Differential Synthesis of Methotrexate Polyglutamates in Normal Proliferative and Neoplastic Mouse Tissues in Vivo

Richard G. Poser, Francis M. Sirotnak, and Paul L. Chello

Laboratory for Molecular Therapeutics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

Synthesis of poly-γ-glutamyl metabolites of methotrexate was demonstrated in mouse small intestine, liver, and bone marrow and in L1210 leukemia, Sarcoma 180, and Ehrlich tumor cells after s.c. injections of [3H]methotrexate to tumor-bearing mice. Ion-exchange chromatography of tissue extracts resolved six peaks of radioactivity believed to represent methotrexate and metabolites with up to five additional glutamyl residues. Polyglutamate formation in L1210 cells and small intestine was shown to be independent of dose at least to 400 mg/kg as long as intracellular levels of drug in excess of the dihydrofolate reductase-binding capacity (exchangeable) were maintained. Both the total amount of polyglutamates and the average length of the polyglutamyl chain increased with time as long as exchangeable level of drug was present intracellularly. The results also showed differences in the extent of metabolism of methotrexate polyglutamates among the tissues examined. Although these differences were at times very large, there was no consistent correlation between these differences and other pharmacological parameters or cytotoxicity. Tumor cells appeared to synthesize more polyglutamates than did the normal tissues examined. However, differences in total drug persistence and sensitivity to drug among tumor cells and among normal tissues did not reflect the relative extent of polyglutamate synthesis in each group. It is concluded that the extent of polyglutamate synthesis per se may not be a determinant of drug sensitivity in murine tissues. However, the accumulation of these metabolites may contribute in some way to overall therapeutic response or relative cytotoxicity.

INTRODUCTION

The biosynthesis of poly-γ-glutamyl metabolites of methotrexate has been demonstrated in a variety of normal (1, 7, 25, 26) and neoplastic (5, 17, 26) mammalian tissues. The addition of one or more glutamyl residues in peptide linkage at the γ position of methotrexate apparently causes little, if any, change in the binding of the drug to dihydrofolate reductase (5, 9, 10, 19, 26) which is necessary for its cytotoxic effect (24). Intra- and extracellular polyglutamyl derivatives should therefore be as potent as methotrexate itself in producing inhibition of folate metabolism. Methotrexate polyglutamates, however, are transported into L1210 cells (19) and microbial cells (11) at substantially lower rates than is methotrexate. The reduction in this flux tends to explain the comparative inability of these polyglutamates to inhibit growth of these cell types in culture (11, 19). Whether conjugation at the γ-carboxyl position of methotrexate produces any decrease in the efflux of this drug is less certain. Measurements of the changes in the intracellular levels with time during efflux from L1210 cells have been reported from our laboratory (19), which indicated that at least the diglutamate form is transported out of these cells as rapidly as is methotrexate. Extracellular recovery of methotrexate and methotrexate polyglutamates, which was suggestive of the same relative efflux in similar experiments with L1210 cells, has also been reported (13) from our laboratory. Other investigations have also postulated that this intracellular metabolism may produce a "retentive" form of methotrexate (5–7). Since the formation of methotrexate polyglutamates occurs to varying relative degrees in several tissues (25, 26), it has been proposed that this might account for the differences in methotrexate sensitivity observed among various normal proliferative and neoplastic tissues. If methotrexate polyglutamates do in fact efflux more slowly than does methotrexate, then increased persistence of total drug might be expected in those tissues which demonstrate a higher capacity for polyglutamate formation. Greater persistence of methotrexate at pharmacologically effective levels in responsive tumors cells versus normal proliferative tissues has been associated (reviewed in Ref. 18) with relative cytotoxicity among cell types (23) and the selective antitumor action (21) of this agent in murine tumor models. Although these pharmacokinetic differences appear to be explained by tissue-specific differences in carrier-mediated influx of methotrexate (reviewed in Ref. 18), a role for polyglutamate formation has not been excluded.

