Antifolate Polyglutamylation and Competitive Drug Displacement at Dihydrofolate Reductase as Important Elements in Leucovorin Rescue in L1210 Cells

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ABSTRACT

Previous studies from this laboratory have shown that the addition of leucovorin to tumor cells dissociates methotrexate, but not methotrexate polyglutamates, from dihydrofolate reductase (L. H. Matherly, D. W. Fry, and I. D. Goldman, Cancer Res., 43: 2694-2699, 1983). To further assess the importance of these interactions to leucovorin rescue, antifolate growth inhibition toward L1210 cells in the presence of leucovorin was correlated with the metabolism of (6S)-5-formyl tetrahydrofolate to dihydrofolate as a measure of dihydrofolate reductase activity. Growth inhibition (greater than 95%) by methotrexate (5-10 µM) following its intracellular polyglutamylation during a 3-h preexposure, or by continuous treatment with high levels of the lipophilic antifolate, trimetrexate (1 µM), was only slightly diminished by 10 µM leucovorin (15-25%). High-pressure liquid chromatographic analyses of the derivatives formed from radiolabeled (6S)-5-formyl tetrahydrofolate under these conditions showed an incomplete conversion to dihydrofolate and metabolism to predominantly 10-formyl tetrahydrofolate. Neither of the antifolates interfered appreciably with the metabolism of the folate derivatives to polyglutamates. Growth inhibition in the presence of leucovorin correlated with the accumulation of dihydrofolate (1.5-2.2 nmol) from radiolabeled (6S)-5-formyl tetrahydrofolate, reflecting continued suppression of dihydrofolate reductase activity at these drug concentrations. With lower equitoxic levels of the trimetrexate (7.5 nM), the provision of leucovorin allowed for a restoration of cell growth to a level greater than 90% of control. Under these conditions, control levels of dihydrofolate (0.2 nmol) were formed from radiolabeled cofactor, consistent with sustained dihydrofolate reductase activity.

These findings support a role for the activation of dihydrofolate reductase as an important component of the reversal of the effects of diaminoantifolates by leucovorin, presumably by a competitive displacement of drug from the enzyme. Since no displacement occurs in cells which have accumulated methotrexate polyglutamates, or in the presence of high levels of trimetrexate, it appears that the concentration of unbound drug within cells is a significant determinant of the extent of this competitive binding interaction. From these considerations, the high levels of methotrexate polyglutamates that accumulate in sensitive tumors relative to bone marrow and gastrointestinal cells would appear to represent an important factor for the selectivity of leucovorin rescue in vivo.

INTRODUCTION

The administration of MTX in conjunction with a source of reduced folate cofactors enhances the therapeutic efficacy of this agent in tumor-bearing animals (1, 2). The original pharmacological premise of this rescue of host tissues from drug toxicity was that the added tetrahydrofolate would circumvent the MTX block at the level of the DHFR, allowing for continued purine and pyrimidine biosynthesis for cell division. While no obvious basis for the demonstrated therapeutic selectivity of this approach is apparent from these considerations alone, the success of these regimens in experimental systems has nonetheless led to their clinical implementation (3, 4).

Primarily because of its chemical stability, rescue protocols have generally used calcium leucovorin [(6R,S)-5-CHO-FH4]. While not a natural cellular constituent, the (6S)-isomer of this compound is readily transported into cells by the MTX-tetrahydrofolate cofactor carrier (5), and, as depicted in Fig. 1, can be assimilated into cellular folate pools following its metabolism initially to (6) 5,10-CH2-FH4 and, subsequently, to (a) 10-CHO-FH4, the one carbon donor for purine biosynthesis, or (c) 5,10-CH2-FH4, the cofactor for thymidylate biosynthesis. While (6S)-5-CHO-FH4 is rapidly metabolized in tumor cells in the presence (6) and absence (7) of MTX, and can readily support cell division (8), it is unclear whether the rates for these processes for cofactor uptake and interconversion are sufficient for cellular replication when DHFR is inactivated by antifolates (9).

Several laboratories have reported that MTX and reduced folates interact competitively during rescue, a finding that cannot easily be reconciled solely on the basis of an anabolic role for the exogeneously supplied cofactor. While this competitive relationship between these folate derivatives has been attributed to direct binding interactions at the level of their shared membrane transport carrier (5, 10), similar interactions have been demonstrated between tetrahydrofolate cofactors and lipophilic 2,4-diaminoantifolates which bind to DHFR but penetrate cells by mechanisms distinct from the MTX-tetrahydrofolate cofactor carrier (8, 11). This suggests that other interactions at common cellular loci are also involved during the reversal of antifolate action by tetrahydrofolates.

