly growing tumor cells, intestinal mucosa, and bone marrow, respectively. It was important to note that the lower level of total MTX in slowly growing tumor cells compared to log phase tumor cells was entirely the result of an 8-fold decrease in MTX-Glu4 since no decrease in unmetabolized MTX was observed. The diminished capacity of slowly growing tumor cells to accumulate MTX-Glu4 was not the result of a general increase in plasma membrane permeability and loss of MTX-Glu4 from the cells, since 97 ± 1% (SD) and 97 ± 1% (SD) of the cells harvested on days 4 and 7 after transplantation were able to exclude trypan blue.

Previous studies have shown that the more highly charged MTX-Glu4 were preferentially retained by tumor cells compared to unmetabolized MTX (26, 27). The results presented in Table 4 were consistent with these observations. In both log phase cells (day 4) and slowly growing cells (day 7), drug loss was most rapid during the first hour after incubation of cells in drug-free medium. However, day 4 tumor cells lost only 16% of the total intracellular drug after 1 h, in contrast to 65% efflux from day 7 cells during the same time period. The more extensive loss of total intracellular MTX from day 7 cells was directly related to the decreased accumulation of MTX-Glu4 in these cells, since unmetabolized MTX was selectively effluxed compared to MTX-Glu4 (Table 4). In log cells, 35% of the total intracellular drug was unmetabolized MTX at the start of the efflux period compared to 81% unmethylated MTX in the slowly growing cells. These data suggest that the enhanced sensitivity of logarithmically growing L5178Y cells to MTX was related, at least in part, to the greater ability of these cells

Table 3 Accumulation of MTX and MTX-Glu4 in L5178Y cells, intestinal mucosa, and bone marrow

Groups of 5–6 mice were inoculated with 106 L5178Y cells on day 0. On days 4 or 7 after tumor transplantation, the mice received an i.p. injection of [3H]MTX (2.5 mg/0.5 mCi/kg). The tumor cells from each group of mice were harvested 4 h later and MTX-Glu4 accumulation was measured as described in “Materials and Methods.” MTX-Glu4 accumulation in intestinal mucosa and bone marrow was quantitated in non-tumor-bearing mice at 4 h after [3H]MTX administration. Values are the average results of either 3 experiments (L5178Y) or 2 experiments (normal tissues).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>MTX</th>
<th>MTX-Glu4</th>
<th>MTX-Glu3</th>
<th>MTX-Glu4</th>
<th>MTX-Glu3</th>
<th>MTX-Glu4</th>
</tr>
</thead>
<tbody>
<tr>
<td>L5178Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>67</td>
<td>63</td>
<td>38</td>
<td>30</td>
<td>9</td>
<td>207</td>
</tr>
<tr>
<td>Day 7</td>
<td>76</td>
<td>7</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>94</td>
</tr>
<tr>
<td>Intestinal mucosa</td>
<td>60</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>3</td>
<td>1</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>4</td>
</tr>
</tbody>
</table>

* ND, none detectable.
to accumulate MTX-Glu₃ and thereby retain the drug for longer time periods.

MTX Transport and Efflux and MTX-Glu₃ Hydrolysis. Various cellular processes could influence the accumulation of MTX-Glu₃, including MTX transport, MTX-Glu₃ synthesis and degradation, and MTX efflux. MTX transport was studied at a relatively low extracellular concentration of MTX (0.1 μM) in order to increase the likelihood that the transported MTX would be trapped intracellularly by binding to dihydrofolate reductase and not effluxed from the cell. Although the rate of MTX transport was somewhat slower in day 7 cells [12.3 ± 1.1 (SE) nmol/liter of cell water/min] than in day 4 cells [19.1 ± 1.1 (SE) nmol/liter of cell water/min] (Fig. 2), the diminished transport did not appear to limit the conversion of MTX to MTX-Glu₃ in day 7 cells. Tables 3 and 4 showed that there were no significant differences in the levels of unmetabolized MTX between log phase and slowly growing tumor cells following a 4-h treatment of mice with MTX. The levels of unmetabolized MTX were also similar in log phase [483 ± 42 (SD) pmol/mg protein] and slowly growing cells [489 ± 51 (SD) pmol/mg protein] after a 30-min MTX treatment of mice. This observation suggested that alterations in the transmembrane efflux of MTX likewise did not limit the accumulation of MTX-Glu₃ in stationary cells. The cellular efflux of MTX was directly examined by treating tumor-bearing mice with [³H]MTX for a short time period (15 min) in order to decrease the accumulation of MTX-Glu₃. In log phase and slowly growing cells, 80 and 83%, respectively, of the total intracellular drug was unmetabolized MTX at 15 min. The unmetabolized MTX effluxed at similar rates when the log phase and slowly growing cells were placed in drug-free medium. After 15- and 30-min efflux periods, 76 and 87% of the unmetabolized MTX effluxed from log phase cells, and 78 and 88% of the unmetabolized MTX effluxed from the slowly growing cells during these time periods.