In the present studies, we have examined the formation of methotrexate polyglutamates in vivo in murine tumors and in mouse small intestine, liver, and bone marrow at various times after mice were given individual s.c. doses of [3H]methotrexate. Analysis of tissue extracts by ion exchange chromatography resulted in quantitative resolution of radioactive peaks for intracellular methotrexate and metabolites with up to 5 additional glutamyl residues. Our results indicate that there are significant differences in the extent of metabolism of methotrexate to polyglutamyl derivatives among the different tissues examined. However, no general correlation between these differences and relative drug persistence or sensitivity associated with these tissues could be demonstrated.

MATERIALS AND METHODS

General. Used in these studies were female C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) mice weighing 20 ± 0.5 g (S.D.) obtained from ARS/Sprague-Dawley, Madison, Wis. The method of transplantation of the ascites forms of the L1210, Sarcoma 180, and Ehrlich tumors has been described (9). Samples of [3',5',9-3H]methotrexate (specific activity, 44 to 66 μCi/μg; Moravek Biochemicals, City of

Received February 2, 1981; accepted August 11, 1981.

1 This work was supported in part by Grants CA-08748, CA-22764, and CA-09207 from the National Cancer Institute and CH-26 from The American Cancer Society. Presented in part at the 71st Annual Meeting of the American Association for Cancer Research, Inc., San Diego, Calif., May 1980 (14).

2 To whom requests for reprints should be addressed.

NOVEMBER 1981

4441
Industry, Calif.) were purified by paper chromatography (20). Radiochemical purity of the final preparation was >98%. Purified nonradioactive methotrexate was supplied by the Drug Synthesis Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., and was used to dilute the radiolabeled drug to a specific activity of between 3 and 0.01 μCi/μg (depending on dose administered to mice).

Recovery of Methotrexate Polyglutamates from Tissues of Tumor-bearing Mice. After s.c. administration of drug to 4 to 6 tumor-bearing animals, mice were sacrificed by cervical dislocation, and tumor cells were harvested from the periportal cavity by lavage. The abdominal wall of the animal was exposed, and 2 ml of a cold (0°C) solution of 0.14 M NaCl:0.02 M potassium phosphate (pH 7.4) were injected i.p. The cavity was opened and drained, and the organs were rinsed with the same solution. Viability of surviving tumor cell populations harvested at varying times after treatment was 90 to 95% as determined by vital staining (18) with trypan blue. The harvested ascitic cells were washed by centrifugation with the same solution (0°C) and resuspended and held in 0.175 M NH₄Cl for 5 min at 0°C to lyse mature RBC. After an additional wash in cold (0°C) buffered NaCl solution, the cells were again resuspended in 2 ml of this solution, and cell density was determined by absorbance at 600 nm, A₆₀₀_of 3.0 = 2.6 ± 0.3 × 10⁶ cells/ml = 0.0219 ± 0.0015 g, wet weight (2, 18). The intracellular drug was then extracted by heating the samples for 15 min at 100°C, which also inactivates folate conjugase (carboxypeptidase) and polyglutamyl synthetase (26). The boiled samples were centrifuged to remove cellular debris, and the cleared extracts were stored at −20°C until analyzed. After removal of tumor cells, the small intestines were surgically excised and placed in a large volume of cold (0°C) phosphate:0.14M NaCl solution. The organ was opened longitudinally and cleaned, and it then was held in the cold buffer for precisely 5 min to allow maximal drug loss from interstitial space and blood vessels (18). After blotting to remove external liquid, the organ was weighed, homogenized, and heated to extract drug as described previously (12).

