Synthesis of Methotrexate Polyglutamates in L1210 Murine Leukemia Cells²

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SUMMARY

The ability of L1210 mouse leukemia cells and of a mutant methotrexate-resistant cell line (L1210/MTX) to synthesize methotrexate polyglutamates was studied. Host DBA/2 mice were treated with methotrexate, after which leukemic cells were harvested from ascitic fluid and levels of methotrexate and metabolites in them were determined by Sephadex G-15 gel chromatography. The level of methotrexate in L1210/MTX cells was 12.5 times greater than that in L1210 cells, reflecting the increased level of dihydrofolate reductase that characterizes this mutant cell line. Synthesis of methotrexate polyglutamates in each cell line required a dose of methotrexate (2.4 mg/kg) 10 times greater than the dose that yielded extensive methotrexate polyglutamate synthesis in rat liver and kidney in previous studies. Total methotrexate polyglutamates synthesized in 4 hr with this dose were the same in each cell line, demonstrating that this metabolism was not affected by differences in the level of dihydrofolate reductase. Methotrexate polyglutamates comprised 47 ±20% of the total methotrexate in L1210 cells. Methotrexate diglutamate was the predominant form. Levels of methotrexate monoglutamate and diglutamate were similar in L1210/MTX cells, whereas methotrexate monoglutamate was the predominant metabolite in host liver, kidney, and small intestine. These differences may reflect differences in substrate preference of pteroylpolyglutamate synthetase in these different tissues. Twenty-four hr after methotrexate administration, total methotrexate in L1210 cells was one-third of that at 4 hr; but the proportion of metabolites was the same, presumably reflecting cell death and division rather than loss of a freely exchangeable portion of intracellular methotrexate present at the earlier time. The affinity of methotrexate polyglutamates for dihydrofolate reductase was found to be similar to that of methotrexate, providing evidence that these metabolites may be as potent antagonists of folate metabolism as is methotrexate itself. Recent studies indicate that inhibition of folate metabolism in cells requires their exposure to high levels of methotrexate in order to achieve intracellular levels of methotrexate greater than needed to bind to dihydrofolate reductase. Such conditions conform to those required for synthesis of methotrexate polyglutamates. Thus, these metabolites may play a specific role in inhibiting folate metabolism, distinct from the antifolate potential that they appear to share with methotrexate.

INTRODUCTION

MTX,³ an analog of the vitamin folic acid (pteroylglutamic acid), is an important anticancer agent. It has proved to be particularly effective in the treatment of choriocarcinoma (18) and of acute lymphoblastic leukemia in children (11). It has also proved to be of benefit in the treatment of a wide variety of other malignant diseases. Recently, adjuvant therapy of osteogenic sarcoma in children using exceedingly large doses of MTX followed by citrovorum factor rescue has been rewarded with long-term remissions (14).

MTX is a potent inhibitor of the enzyme dihydrofolate reductase (EC 1.5.1.3) and thereby of folate metabolism. Classically, its cytotoxic action has been attributed to this inhibition (3). However, recent studies have identified a role for a component of intracellular MTX in excess of that portion which is tightly bound (presumably to dihydrofolate reductase) in contributing to the inhibition of folate metabolism (9, 12, 21). It has been suggested that binding of MTX to targets other than dihydrofolate reductase (9, 16) or the attainment of more complete inhibition of dihydrofolate reductase by this excess intracellular MTX (12) may be critical to the action of this agent and explain the added benefit seen clinically with high-dose MTX treatment (14).

Synthesis of poly-γ-glutamyl metabolites of MTX has been reported from a number of laboratories (2, 5, 10, 13, 19, 24, 25). Detailed study of this metabolism of MTX has been carried out in rat tissues (2, 24, 25). In this animal, dose-dependent synthesis of both MTX(+G₁) and MTX(+G₂) has been observed in liver and kidney (24, 25), whereas almost no such synthesis occurred in small intestine and thymus. In this report, these studies have been extended to the metabolism of MTX in L1210 murine leukemia cells, since these cells are sensitive to MTX (21) and have served often for the study of the activity of MTX. In addition, MTX metabolism was studied in a MTX-resistant mutant strain, L1210/MTX, characterized by a high level of dihydrofolate reductase (7), to see whether differences in its metabolism were present. A preliminary report of these studies has been presented (23).

