Effects of Folinic Acid on Hepatoma Cells Containing Methotrexate Polyglutamates

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ABSTRACT

The effects of folic acid on a toxic pulse exposure of cultured hepatoma cells to methotrexate (4-amino-10-methylpteroylglutamic acid) is reported. Inclusion of folic acid (5-formyl-5,6,7,8-tetrahydropteroylglutamic acid) (10 μM) with the 2-hr pulse of methotrexate (10 μM) nearly completely prevents the uptake and γ-glutamylation of methotrexate and prevents toxicity. Addition of folic acid after methotrexate results in a partial rescue that is time and concentration dependent. Restoration of cell growth in the presence of increasing amounts of folic acid is accompanied by a concentration-dependent elevation in tritium release from [5-3H]deoxyuridine. In the absence of folic acid, the release of tritium from [5-3H]deoxyuridine remains inhibited for three days after exposure to methotrexate, which can be related to the cellular formation and retention of methotrexate polyglutamates. Following the 2-hr pulse of methotrexate, the cellular pool consists of 70% polyglutamates of which the predominant species has three glutamate residues (4-NH2-10-CH3PteGlu3). When methotrexate is removed from medium, following the pulse, unmetabolized methotrexate rapidly leaves the cells, and 4-NH2-10-CH3PteGlu3 is converted to methotrexate polyglutamates containing four to six glutamate residues. Addition of folic acid after the methotrexate pulse prevents the conversion of 4-NH2-10-CH3PteGlu3 to the higher-chain-length derivatives and causes a reduction in the total methotrexate cell pools over the next 48 hr. These results suggest that the effects of folic acid on methotrexate polyglutamates may play a role in the rescue of cells containing these derivatives.

INTRODUCTION

Following the discovery that methotrexate (4-amino-10-methylpteroylglutamic acid) is converted to polyglutamate derivatives in liver (3), these derivatives have been detected in numerous tissues and cell lines. These lines include rat hepatocytes (2, 12, 16), rat hepatoma cells (1, 2, 13, 14), L1210 cells (26, 33), human fibroblasts (27), MCF-7 human breast cancer cells (30), and Ehrlich ascites cells (11). The importance for the target enzyme dihydrofolate reductase (EC 1.5.1.3) at least equal to that of methotrexate (11, 14, 30) and are retained by cultured cells for more avidly than is methotrexate, due to their reduced permeability (1, 2, 11).

Selective retention of an equally inhibitory species of methotrexate, such as the polyglutamates, endows the chemotherapeutic agent with much greater cytotoxic potential. This is due to the fact that the intracellular concentration of methotrexate must exceed that bound by dihydrofolate reductase in order to inhibit cell growth effectively (17, 31). Removal of methotrexate from the extracellular space results in a rapid loss of free methotrexate from the intracellular space. Under such conditions, however, the polyglutamate derivatives are retained for hours, and even days, against large chemiosmotic gradients (1, 2, 11). Thus, intracellular pools of methotrexate polyglutamates in excess of dihydrofolate reductase can be maintained in the absence of methotrexate in the extracellular space.

Folinic acid (5-formyl-5,6,7,8-tetrahydropteroylglutamic acid) has profound effects on methotrexate-caused cytotoxicity. These effects can be obtained either by adding folic acid with the methotrexate to impair uptake and γ-glutamylation (2, 29) or by adding it after methotrexate to rescue the cells from the effects of the antifolate (9, 18, 23, 25). Numerous clinical studies have demonstrated the advantages of rescue in conjunction with chemotherapy to lessen selectively the toxic effects of methotrexate on host tissue (4, 7).

Little attention has been paid to the effects of folic acid on cells which have a recognized high level of conversion of methotrexate to polyglutamates. In the present study with hepatoma cells in monolayer culture, we have investigated the effects of such addition on cell growth, de novo thymidylate biosynthesis, and the composition of the cellular methotrexate polyglutamate pool.