Recovery of Methotrexate Polyglutamates from Tissues in Normal Mice. The formation of methotrexate polyglutamates in mouse bone marrow, liver, and small intestine was determined in normal mice to avoid the complication of tumor infiltration of these tissues (18). Because of the small amount of marrow which can be recovered from a mouse, a 400-mg/kg dose of high-specific-activity [³H]methotrexate was given to 18 mice, and the tissues were harvested at 3 hr. Small intestine and liver samples were taken from 3 mice and processed in the manner described above. Marrow was collected by aspiration from the long bones (i.e., femur, tibia, and humerus) after surgical removal from the animals. Cold (0°C) phosphate:0.14M NaCl solution was forced through one end of the bone with the aid of a syringe, and the cell suspension extruded from the opposite end into a chilled 30-ml test tube. Marrow from the 18 mice was pooled and centrifuged, and mature RBC were lysed by resuspending the marrow in cold 0.17 M NH₄Cl. The remaining cells were resuspended in 2 ml phosphate NaCl:0.14M solution. The small intestine, liver, and marrow samples were processed in preparation for column chromatography as described above.

Chromatographic Analysis. The cleared tissue extracts were analyzed by anion exchange chromatography, using techniques adapted from the separation of natural folate polyglutamates (3). Chromatographic material, columns, and gradient apparatus were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Aliquots (1.0 to 3.0 ml) were brought to an ion strength of 0.3 M NaCl with 1.0 M NaCl and applied to 0.9- × 30-cm DEAE-Sephadex A-25 columns along with authentic methotrexate and either methotrexate diglutamate, methotrexate tetratglutamate (supplied by D. G. Montgomery, Southern Research Institute, Birmingham, Ala.), or methotrexate triglutamate (supplied by Dr. M. G. Nair, University of Alabama, Birmingham, Ala.) included as optical markers. Following an initial wash of 20 ml of 0.3 M NaCl in phosphate buffer (0.05 M potassium phosphate, pH 7.0), the chromatographic columns were developed with a linear gradient of 0.3 to 0.6 M NaCl in phosphate buffer, collecting 2.0-ml fractions of a total elution volume of 250 ml at a flow rate of 8 to 10 ml/hr. This was followed by a wash of 25 ml of 1.0 M NaCl in phosphate buffer. UV absorption of each fraction at 300 nm was measured to monitor the elution positions of the authentic standards. The radioactivity of 1.0 ml of each fraction was assayed in 10 ml of Scintisol (Isolab, Akron, Ohio) by scintillation spectrophotometry.

Identification of Polyglutamate Fractions. To verify the identity of radioactive species eluted during chromatography (which were believed to be methotrexate polyglutamates), the peak fractions were assayed following 1:1,000 × 1:1,000 dilution in 0.05 M potassium phosphate (pH 7.3) for their ability to inhibit dihydrofolate reductase by methods described previously (22). Lability to folate carboxypeptidase was determined by diluting a portion of boiled extract (obtained from L1210 cells harvested 16 hr after tumor-bearing mice received 12 mg [³H]methotrexate per kg) with H₂O to 0.14 M with respect to NaCl and adding an equal volume of fetal calf serum (Microbiological Associates, Walkersville, Md.) with folylcarboxypeptidase activity (19) at 37°C for 7 hr at pH 7. This was then processed and analyzed by column chromatography as before, and the results were compared with data obtained from the reserved portion not treated with fetal calf serum.

Analysis of Drug Bound to Intracellular Dihydrofolate Reductase. At 16 and 24 hr after [³H]methotrexate (12 mg/kg) was administered s.c. to L1210-bearing mice, the ascitic cells were harvested, washed in cold (0°C) buffered NaCl solution, and resuspended in 0.05 M potassium phosphate at pH 6.2. Absorbance at 600 nm was measured to determine cell number, and the samples were sonicated at 0°C for 30 sec (Model W200R sonicator cell disruptor; Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). After centrifugation, the supernatants were dialyzed against 0.05 M potassium phosphate buffer (pH 7.0) for 48 hr. The content of the dialysis tubing was processed and analyzed for methotrexate polyglutamates by column chromatography as described above. Supernatants were also chromatographed (22) on Sephadex G-25, and the contents of the void volume were analyzed by DEAE chromatography. The loss of polyglutamates, which were not enzyme bound, from inside to dialysate bag was shown by dialyzing the tetraglutamate derivative (methotrexate + G₄) and cell extracts containing polyglutamates in excess of the dihydrofolate reductase binding capacity.