¹ This work was supported by grants from the Medical Research Council of Canada and the Cancer Research Society.
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³ Received July 8, 1976; accepted November 1, 1976.
MATERIALS AND METHODS

Male adult DBA/2 mice bearing L1210 and L1210/MTX leukemias in ascitic form were obtained from Dr. I. Wodinsky, Arthur D. Little, Inc., Cambridge, Mass. They were replaced after 6 months. Leukemias were maintained by weekly transfer to DBA/2 mice (8). For study, mice were usually killed with ether on Day 5, and cells were harvested from the peritoneal cavity by rinsing it with 2 to 3 ml of sterile 0.9% NaCl solution. Cells from 2 to 4 mice were pooled. Grossly hemorrhagic specimens were discarded. Cells were washed 3 times with sterile 0.9% NaCl solution and finally suspended in 4 volumes of 100 mM sodium phosphate buffer, pH 7.0. Duplicate cell counts were made in a Coulter Counter, Model S (Coulter Electronics, Hialeah, Fla.) and were usually in the range of 1 to 4 x 10^8 cells/ml. Cells frequently clumped together during cell washing. This was not corrected by addition of heparin to cell suspensions. Therefore, cell counts may sometimes have been falsely low. Combined cytacnics and cell counts were carried out on suspensions of L1210 and L1210/MTX cells on 9 and 16 occasions, respectively. The mean cell volume of L1210 cells (553 ± 162 fl) did not differ significantly from that of L1210/MTX cells (737 ± 272 fl). The mean cell volume of L1210 and L1210/MTX together was 671 ± 254 fl. Final cell suspensions were heated in boiling water for 10 min and centrifuged, and the supernatant was stored at −20°. Preparation of extracts of host mouse tissues was carried out as previously described (24).

3',5',-9-[3H]MTX (Amersham/Searle Co., Don Mills, Ontario, Canada) was mixed with MTX (Lederle Products Dept., Cyanamid of Canada Ltd., Montreal, Quebec, Canada) to obtain a specific activity of 0.1 to 0.3 μCi/μg, and the mixture was purified by Sephadex G-15 gel chromatography (Pharmacia, Uppsala, Sweden) as previously described (24). [3H]MTX was usually administered to mice as a single i.p. injection 4 or 24 hr prior to harvesting of leukemic cells. MTX and metabolites in heated extracts of leukemic cells and of host mouse tissues were separated by chromatography on columns of Sephadex G-15 (0.9 x 55 cm), and were identified by cochromatography with authentic standards. MTX(+G_2), MTX(+G_3), and MTX(+G_4) were kindly supplied by Dr. C. M. Baugh and Dr. M. G. Nair of the Department of Biochemistry, University of South Alabama, Mobile, Ala. (17). Results were expressed as ng MTX or MTX equivalent per g tissue or 10^8 cells. Unlike rat tissues (24, 25), chromatograms of extracts of mouse liver, kidney, and small intestine contained a number of 3H-labeled materials in addition to but largely distinct from MTX and MTX polyglutamates. These materials were not present in the injected [3H]MTX. They were not identified. These contaminants accounted for as much as 20% of the 3H in some chromatograms, and they affected quantitation of MTX and MTX polyglutamates from time to time. Fortunately, they were never detected in chromatograms of L1210 and L1210/MTX cells (Chart 1).

The binding affinity of MTX polyglutamates for dihydrofolate reductase was compared to that of MTX using the single-step variation of a new ligand-binding radioassay for MTX (15). This assay used dihydrofolate reductase as binder, [3H]MTX as label, and Dextran T-10-coated charcoal (Pharmacia) to separate bound from unbound reactants. The ability of MTX polyglutamates to compete with [3H]MTX for binding to dihydrofolate reductase was measured by substituting them for MTX in standard curves performed by direct competitive binding (15). Studies were carried out on dihydrofolate reductase partially purified from guinea pig liver (4, 15) and on dihydrofolate reductase present in lysates of L1210 and L1210/MTX cells (1).