MATERIALS AND METHODS

Materials. Swin’s Medium S-77, folic acid-free Swim’s Medium S-77, fetal calf serum, and horse serum were obtained from Grand Island Biological Co. (Grand Island, N. Y.). Methotrexate and [3′5′7-3H]methotrexate were obtained from Lederle Laboratories (Pearl River, N. Y.) and Amersham/Searle Corp. (Arlington Heights, Ill.), respectively. Both compounds were purified by DEAE-cellulose column chromatography as described previously (12, 13). The concentration of methotrexate was determined from the extinction coefficient at 302 nm (6). The final specific radioactivity of [3H]methotrexate in all experiments ranged from 1 to 80 × 105 dpm/nmol. Methotrexate polyglutamate standards (4-NH2-10-CH3PteGlu3 to -Glu5) were kindly provided by Dr. John Montgomery, Southern Research Institute, Birmingham, Ala., and Dr. James Coward, Department of Chemistry, Rensselaer Polytechnic Institute, Troy, N. Y. (+)-5-Methyl-5,6,7,8-tetrahydropteroylglutamic acid was synthesized and purified as described previously (24), and folic acid was purchased from ICN Pharmaceuticals (Plainview, N. Y.). DEAE-cellulose was obtained from Schleicher and Schuell (Keene, N. H.), and Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Inc., (Piscataway, N. J.).

Cell Culture. H-11-EC3 cells (hereafter called H35 cells) derived

1 Recipient of NIH Grant CA 25933 awarded by the National Cancer Institute, USPHS; Department of Health and Human Services. To whom requests for reprints should be addressed.

Received May 10, 1982; accepted November 1, 1982.

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from the Reuber H35 rat hepatoma were cultured as described previously (13). For all the studies described in this report, the cells were seeded at a density of $2 \times 10^5$ cells/ml on a 60-mm culture dish or $7.5 \times 10^5$ cells/ml on a 100-mm culture dish. After 24 hr, methotrexate was added for 2 hr at a $10 \mu M$ concentration. The medium was then replaced with the same medium lacking methotrexate and containing the indicated concentration of folinic acid. Cells were released from the plates with 0.05% trypsin and were counted, as described previously, with a ZB1 Coulter Counter (13, 14).

**Analytical Procedures.** *De novo* synthesis of thymidylate was measured by a modification (14) of the procedure for measuring tritium released from [5-3H]deoxyuridine (20, 32).

The intracellular concentration of methotrexate was measured by procedures already described (12, 13). [3H]Methotrexate was added for a 2-hr pulse after the cells had been in culture for 24 hr. The cells were sampled at the end of the pulse or were placed back in the serum-enriched Swim’s medium in the presence or absence of folinic acid and sampled at various times. To terminate the incubation, the plates were cooled; washed 4 times with 4 ml of ice-cold 0.85% NaCl solution with 0.01 M potassium phosphate, pH 7.4; and scraped with two 1-ml aliquots of ice-cold 0.85% NaCl solution with 0.01 M potassium phosphate, pH 7.4. All samples were placed in a boiling bath for 10 min and centrifuged at 10,000 x g in a Sorval RC-2B centrifuge. The composition of the cellular methotrexate and methotrexate polyglutamates was established by DEAE-cellulose chromatography of cellular extracts (2). For chromatography, the DEAE-cellulose was equilibrated with 0.02 M NH₄HCO₃. Cell extracts with synthetic standards of methotrexate polyglutamates (approximately 0.2 µmol of each) and methotrexate were loaded on the columns (1 x 25 cm), which were then washed with 100 ml of 0.02 M NH₄HCO₃, followed by 40 ml of 0.1 M NH₄HCO₃. The radioactivity and standards were eluted with a gradient of 200 ml each of 0.2 to 0.6 M NH₄CHO. The eluate was collected in 4-ml fractions, and the standards were identified by their spectra (6). Methotrexate and each of the polyglutamates were quantitated by measuring the radioactivity in each peak on a Beckman (Irvine, Calif.) LS-250 liquid scintillation spectrometry system.