The hydrolysis of MTX-Glu₃ was monitored by incubating cell-free extracts from day 4 and day 7 L5178Y cells with 25 μM MTX-Glu₃. The assays were carried out at either pH 4.5 or 7.2 in order to detect the lysosomal and cytosolic activities of γ-glutamyl hydrolase (14). MTX-Glu₁ and unmetabolized MTX were the only forms of MTX detected following incubation of the cell extracts at either pH 4.5 or 7.2 for up to 180 min with MTX-Glu₃. Therefore, any disappearance of MTX-Glu₃ in the cell extract could not be attributable to the conversion of MTX-Glu₃ to higher MTX-Glu₄. The rates of MTX-Glu₃ hydrolysis at pH 4.5 and 7.2 in extracts of day 4 L5178Y cells were 500 ± 70 (SE) and 270 ± 30 (SE) pmol/min/mg protein, respectively. In extracts of day 7 cells the rates of hydrolysis were 60 ± 30 (SE) and 131 ± 3 (SE) pmol/min/mg protein at pH 4.5 and 7.2, respectively. These results indicated that the impaired accumulation of MTX-Glu₃ in the slowly growing cells was not related to an enhanced degradation of MTX-Glu₃, since MTX-Glu₃ hydrolysis at either pH was actually more rapid in extracts of proliferating cells compared to slowly growing cells. Samuels et al. (14) have likewise reported that MTX-Glu₃ hydrolysis was faster in extracts prepared from proliferating mouse ascites tumors versus quiescent cells, and that unmetabolized MTX was the only significant product detected from hydrolysis of MTX-Glu₃. Taken together, the above data imply that the 8-fold lower level of MTX-Glu₃ in the slowly growing cells (Table 3) resulted from decreased MTX-Glu₃ synthesis, since the diminished accumulation of MTX-Glu₃ could not be attributable to either impaired MTX transport, elevated γ-glutamyl hydrolase activity, or enhanced transmembrane efflux of MTX.

dTMP Synthase Activity and Levels of 5,10-CH₂H₄PteGlu₃. The activity of dTMP synthase in tumor cells is probably a critical determinant of MTX cytotoxicity, since dTMP synthase catalyzes the oxidation of the reduced folate cofactor, 5,10-CH₂H₄PteGlu₃. In cells that have low rates of dTMP synthase activity the requirement for dihydrofolate reductase should be diminished because of the slower oxidation of 5,10-CH₂H₄PteGlu₃. Thus, the antiproliferative effect of MTX should also be diminished. For this reason it was of interest to determine if dTMP synthase activity differed significantly in...
logarithmically growing L5178Y cells compared to slowly growing cells. Enzyme activity was monitored in in situ and in cell extracts by quantitating the rates of $^3$H released from [5-$^3$H]dUrd and [5-$^3$H]dUMP, respectively. The activities in the intact cell were normalized to cell volume instead of cell number in order to account for possible differences in cell volumes between exponentially and slowly growing tumor cells. It can be seen in Fig. 3 that at all time points the amount of $^3$H released per μl of cell water was approximately 4-fold higher in logarithmically growing cells as opposed to slowly growing cells. dTMP synthase activity was also significantly higher in extracts of logarithmically growing cells [0.49 ± 0.04 (SD) mmol/min/mg protein] than in extracts prepared from day 7 tumor cells [0.24 ± 0.02 (SD) mmol/min/mg protein, $P < 0.01$], although the magnitude of the difference was less than that observed in situ.