3H in samples was counted in an Isocap/300 Liquid Scintillation System (Nuclear-Chicago Corp., Des Plaines, Ill.) The chromatographic behavior of unlabeled standards was determined spectrophotometrically on a Beckman Model DK2 recording spectrophotometer. The inhibitory biological activity of MTX and metabolites was measured with the folate assay organism Lactobacillus casei (American Type Cell Culture 7469) (26).

RESULTS

Synthesis of MTX Polyglutamates in L1210 and L1210/MTX Cells and in Host DBA/2 Mouse Tissues. Sephadex G-15 gel chromatograms of heated extracts of L1210 and L1210/MTX cells revealed 2 3H-labeled fractions in addition to MTX (Chart 1). These fractions were inhibitory to growth of L. casei. They corresponded in volume of elution to metabolites of MTX previously characterized as MTX(+G_2) and MTX(+G_3) in rat liver and kidney (24, 25). The identity of these fractions as MTX(+G_2) and MTX(+G_3) was confirmed by cochromatography with authentic standards (17) (Chart 1).

Characteristic of MTX metabolism in L1210 cells was the large proportion of MTX polyglutamates and the predominance of MTX(+G_2) (Chart 1A). In 6 studies of pooled L1210 cells, MTX polyglutamates comprised 47 ± 20% of the total intracellular MTX, and the level of MTX(+G_2) was 4 times greater than that of MTX(+G_3) (p < 0.05) (Table 1). The level of MTX in L1210/MTX cells was 12.5 times greater than that in L1210 cells (p < 0.001) (Chart 1B; Table 1). The level of...
total MTX polyglutamates in L1210/MTX cells (278 ± 197 ng/10^6 cells), however, did not differ significantly from the total in L1210 cells (243 ± 161 ng/10^6 cells) (Table 1). Thus both MTX-sensitive and -resistant L1210 cells appeared to have a similar capacity to synthesize MTX polyglutamates, although in the latter case both MTX(+G1) and MTX(+G2) were present in roughly equal amounts. In host liver the level of MTX(+G2) was about 6 times that of MTX(+G1) (p < 0.001). Total MTX and metabolites in mouse kidney were about one-third of those in liver (p < 0.001). In mouse small intestine, despite a mean level of intracellular MTX comparable to that in liver (Table 1), total MTX polyglutamates constituted only 6 ± 3% of the total. Again, MTX(+G1) was the larger component (p < 0.01). Thus metabolism of MTX appeared to be distinctive in each of the 5 tissues studied (Table 1).

Synthesis of MTX polyglutamates in L1210 and L1210/MTX cells was dose dependent. The dose of MTX required to appear to be 10 times greater than that which resulted in extensive synthesis of MTX polyglutamates in rat liver and kidney (24, 25). Thus with divided s.c. doses of [3H]MTX totaling 0.16 and 0.24 mg/kg, almost no metabolism of MTX was noted, whereas a divided s.c. dose of 2.4 mg/kg yielded large quantities of both MTX(+G1) and MTX(+G2) (Table 2). These experiments served to define an appropriate dose of [3H]MTX for subsequent studies.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of studies</th>
<th>MTX</th>
<th>MTX(+G1)</th>
<th>MTX(+G2)</th>
<th>Total</th>
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<tr>
<td>L1210</td>
<td>6</td>
<td>281 ± 162</td>
<td>47 ± 22</td>
<td>196 ± 145</td>
<td>524 ± 237</td>
</tr>
<tr>
<td>L1210/MTX</td>
<td>7</td>
<td>3511 ± 1483</td>
<td>166 ± 97</td>
<td>112 ± 104</td>
<td>3789 ± 1554</td>
</tr>
<tr>
<td>Liver</td>
<td>6</td>
<td>1130 ± 270</td>
<td>370 ± 96</td>
<td>64 ± 48</td>
<td>1564 ± 312</td>
</tr>
<tr>
<td>Kidney</td>
<td>4</td>
<td>484 ± 108</td>
<td>67 ± 16</td>
<td>0^a</td>
<td>537 ± 125</td>
</tr>
<tr>
<td>Small intestine</td>
<td>6</td>
<td>1131 ± 710</td>
<td>35 ± 13</td>
<td>18 ± 8</td>
<td>1184 ± 706</td>
</tr>
</tbody>
</table>