RESULTS

Formation of Polyglutamates. Similar to that shown in our earlier (13) report, a high degree of resolution was obtained using ion exchange chromatography for analysis of cellular extracts containing radiolabeled methotrexate and its polyglutamate metabolites. From extracts of L1210 cells and small intestine harvested after leukemic mice were given [³H]methotrexate (12 mg/kg s.c.), the longer chain polyglutamates with up to 5 additional glutamyl residues appeared to be separated. Authentic standards have been obtained only for methotrexate and its metabolites with one (methotrexate diglutamate), 2 (methotrexate triglutamate), and 3 (methotrexate tetratglutamate) additional γ-glutamyl residues. All radioactive peaks, however, were found to be eluted at equidistant-ionic-concentration, titration inhibitors of dihydrofolate reductase and carboxypeptidase labile, i.e., converted to methotrexate after treatment with fetal calf serum, a source (2) of carboxypeptidase. Table 1 shows the percentage of methotrexate given to tumor-bearing mice. This was determined by integrating the area under each peak of the elution profiles as shown earlier (13).

From these data, it can be seen that both the total amount of polyglutamates and the peptide chain length of each form

Downloaded from cancerres.aacrjournals.org on April 12, 2017. © 1981 American Association for Cancer Research.
The major period of polyglutamate synthesis in both tissues occurred at different rates in each tissue, the data show that are shown in Chart 1. Although total polyglutamate formation drug and polyglutamates in both L1210 cells and small intestine course for accumulation and persistence of total intracellular trexate in vitro. The pharmacokinetic data depicting the time total amount of polyglutamates recovered in small intestine was drug well in excess of the dihydrofolate reductase-binding coincides with the presence of intracellular concentrations of drug well in excess of the dihydrofolate reductase-binding capacity. In both tissues, after the peak period of synthesis was reached, a reduction in total polyglutamates occurred which paralleled the reduction in total intracellular drug. Negligible reduction of total polyglutamates occurred from 16 to 24 hr following this dose in either tissue, when essentially all remaining intracellular drug appeared to be bound to dihydrofolate reductase. Dialysis of the L1210 cell extracts obtained 16 or 24 hr after [3H]methotrexate was administered resulted in no change in either the distribution profiles or total amounts of methotrexate and each metabolite. A similar result was obtained with material excluded on Sephadex G-25. In agreement with earlier (5, 16) reports, these results imply that drug bound to dihydrofolate reductase is not a substrate for polyglutamyl synthetase. This is substantiated for this enzyme by the data in Table 2, which shows the lack of significant polyglutamate formation in either L1210 cells or small intestine following administration of [3H]methotrexate (0.6 mg/kg) to leukemic mice. This low dose produces almost no free (i.e., not reductase-bound) intracellular drug in L1210 cells. A similar result (data not shown) was found in the case of small intestine. When the dosage of methotrexate administered is sufficiently high to maintain intracellular levels of methotrexate in excess of the dihydrofolate reductase-binding capacity for a significant period of time, the synthesis of polyglutamates appears to be independent of dose. As seen in Table 3, the percentage of methotrexate and each polyglutamate metabolite was the same in L1210 cells harvested 4 hr after tumor-bearing mice were given 3, 12, 24, 48, or 400 ng [3H]methotrexate per kg. The same was true for small intestine at the 4 higher dosages. We compared the extent of polyglutamate formation in 2 other recovered increased with time. A similar result was obtained (data not shown) in the case of small intestine. However, the total amount of polyglutamates recovered in small intestine was substantially lower. Similar results were obtained (15, 16) in human fibroblasts during prolonged exposure to [3H]methotrexate in vitro. The pharmacokinetic data depicting the time course for accumulation and persistence of total intracellular drug and polyglutamates in both L1210 cells and small intestine are shown in Chart 1. Although total polyglutamate formation occurred at different rates in each tissue, the data show that the major period of polyglutamate synthesis in both tissues coincides with the presence of intracellular concentrations of drug well in excess of the dihydrofolate reductase-binding