**RESULTS**

The effect of folinic acid on the growth of H35 cells, pulsed with 10 µM methotrexate for 2 hr and counted after 72 hr in culture, is shown in Chart 1. Inclusion of 10 µM folinic acid during the pulse, or during the pulse and after, caused the cells to grow with folinic acid as well as untreated cultures to grow. Folic acid concentrations <1 µM had little effect in reversing the methotrexate inhibition. Addition of folinic acid after the methotrexate pulse caused a partial recovery of cell growth with little rescue below 5 µM folinic acid, increasing rescue between 5 and 50 µM, and a maximum of just under 60% recovery at 100 µM. Concentrations up to 500 µM did not result in further increases in cell growth.

The kinetics of the rescue of methotrexate-pulsed H35 cells by various concentrations of folinic acid is shown in Chart 2. In all cases where recovery was effected (>5 µM), it was time and concentration dependent. Although the growth rate with 10 and 100 µM folinic acid was nearly as great as in untreated cultures (73 and 94%, respectively), a lag of 24 hr occurred before the cultures regained this rate. A 48-hr lag was observed with 5 µM folinic acid rescue, and after that the rate was only 25% of control. With 1 µM folinic acid, no recovery was noted for 72 hr following the methotrexate pulse.

The primary site of action of methotrexate and its polyglutamate derivatives is assumed to be dihydrofolate reductase (5, 17, 31). Inhibition of this enzyme causes the cell to be depleted of the reduced folate coenzymes which are then insufficient to support the terminal step in the synthesis of thymidylate and other one-carbon transfer reactions. We have shown that de novo thymidylate synthesis is inhibited in H35 cells after a pulse dose of methotrexate (14). This was demonstrated by measuring the loss of tritium from [5-3H]deoxyuridine in intact cells, yielding a relative measurement of thymidylate synthase (EC 2.1.1.45) activity (20, 32).

The same assay was applied to H35 cells to determine if folinic acid rescue is accompanied by an increase in thymidylate synthesis. Just after the methotrexate pulse (26 hr), there was a marked inhibition of tritium release, and the presence of folinic acid did not greatly relieve this inhibition (Chart 3). Over the next 72 hr, the folinic acid-rescued cells showed an elevation in tritium release that was directly related to the concentration of folinic acid in the medium, whereas little tritium was...
Folinic Acid Rescue of Hepatoma Cells

Chart 3. Effect of folic acid on de novo thymidylate biosynthesis in methotrexate-pulsed H35 cells. H35 cells were cultured as described in "Materials and Methods." After 24 hr in culture, all plates except control cultures (C) were exposed to a 2-hr pulse of 10 μM methotrexate. The pulsed cultures were then placed in medium lacking methotrexate and containing the following concentrations of folic acid: none (O); 1 μM (C); 10 μM (Q); 100 μM (□). The conversion of deoxyuridylate to thymidylate was measured by adding 20 μM [5-3H]deoxyuridine (2.8 × 10^3 dpm/mmol) to the cultures and measuring the release of tritium as described in "Materials and Methods." The assays were conducted just after the pulse (26 hr) and thereafter on a daily basis (at 72, 96, and 196 hr).

released by pulsed cells that received no folic acid. At 96 hr in culture, control cells had reached confluence (Chart 2), and tritium release had decreased markedly, owing to the marked reduction of thymidylate synthase activity in the confluent cells (13). However, tritium release remained elevated in cells treated with folic acid, and a slight increase was noted even at a folic acid concentration (1 μM) that did not support rescue.

Methotrexate is extensively converted by H35 cells to γ-glutamyl derivatives which contain primarily 3 to 5 glutamate residues (1, 2, 13, 14). Because of the involvement of these derivatives in the cytotoxicity of methotrexate, the effect of folic acid on the methotrexate polyglutamate composition of the cells was examined.

A 2-hr pulse of 10 μM [3H]methotrexate to H35 cells that had been in culture for 24 hr resulted in approximately 70% of the material being converted to polyglutamates, predominantly 4-NH2-10-CH3PteGlu3 and -Glu4 (Table 1). The intracellular concentration of the total methotrexate polyglutamates (4-NH2-10-CH3PteGlu2 and higher) was 17.5 ± 4.7 (S.D.) μM (n = 6). Inclusion of 10 μM folic acid during the pulse resulted in a 90% reduction of all methotrexate species.