^a MTX(+G2) was absent from 3 pooled kidney extracts and present (22 ng/g) in 1.

Effects of dose of MTX on synthesis of MTX polyglutamates in L1210

The dose of [3H]MTX was divided and administered as 6 or 9 injections at 15-min intervals. Four hr after the first injection, cells from 2 to 4 mice were harvested and pooled and extracts were prepared (see "Materials and Methods"). Results are expressed as ng MTX or MTX equivalent per 10^8 cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Dose of [3H]MTX (mg/kg)</th>
<th>MTX (-G2)</th>
<th>MTX (+G1)</th>
<th>MTX (+G2)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>0.16</td>
<td>93</td>
<td>5</td>
<td>3</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td>277</td>
<td>12</td>
<td>8</td>
<td>297</td>
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<tr>
<td></td>
<td>2.40</td>
<td>282</td>
<td>68</td>
<td>290</td>
<td>640</td>
</tr>
<tr>
<td>L1210/MTX</td>
<td>0.16</td>
<td>723</td>
<td>4</td>
<td>1</td>
<td>728</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td>4632</td>
<td>8</td>
<td>7</td>
<td>4647</td>
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<tr>
<td></td>
<td>2.40</td>
<td>1754</td>
<td>215</td>
<td>366</td>
<td>2335</td>
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</table>

Comparison of the Affinity of MTX and MTX Polyglutamates for Dihydrofolate Reductase. The powerful antifolate activity of MTX has been equated with its exceptionally high affinity for the enzyme dihydrofolate reductase (3). To obtain information regarding the relative potency of MTX polyglutamates as folate antagonists, their binding affinity for dihydrofolate reductase was compared to that of MTX. Results obtained with dihydrofolate reductase contained in lysates of L1210 and L1210/MTX cells are shown in Chart 2. They have been expressed as 1/3 fraction of [3H]MTX bound, related to the quantity of unlabelled MTX or MTX polyglutamates present. With lysates from L1210 cells, 50% reduction in binding of [3H]MTX was obtained with 1.1 pmoles MTX and 1.2 pmoles MTX(+G2) [ratio of 2 (Chart 2A)]. With lysate from L1210/MTX cells, 50% reduction in binding of [3H]MTX was obtained with 1.0 pmoles MTX, 1.4 pmoles MTX(+G1), 1.6 pmoles MTX(+G2), and 1.0 pmoles MTX(+G3). These results showed that the affinities of MTX polyglutamates for dihydrofolate reductase were similar if not identical to that of MTX. Similar results were obtained using dihydrofolate reductase partially purified from guinea pig liver (not shown).
Table 3
Levels of MTX and metabolites in L1210 and L1210/MTX cells 4 and 24 hr after a single i.p. injection of [3H]MTX (2.4 mg/kg) into host DBA/2 mice

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of studies</th>
<th>Time (hr)</th>
<th>Levels of MTX and metabolites in cells (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>3</td>
<td>4</td>
<td>387 ± 143, 66 ± 6, 275 ± 169, 727 ± 81</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>24</td>
<td>149 ± 28, 20 ± 8, 77 ± 31, 246 ± 66</td>
</tr>
<tr>
<td>L1210/MTX</td>
<td>2</td>
<td>4</td>
<td>3602 ± 765, 277 ± 29, 248 ± 50, 4127 ± 844</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24</td>
<td>2199 ± 4, 154 ± 36, 211 ± 52, 2563 ± 84</td>
</tr>
</tbody>
</table>