---

**Table 1**

<table>
<thead>
<tr>
<th>Interval (hr)</th>
<th>Total intracellular drug (mg/g)</th>
<th>MTX&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MTX + G&lt;sub&gt;i&lt;/sub&gt;</th>
<th>MTX + G&lt;sub&gt;2&lt;/sub&gt;</th>
<th>MTX + G&lt;sub&gt;3&lt;/sub&gt;</th>
<th>MTX + G&lt;sub&gt;4&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.10 ± 0.16&lt;sup&gt;d&lt;/sup&gt;</td>
<td>88.3 ± 13</td>
<td>85.6 ± 0.6</td>
<td>4.9 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>1.04 ± 0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>77.1 ± 8</td>
<td>6.8 ± 0.6</td>
<td>19.5 ± 3.4</td>
<td>2.6 ± 0.3</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>0.60 ± 0.17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.3 ± 7</td>
<td>8.3 ± 0.3</td>
<td>34.6 ± 4.2</td>
<td>1.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>0.54 ± 0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17.0 ± 2</td>
<td>4.8 ± 0.2</td>
<td>40.1 ± 5.6</td>
<td>3.4 ± 0.5</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>14</td>
<td>0.29 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>15.0 ± 1</td>
<td>9.7 ± 1.2</td>
<td>44.2 ± 6.5</td>
<td>25.3 ± 2.2</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>24</td>
<td>0.25 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>15.0 ± 1</td>
<td>7.5 ± 1.8</td>
<td>35.0 ± 2.4</td>
<td>29.3 ± 4.6</td>
<td>7.4 ± 1.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total methotrexate equivalents.
<sup>b</sup> Average ± S.E.
<sup>c</sup> MTX, methotrexate; G<sub>i</sub>, number of additional glutamyl residues attached to methotrexate, i.e., MTX + G<sub>i</sub> is methotrexate diglutamate.
<sup>d</sup> Average ± S.E.

---

**Table 2**

<table>
<thead>
<tr>
<th>Interval (hr)</th>
<th>Total intracellular drug (mg/g)</th>
<th>MTX&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MTX + G&lt;sub&gt;i&lt;/sub&gt;</th>
<th>MTX + G&lt;sub&gt;2&lt;/sub&gt;</th>
<th>MTX + G&lt;sub&gt;3&lt;/sub&gt;</th>
<th>MTX + G&lt;sub&gt;4&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.653</td>
<td>91.80</td>
<td>5.21</td>
<td>2.72</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.204</td>
<td>89.41</td>
<td>4.12</td>
<td>5.33</td>
<td>5.85</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.200</td>
<td>90.52</td>
<td>3.85</td>
<td>5.85</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Total methotrexate equivalents.
<sup>b</sup> MTX, methotrexate; G<sub>i</sub>, number of additional glutamyl residues attached to methotrexate, i.e., MTX + G<sub>i</sub> is methotrexate diglutamate.
Table 3
Formation of methotrexate polyglutamates in L1210 cells and small intestine 4 hr after administration of different doses of methotrexate to tumor-bearing mice

Cleared extracts of L1210 cells and small intestine harvested 4 hr after tumor-bearing mice received \([^{3}H]\)methotrexate s.c. at the indicated doses were analyzed by ion-exchange chromatography. Pooled tissue from 2 or 3 mice was used to prepare each extract. See legend of Table 1 for method of calculation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Dose (mg/kg)</th>
<th>Total intracellular drug (µg/g)</th>
<th>% of total drug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTX</td>
<td>MTX + G₁</td>
<td>MTX + G₂</td>
</tr>
<tr>
<td>L1210 cells</td>
<td>0.3</td>
<td>0.393</td>
<td>94.10</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.488</td>
<td>74.40</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.524</td>
<td>48.62</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.804</td>
<td>46.35</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.724</td>
<td>42.72</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2.184</td>
<td>44.82</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>8.830</td>
<td>47.83</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.3</td>
<td>0.120</td>
<td>95.30</td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td>0.112</td>
<td>91.81</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.416</td>
<td>93.73</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.578</td>
<td>85.16</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.935</td>
<td>87.23</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.759</td>
<td>87.56</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>3.048</td>
<td>88.73</td>
</tr>
</tbody>
</table>

*a* Total methotrexate equivalents.