Because of the greater dTMP synthase activity in the rapidly proliferating L5178Y cells versus the slowly growing cells, we reasoned that the depletion of the 5,10-CH$_2$H$_4$PteGlu" pool by MTX should likewise be greater in log phase cells than in slowly growing cells. Table 5 shows that untreated day 4 and day 7 cells had similar levels of 5,10-CH$_2$H$_4$PteGlu". A 4-h treatment of mice bearing day 4 growing tumor cells with 2.5 mg/kg MTX reduced the level of 5,10-CH$_2$H$_4$PteGlu" to 37% of the untreated control cells. In contrast, the level of 5,10-CH$_2$H$_4$PteGlu" in day 7 cells treated with this dose of MTX was still at 74% of control. It might be argued that the greater depletion of 5,10-CH$_2$H$_4$PteGlu" in the rapidly proliferating cells was unrelated to dTMP synthase activity, but was the result of the greater accumulation of MTX-Glu" in the rapidly proliferating cells. In an attempt to address this possibility, mice bearing day 7 tumor cells were also treated with 60 mg/kg MTX. This higher dose of MTX reduced the 5,10-CH$_2$H$_4$PteGlu" level to only 60% of control (Table 5), despite the fact that the levels of total intracellular MTX [433 ± 146 (SD) pmol/mg protein] and MTX-Glu" [97 ± 31 (SD) pmol/mg protein] were comparable to those of log phase cells that had been treated with 2.5 mg/kg MTX (Tables 2 and 3). These results suggested that in L5178Y cells the depletion of 5,10-CH$_2$H$_4$PteGlu" by MTX was influenced not only by the cellular accumulation of MTX-Glu", but also by the activity of dTMP synthase.

**DISCUSSION**

The present study explored various cellular processes important to the therapeutic activity of MTX in leukemic mice with the aim of providing insight for improved MTX scheduling in subsequent clinical trials. It was likely that MTX polyglutamylation and dTMP synthase activity had critical and interrelated roles in determining the selectivity of MTX against rapidly growing tumors. The proliferation-dependent cytotoxicity of MTX was associated with a markedly greater accumulation of MTX-Glu" in exponentially growing L5178Y ascites cells compared to slowly growing tumor cells. These results strongly suggested that the increased capacity of the rapidly proliferating tumor cells to accumulate MTX-Glu" resulted from a more rapid rate of MTX-Glu" synthesis. Other cellular processes which could possibly influence the intracellular levels of MTX-Glu", such as MTX transport, MTX-Glu" hydrolysis, and MTX efflux did not appear to contribute significantly to the differences in MTX-Glu") accumulation between log phase and near stationary cells. Direct evidence which supported the concept of a proliferation-linked synthesis of MTX-Glu" came from the work of Gali\'van's laboratory, which demonstrated that folypolyglutamatase synthetase activity (28) and the rate of MTX-Glu") synthesis (13) in cultured hepatoma cells varied directly with the tumor growth rate. Because of the slow intracellular metabolism of MTX to polyglutamate derivatives compared to most natural folates, a modest decrease in the expression of folypolyglutamatase synthetase activity in slowly growing cells would be expected to have a significant effect on MTX-Glu") synthesis (29).

MTX polyglutamylation could affect the cytotoxicity of the drug in several ways. In this study and in those of others (26, 27), the metabolism of MTX to MTX-Glu" was shown to prolong the cellular retention of MTX. Furthermore, MTX-Glu", as opposed to unmetabolized MTX, were fairly potent inhibitors of purified human dTMP synthase (30–32) and aminoimidazolecarboxamide ribonucleotide transformylase (33). This suggested that MTX, especially after high dose therapy, could have sites of action in addition to dihydrofolate reductase as a consequence of the extensive accumulation of MTX-Glu", in rapidly proliferating cells. The dose and schedule of MTX needed to invoke these effects either in situ or in the clinical setting have not yet been determined. At the relatively low dose of MTX used in this study, it appeared unlikely that direct inhibition of dTMP synthase was the major determinant of the proliferation-dependent cytotoxicity of the drug. Following MTX administration, the depletion of 5,10-CH$_2$H$_4$PteGlu" was greater in rapidly proliferating than in slowly growing cells. One would expect that direct inhibition of dTMP synthase by