Chart 2. Comparison of the affinity of MTX and MTX polyglutamates for dihydrofolate reductase present in lysates of (A) L1210 cells and (B) L1210/MTX cells, using a radiochemical ligand-binding assay for MTX (1, 15). Incubation mixtures consisted of 0.9 ml of 0.01 M potassium phosphate, 0.15 M KCl buffer (pH 6.0) containing 1 mg albumin and 0.1 μmole NADPH per ml, 0.1 ml MTX or MTX polyglutamate solution containing 2.2 to 90.0 pmoles/ml, and 0.1 ml of [3H]MTX containing 15 pmoles/ml. Incubation was started with the addition of 0.1 ml binder (lysate) preparation (binding capacity, 1 to 1.2 pmoles MTX). After 10 min of incubation at 4°C, the reaction was stopped by the addition of 1.0 ml of a suspension of 2.5% charcoal and 0.5% Dextran T-10. Standard curves prepared with MTX (—, ●), with MTX(+G1) ( - - - - ), with MTX(+G2) (---), and with MTX(+G3) (-----, ○) are shown.

These studies were carried out at pH 6.0. Essentially identical results were obtained when the affinities of MTX and MTX(+G) for dihydrofolate reductase from L1210/MTX and guinea pig liver were compared at pH 7.0 and 8.0.

DISCUSSION

MTX polyglutamates were previously identified in rat tissues (2, 24, 25). In these studies, MTX(+G1) and MTX(+G2) were recognized as 3H-labeled materials that produced folate-reversible inhibition of growth of the folate assay organisms L. casei and Streptococcus fecalis. They were identified as polyglutamates by the demonstration that they chromatographed as MTX after incubation with a source of pteroylpolyglutamate hydrolase (γ-glutamyl carboxypeptidase, folate conjugase) (2, 24, 25). Their identities were established by cochromatography using authentic standards of MTX(+G1) and of MTX(+G2) (17). The demonstration of synthesis of MTX(+G1) and of MTX(+G2) in both L1210 and L1210/MTX cells (Chart 1) extends the number of tissues in which such metabolism of MTX has been found to occur (2, 5, 10, 13, 19, 24, 25). Not surprisingly, synthesis of MTX polyglutamates was also found in host mouse tissues (Table 1), confirming previous observations (22).

The present results were not entirely anticipated. Because rat small intestine did not synthesize MTX polyglutamates (24, 25) and small intestine is exquisitely sensitive to MTX toxicity, it appeared likely that L1210 cells, themselves sensitive to MTX toxicity, might lack this metabolic pathway as well. Indeed, almost no metabolism of MTX occurred in either L1210 or L1210/MTX cells with doses previously used in the rat (Table 2). However, this may have been due in part to failure of MTX to accumulate in sufficient amount in ascitic fluid after s.c. injection (20). When a dose of MTX (2.4 mg/kg) was used that was comparable to the dose recommended for the reduction of the L1210 leukemia cell mass (3.0 mg/kg host weight) (8), extensive metabolism of MTX was indeed seen, both with divided s.c. administration and with single i.p. doses. Synthesis of MTX polyglutamates also occurred in mouse small intestine with these doses. Thus, sensitivity to MTX toxicity did not equate with failure to synthesize MTX polyglutamates. Nor could resistance to MTX toxicity, which characterizes the L1210/MTX mutant cell line, be attributed to differences in MTX polyglutamate synthesis, since the total quantity of MTX polyglutamates was the same in both cell lines.

MTX(+G2) was the predominant metabolite in L1210 cells. However, MTX polyglutamates larger than MTX(+G2), e.g., MTX(+G3), did not separate well from it under the conditions of chromatography used in the present study. These require a large column to be visualized (unpublished observation). Therefore, it is possible that L1210 cells synthesized some MTX polyglutamates containing more than 2 additional γ-glutamyl residues (Chart 1A). In contrast, the major metabolite of MTX in other tissues studied to date has been MTX(+G). Each of the tissues studied here showed a distinctive pattern of distribution of MTX and metabolites. It may be speculated that the predominance of MTX(+G) in L1210 cells reflects a difference in the enzyme pteroylpolyglutamate synthetase in this cell with regard to substrate preference. Further, it is possible that differences in metabolism of MTX in different tissues may reflect differences in pteroylpolyglutamate synthesis in these tissues as well. Levels of total MTX polyglutamates in L1210 and L1210/MTX cells were the same despite the high level of MTX in the latter, reflecting no doubt the high level of dihydrofolate reductase that characterizes this mutant (7). It was concluded that synthesis of MTX polyglutamates was not noticeably affected by this large difference in level of dihydrofolate reductase.