*b* MTX, methotrexate; G₁, number of glutamyl residues attached to methotrexate, i.e., MTX + G₁, is methotrexate diglutamate.

Table 4
Formation of methotrexate polyglutamates in normal mouse tissue 3 hr after administration of methotrexate (400 mg/kg)

Cleared extracts of normal proliferative mouse tissues harvested 3 hr after mice received \([^{3}H]\)methotrexate (400 mg/kg s.c.) were analyzed by ion-exchange chromatography. The marrow from 18 mice and other tissues from 3 mice were pooled to prepare each extract. See legend of Table 1 for method of calculation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total intracellular drug (µg/g)</th>
<th>% of total drug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTX*a</td>
<td>MTX + G₁</td>
</tr>
<tr>
<td>Small intestine</td>
<td>58.391</td>
<td>93.43</td>
</tr>
<tr>
<td>Liver</td>
<td>137.289</td>
<td>92.40</td>
</tr>
<tr>
<td>Marrow</td>
<td>0.634</td>
<td>80.67</td>
</tr>
</tbody>
</table>

*a* Total methotrexate equivalents.

*b* MTX, methotrexate; G₃, number of glutamyl residues attached to methotrexate, i.e., MTX + G₃, is methotrexate triglutamate.

DISCUSSION

The present studies demonstrate significant differences in the rate and extent of formation of polyglutamyl derivatives of methotrexate by several murine tissues. Polyglutamate formation in mouse small intestine, Sarcoma 180, and L1210 cells was in the same ascending order as the degree of persistence of total drug in these different tissues as well as their relative sensitivity to the cytotoxic action of methotrexate (18). This correlation, however, did not hold true in the case of Ehrlich carcinoma, which exhibited (18) little persistence and a low level of methotrexate sensitivity similar to Sarcoma 180 cells, despite a comparatively high level of polyglutamate formation found to be more like L1210 cells. Similarly, although polyglutamates were formed somewhat more readily in normal mouse marrow than in small intestine and liver, this result does not correlate with the markedly greater persistence and sensitivity of murine tumors (Sarcoma 180 and Ehrlich carcinoma) to that observed in L1210 cells and small intestine at 4 hr after tumor-bearing mice received 48 mg \([^{3}H]\)methotrexate per kg s.c. The results are presented in Chart 2. They show that, after equal dosage and exposure time, metabolism of methotrexate to polyglutamates occurred to a different extent in each tissue. The percentage of total intracellular drug in the form of methotrexate polyglutamates was lowest in small intestine (12%), higher in Sarcoma 180 cells (22%), and highest in Ehrlich carcinoma (44%) and L1210 cells (55%). Polyglutamate formation in tissues from normal mice is shown in Table 4. The data show that marrow metabolizes methotrexate 2- to 3-fold faster than either small intestine or liver. Since marrow metabolized 20% of total intracellular drug to polyglutamate forms by 3 hr (in contrast to 7 to 8% for liver and small intestine), while L1210 cells converted 30% by 2 hr (Table 1), we may infer that the ability of marrow to form polyglutamates is intermediate between L1210 cells and small intestine or liver.

44444
CANCER RESEARCH VOL. 41

---

R. G. Poser et al.
Synthesis of Methotrexate Polyglutamates

NOVEMBER 1981

4445

to methotrexate exhibited by small intestine versus marrow (23). Viewed in the light of results showing (13) the equivalent efflux of methotrexate and methotrexate polyglutamates from L1210 cells, we conclude from these data that this form of metabolism per se may not contribute to drug sensitivity by increasing persistence of total drug in each tissue.