After the pulsed cells had been placed in methotrexate-free medium for 4 hr, marked changes occurred in the profile of the methotrexate species (Table 1). As expected, the methotrexate rapidly equilibrated with the medium (1, 2) and, as a result, very low cellular concentrations of methotrexate were observed. Among the polyglutamates, there was a dramatic loss of 4-NH2-10-CH3PteGlu3, accompanied by increases in -Glu4, -Glu5, and -Glue. The balance of the methotrexate pools during early efflux is shown in Table 1. A total of 1.35 pmol was lost by the cells; this loss could be related to reductions in methotrexate and 4-NH2-10-CH3PteGlu4 and to the appearance of small amounts of longer-chain-length polyglutamates in the medium. This result is in agreement with earlier studies that indicated the ability of small amounts of polyglutamates to escape the cell (1, 2) and that the extent of retention is directly related to the glutamate chain length (2). In this experiment, the -Glu4 and -Glu5 derivatives were not detected in the medium, and the total pool of 4-NH2-10-CH3PteGlu3 and higher did not decrease over the 4-hr efflux. The 4-NH2-10-CH3PteGlu3 peak was reduced by 1.54 pmol, but this was offset by an increase in -Glu4, -Glu5, and -Glue of 1.59 pmol. Although it could be conjectured that the increases were derived from methotrexate, this seems unlikely because: (a) methotrexate rapidly leaves the cell and is not available in sufficient amounts after the first 15-30 min of efflux to account for extensive polyglutamate synthesis (1, 2); (b) 4-NH2-10-CH3PteGlu3 is retained by the cells (1, 2); and (c) in repeated experiments, the amount of 4-NH2-10-CH3PteGlu4, -Glu5, and -Glue appearing during efflux consistently paralleled with the reduction in 4-NH2-10-CH3PteGlu3.

The effect of folic acid added after the methotrexate pulse on the distribution and concentration of methotrexate polyglutamates is depicted in Chart 4. Methotrexate rapidly equilibrated with the medium in the presence or absence of folic acid. The major difference caused by the presence of folic acid was that 4-NH2-10-CH3PteGlu3 was not converted to longer-chain-length polyglutamates. Instead, there was a loss of all polyglutamate species, and the extent of loss at 4 hr was

<table>
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<th>Sample</th>
<th>Glu1</th>
<th>Glu2</th>
<th>Glu3</th>
<th>Glu4</th>
<th>Glu5</th>
<th>Glu6</th>
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<td>Cells, 2-hr uptake</td>
<td>1.04</td>
<td>0.38</td>
<td>1.67</td>
<td>1.03</td>
<td>0.23</td>
<td>ND</td>
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<td>Cells, 4-hr efflux</td>
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<td>ND</td>
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<td>1.86</td>
<td>0.72</td>
<td>0.27</td>
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<tr>
<td>Medium, 4-hr efflux</td>
<td>1.02</td>
<td>0.14</td>
<td>0.16</td>
<td>0.13</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Standards for 4-NH2-10-CH3PteGlu6 are not available. The identity of this peak is assumed, based upon its migration and sensitivity to conjugase.

ND, these species could not be detected.

Chart 4. Cellular concentration of methotrexate polyglutamates after pulse dose of [3H]methotrexate. H35 cells were cultured and pulsed with [3H]methotrexate as described in "Materials and Methods," and then incubated for the time shown in the absence (A) or presence (B) of 100 μM folic acid. The cellular concentrations of the individual methotrexate polyglutamate species were measured as described in "Materials and Methods." The concentrations of 4-NH2-10-CH3PteGlu3, -Glu4, -Glu5, and -Glue are shown. Although standards for Glu6 are not available, its identity is assumed from its elution position and susceptibility to conjugase.