The concept of "free" intracellular MTX, or of levels of MTX in cells in excess of that required to bind to dihydrofolate reductase (9, 12, 21), has taken on importance as a result of new therapeutic initiatives, with the use of high-dose MTX (14), and following a number of studies which have credited this intracellular portion of MTX with a critical role in the inhibition of folate metabolism (9, 12, 16, 21).
Sirotnak and Donsbach (20) reported levels of MTX in L1210 cells 4 hr after an i.p. dose of MTX (3.0 mg/kg) to be greater than the level of dihydrofolate reductase. This level had fallen by 24 hr, and the fall was attributed to the loss of freely exchangeable MTX present within these cells at the earlier time. If this were so, one would have expected a disproportionate fall in level of MTX compared to MTX polyglutamates in L1210 cells at 24 hr in this study. In fact, the proportion of MTX and metabolites was the same at both times. These results do not define a so-called "labile" portion of MTX at 4 hr, nor do they explain the subsequent fall. It is possible but unlikely that MTX and metabolites were released from living L1210 cells at the same rate. However, this was not seen with rat liver (24, 25). It is more likely that the fall in level of MTX and metabolites reflected the combined effect of cell death and of division and growth of the surviving population.

Jacobs et al. (13) studied the ability of MTX(+G) to inhibit the activity of dihydrofolate reductase purified from L1210 cells. They found it to be as potent as MTX. In fact, it appeared to be a more potent inhibitor than MTX when studied at pH 8.5. Further, they showed that growth of L1210 cells in culture was inhibited equally by MTX and MTX(+G), and that MTX(+G) had antitumor activity similar to that of MTX in vivo in prolonging the life-span of BALB/c x DBA/2 F1 mice bearing the L1210 leukemia. The demonstration in this study that MTX polyglutamates bound as tightly to dihydrofolic reductase as did MTX (Chart 2) provides further evidence that these metabolites are as potent antifolates as is MTX and that decreased membrane transport of MTX polyglutamates appears to be the major reason for their lesser toxicity in whole-cell systems such as S. fecalis (17). These results also demonstrate the insensitivity of the MTX binding site on dihydrofolate reductase regarding the number of glutamyl residues present. A similar observation has been made regarding this enzyme and poly-γ-glutamyl derivatives of dihydrofolate (6). Lastly, this study showed that measurement by ligand-binding radioassay (15) of total tissue MTX and metabolites can be carried out with a single standard curve and without the need to isolate constituents.

It has been speculated that binding of MTX to intracellular sites of lower binding affinity than that for dihydrofolate reductase is required for inhibition of folate metabolism (9). Suggested targets have been thymidylate synthetase (EC 2.1.1.1.b), and the folate-dependent enzymes involved in purine synthesis. Present results would suggest that MTX polyglutamates comprise part of this component of intracellular MTX.

**ACKNOWLEDGMENTS**

The author thanks Marlene Dupont, Maureen McCormack, and Viviane Nicolet for technical assistance; Charles Baugh and M. G. Nair for the kind provision of MTX polyglutamates; and Bart Kamen for his advice with the radioassay.

**REFERENCES**

17. Nair, M. G., and Baugh, C. M. Synthesis and Biological Evaluation of Poly-γ-glutamyl Derivatives of Dihydrofolic Acid (6). Lastly, this study showed that measurement by ligand-binding radioassay (15) of total tissue MTX and metabolites can be carried out with a single standard curve and without the need to isolate constituents.

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