Since methotrexate or its metabolites are apparently not suitable substrates for polyglutamyl synthetase when bound to dihydrofolate reductase, the synthesis of polyglutamates occurs only while there is freely exchangeable intracellular drug (5, 6, 15, 16). The duration of this critical period is dictated by the pharmacokinetics for methotrexate in each tissue, which in turn appears to be a function of transport characteristics unique in each cell type (18). This is illustrated in Chart 1. However, the data also suggest that specific differences among mouse tissues in the absolute rate of polyglutamate synthesis must also exist. Although the level of intracellular exchangeable drug in small intestine is close to 3 times that found in L1210 cells for the first 2 hr after methotrexate was administered, the tumor cells synthesize over twice as much methotrexate polyglutamates as the normal proliferative tissue during this initial period. The data on total drug levels in small intestine and L1210 cells at the various times shown in Chart 1 are in close agreement with those reported previously from this laboratory (18, 21), although different from results of an earlier study by Whitehead (26) which showed much greater total drug in small intestine. While our data on the fraction of methotrexate metabolized to polyglutamate forms in L1210 cells and small intestine at 4 hr are in agreement with those reported by this investigator for these tissues, the higher total drug levels found in small intestine in this earlier study may reflect differences in the manner in which this tissue was prepared for analysis. For instance, no indication was given in this earlier study that the drug present in interstitial space or blood vessels was removed from the tissue prior to analysis.

The results presented here also strongly suggest that polyglutamyl forms of methotrexate are not inherently retentive in L1210 cells. There was no selective retention of polyglutamates as compared to methotrexate following their synthesis in vivo (Chart 1). This is in agreement with our prior (13) studies measuring efflux in vitro. Although during a 20-min in vitro incubation the total intracellular drug level fell significantly, the ratio of methotrexate to each polyglutamate remained constant, suggesting equivalent efflux of each form. In this earlier (13) study, we were unable to detect significant quantities of extracellular polyglutamates without the prior addition of purified dihydrofolate reductase to the cell suspension which appeared to prevent their extracellular hydrolysis.

The observation, that the transport of methotrexate polyglutamates into various cell types decreases as the length of the γ-glutamyl chain length increases (11, 19), by itself does not necessarily imply decreased efflux of these forms as well. A previous report from our laboratory (4) suggested that the efflux of methotrexate and other 4-amino folate analogs at pharmacological concentrations may be mediated by a carrier system distinct from that mediating influx. The equivalent efflux of methotrexate and its polyglutamates has also been reported (16) from human fibroblasts in culture.

Although these data and the results of our earlier (13) study suggest that polyglutamate derivatives do not represent a retentive form of methotrexate in mouse tissues, their presence may contribute to overall therapeutic responsiveness in other ways. In this regard, it should be noted that polyglutamate formation was greater in all of the tumors studied than in the small intestine. This is in agreement with the fact that some selective effects of methotrexate can be demonstrated (18) against all of these tumors in mice. While these effects can be largely explained (18) by differences in the transport of methotrexate in tumor versus normal drug-limiting proliferative tissue in host small intestine, it is conceivable that polyglutamates of methotrexate may contribute to the net therapeutic response by, for instance, inhibiting other enzymes of folate metabolism, such as thymidylate synthetase, more effectively than methotrexate. Since the synthesis of polyglutamates and the cytotoxic activity of methotrexate both require the continued presence of free intracellular drug, we will continue to examine whether this relationship is causal or coincidental. Finally, no implications are made from the results of these studies which apply to human or other rodent tissues. Additional studies will be necessary to clarify these issues with respect to mammalian tissues as a whole.

ACKNOWLEDGMENTS

The assistance of Donna M. Moccio during the development of some of the methodology is gratefully acknowledged.

REFERENCES

R. G. Poser et al.


Differential Synthesis of Methotrexate Polyglutamates in Normal Proliferative and Neoplastic Mouse Tissues *in Vivo*

Richard G. Poser, Francis M. Sirotnak and Paul L. Chello


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/41/11_Part_1/4441

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.