J. Galivan, unpublished data.

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Methotrexate-pulsed H35 cells develop a significant proportion of higher methotrexate polyglutamates (Glu4+), which are further enhanced when methotrexate is removed from the medium (Chart 4). The presence of these derivatives in the cells for prolonged periods of time allows the possibility of interaction with sites other than dihydrofolate reductase. Numerous studies have demonstrated that the polyglutamates are generally far better substrates for folate-utilizing enzymes than are the corresponding monoglutamates (22). Similarly, methotrexate polyglutamates are more effective inhibitors of folate-utilizing enzymes, such as thymidylate synthase (8, 21), than is methotrexate itself. If the sustained concentrations of methotrexate polyglutamates are inhibiting folate-requiring enzymes other than dihydrofolate reductase, sufficient concentrations of appropriate substrates must be built up within the cell from folinic acid to overcome methotrexate polyglutamate inhibition and to act as one-carbon donors. Methotrexate polyglutamates could also exert these inhibitory effects indirectly by causing an increase in cellular dihydrofolylpolyglutamates (19) which are known to be formidable inhibitors of thymidylate synthase (8, 10).

By this reasoning, reduction in the concentration of methotrexate polyglutamates by folinic acid could contribute to rescue against their toxicity. When folinic acid is added following a pulse dose of methotrexate, both the glutamyl chain length and the concentration are reduced (Charts 4 and 5). It is possible that this is a cause-and-effect relationship, since retention of methotrexate polyglutamates is directly related to the glutamyl chain length (2). Folinic acid, by preventing the conversion of 4-NH2-10-CH3PteGlu3 to higher polyglutamates, creates a cellular pool consisting of shorter glutamyl chain lengths. The shorter chain lengths differ from the longer-chain-length derivatives in that they may be retained by the cells less efficiently (2), and they are probably poorer inhibitors of other folate-utilizing enzymes (8, 21). The reduction in cellular methotrexate polyglutamates caused by folinic acid addition after methotrexate could cause other alterations in the cells which would favor rescue. These include a reduction in the methotrexate pool (Charts 4 and 5) which favors the appearance of active dihydrofolate reductase. In the presence of active enzyme, intracellular dihydrofolylpolyglutamates would be converted to the tetrahydro derivatives, and the concentration of the potentially inhibitory dihydro species would be lowered. This, along with the reduction in methotrexate polyglutamates, would result in a decrease in potential inhibitors of one-carbon transfer.

Although the mechanism of folinic acid-dependent inhibition of glutamylation (Charts 4 and 5) has not been elucidated, it could be readily accounted for by a higher affinity of folylpolyglutamate synthetase for folinic acid (and its metabolic products) than for 4-NH2-10-CH3PteGlu3. Since folinic acid can reduce the γ-glutamylation of methotrexate (2, 29), it is reasonable to assume that it could inhibit further γ-glutamylation of 4-NH2-10-CH3PteGlu3. Other reduced folates could exhibit similar effects. In fact, (+)-methyl-5,6,7,8-tetrahydropteroylglutamic acid has the same effects on the methotrexate polyglutamate pools as does folinic acid (Charts 4 and 5). The effects of both reduced folates may be indirect and related to other folate coenzymes formed within the cell.

The present study demonstrates that folinic acid can rescue hepatoma cells containing methotrexate polyglutamates at-
though at relatively high folic acid concentrations and with a considerable lag period. It is not known whether a differential capacity for methotrexate polyglutamate synthesis contributes to the selective rescue of host tissue from methotrexate toxicity. However, Poser et al. (26) have suggested that tumor cells are more active in the synthesis of methotrexate polyglutamates than are certain normal cells. Based upon our results, a regimen might be designed which will selectively rescue host cells that synthesize limited amounts of methotrexate polyglutamates (26, 34) but will not rescue a transformed line highly active in methotrexate polyglutamate synthesis. We do not know if the effects of folic acid on cells containing methotrexate polyglutamates are a general phenomenon. However, a report appearing after the submission of this manuscript is consistent with data presented in Charts 1 and 3 (28).

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. James Coward, Department of Chemistry, Rensselaer Polytechnic Institute, Troy, N. Y., and Dr. John Montgomery, Southern Research Institute, Birmingham, Ala., for providing methotrexate polyglutamate substrates. We also thank Dr. Malgorzata Balinska for many helpful discussions of this problem.

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