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**Table 5** Effect of MTX on 5,10-CH$_2$H$_4$PteGlu" levels in logarithmically growing and stationary phase L5178Y cells.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>5,10-CH$_2$H$_4$PteGlu&quot; (pmol/mg protein)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 4</td>
<td>Control</td>
<td>195 (179–211)</td>
</tr>
<tr>
<td></td>
<td>2.5 mg/kg MTX</td>
<td>72 (60–84)</td>
</tr>
<tr>
<td>Day 7</td>
<td>Control</td>
<td>199 (163–235)</td>
</tr>
<tr>
<td></td>
<td>2.5 mg/kg MTX</td>
<td>148 (115–181)</td>
</tr>
<tr>
<td></td>
<td>60 mg/kg MTX</td>
<td>119 (116–122)</td>
</tr>
</tbody>
</table>
PROLIFERATION-DEPENDENT CYTOTOXICITY OF MTX

MTX-Glu_u would have spared the 5,10-CH_2H_4PteGlu_u pool in the logarithmically growing cells.

Two methods were chosen for the determination of dTMP synthase activity as an attempt to obtain an accurate estimate of the intracellular activity of this enzyme. Enzyme activity was monitored in situ and in cell extracts by quantitating the rates of [3H]dUrd and [3H]dUMP, respectively. The in situ assay has the potential advantage over the cell-free assay in that variations in the intracellular dTMP synthase activity as a result of metabolic regulation can be detected (34). However, it was also possible that the results obtained with the in situ assay did not reflect cellular dTMP synthase activity, but were functions of the different rates of [3H]dUrd uptake or the dUMP concentrations in logarithmically and slowly growing cells. Depending on the method used, we obtained estimates of dTMP synthase activity that were between 2- and 4-fold higher in the exponentially growing cells compared to the slowly growing cells. Thus, both methods revealed higher dTMP synthase activity in the rapidly growing cells, although not to the same degree. The basis for the apparently higher dTMP synthase activity in situ than in cell extracts has yet to be determined.

Although MTX polyglutamylation has an important role in mediating the proliferation-dependent cytotoxicity of MTX, the following evidence suggested that the high activity of dTMP synthase in rapidly proliferating cells also contributed to this phenomenon. Since dTMP synthase catalyzed the oxidation of the reduced folate cofactor, 5,10-CH_2H_4PteGlu_u this implied that in logarithmically growing L5178Y cells the rate of tetrahydrofolate oxidation and the requirement for dihydrofolate reductase activity were relatively high. Conversely, in slowly growing cells that have low dTMP synthase activity, the requirement for dihydrofolate reductase activity and the effect of MTX would be decreased. In agreement with this idea, it was observed that MTX induced a 2.4-fold greater depletion of 5,10-CH_2H_4PteGlu_u in proliferating L5178Y cells compared to slowly growing cells. Others have previously provided convincing evidence that dTMP synthase activity was necessary for the development of MTX cytotoxicity. The cellular sensitivity to MTX was reduced in cells that had lower levels of dTMP synthase activity (35-37) and when dTMP synthase was inhibited by pretreatment with fluoropyrimidines (12). However, it should be noted that the results of recent studies are at variance with the classical concept that MTX cytotoxicity is a direct consequence of simple depletion of 5,10-CH_2H_4PteGlu_u (38). Since 5,10-CH_2H_4PteGlu_u and other cellular reduced folate pools have been shown to be metabolically linked (38), it was possible that the proliferation-dependent cytotoxicity of MTX was more closely related to alterations in folate pools other than 5,10-CH_2H_4PteGlu_u. Thus, further studies are required to define the exact relationship among dTMP synthase activity, reduced folate pools, and MTX cytotoxicity.

Previous laboratory studies of the therapeutic effect of MTX against murine leukemia demonstrated important differences in the therapeutic outcome depending on the growth rate of the tumor (1). Intermittent administration of large doses of MTX was effective in early stage disease, while this same dose and schedule was ineffective for mice with advanced disease. The enhanced accumulation of MTX-Glu_u in early (i.e., exponentially growing) L5178Y cells relative to the normal marrow and gastrointestinal mucosa of the mouse reflected the observed selectivity of drug action with an improved therapeutic index.

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