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The abbreviations used are: MTX, methotrexate; 5-CHO-FH4, 5-formyl tetrahydrofolate; 10-CHO-FH4, 10-formyl tetrahydrofolate; 5,10-CH2-FH4, 5,10-methylenetetrahydrofolate; 5,10-CH=CH2-FH4, 5,10-methylene tetrahydrofolate; 5-CH2=CH2-FH4, 5-methyltetrahydrofolate; DHFR, dihydrofolate reductase; FH2, dihydrofolate; FH4, tetrahydrofolate; GAT, glycine (200 µM), adenosine (100 µM), and thymidine (10 µM); TMO, trimetrexate (2,4-diamino-5-methyl-6-(3,4,5-trimethoxynilino)-methyl) quinazoline); HPLC, high-pressure liquid chromatography.

Recent interest has focused on a potential role for competitive interactions between MTX and folate cofactors derived from leucovorin at the level of DHFR as a basis for the reduction of drug activity during rescue (9, 12–14). Studies from this laboratory have shown that exogenous tetrahydrofolates in sufficient concentrations effect a net displacement of MIX bound to DHFR. Cellular responses have shown that exogenous tetrahydrofolates in sufficient concentrations serve as folate cofactors. The present report demonstrates that the presence of leucovorin at the level of DHFR as a basis for the reduction of drug activity by chemical intermediates (13). These findings suggest a potential role for MTX polyglutamylation as a selective cellular determinant of leucovorin rescue in vivo. The present report explores the molecular relationship between antifolate polyglutamylation, binding to DHFR, and the reversal of drug activity by tetrahydrofolate cofactors.

MATERIALS AND METHODS

Chemicals. [3',5',7,9-3H]Folic acid was purchased from Amersham (Arlington Heights, IL). MTX and TMQ (glucuronate salt) were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD. MTX was purified as described previously (15). (6R,5S)-5-CHO-FH4, (6R,5S)-5-CHO-FH4, and (6R,5S)-FH4 were purchased from Sigma Chemical Co. (St. Louis, MO). Standard folates, including 10-CHO-FH4 (16), 5,10-CH2-FH4 (16), and FH2 (17) were prepared as described previously. (3',5',7,9-3H)-(6S)-5-CHO-FH4 and unlabeled (6S)-5-CHO-FH4 were prepared from radiolabeled folic acid and unlabeled FH2, respectively, as described by Moran and Colman (18), except that all procedures were conducted under a nitrogen atmosphere as described below. Other chemicals were obtained from commercial sources.

Cell Culture. The murine L1210 leukemia was grown in a humidified atmosphere at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated dialyzed fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml), all purchased from Gibco Laboratories (Grand Island, NY). Cell folate pools were depleted by culturing the cells for several weeks in RPMI 1640 without folate acid from Gibco Laboratories (Grand Island, NY). Cell folate pools were depleted by culturing the cells for several weeks in RPMI 1640 without folate acid from Gibco Laboratories (Grand Island, NY). Cell folate pools were depleted by culturing the cells for several weeks in RPMI 1640 without folate acid from Gibco Laboratories (Grand Island, NY). Cell folate pools were depleted by culturing the cells for several weeks in RPMI 1640 without folate acid from Gibco Laboratories (Grand Island, NY).

Incubation Techniques and Extraction of Cellular Folates. Cells (approximately 4 x 10^6 cells/ml) were incubated in specially designed flasks under an atmosphere of 95% O2-5% CO2 in complete folate-free medium containing 0.02 mM 2-mercaptoethanol. The centrifuge tube was sealed with a serum stopper and the contents were evacuated with a vacuum pump and flushed with nitrogen through a syringe needle. The cell suspension was heated in a boiling water bath for 90 s. After cooling to 0°C, the extract was incubated overnight at 32°C under nitrogen with a conjugate prepared from chicken pancreas (19). This treatment resulted in an essentially complete (greater than 95%) hydrolysis of the polyglutamate folates to the monoglutamyl forms. A nonenzymatic conversion of 10-CHO-FH4 to 5-CHO-FH4 (25%) and 5,10-CHO-FH4 (less than 10%) occurred during the extraction and hydrolytic procedures. While some loss of FH2 was also encountered (approximately 32%) the recoveries of 5-CH3-FH4 and FH2 were essentially quantitative (greater than 90%). Under these conditions, 5,10-CHO-FH4 appears as FH2. The degradation products detected both intracellularly and extracellularly following HPLC analysis (see below) were particularly prevalent following extended incubations with the radiolabeled folate (generally 20 to 30% of total extracellular and intracellular radiolabel after 3 h). These products were attributable primarily to oxidative degradative processes during the incubations; the extent of this nonenzymatic decomposition was not reduced by decreasing the oxygen levels to 20%.

For analysis of total cellular folate polyglutamates, an additional portion of washed cells was boiled in 50 mM sodium phosphate, pH 7.2, and 1% 2-mercaptoethanol. The centrifuge tube was sealed with a serum stopper and the contents were evacuated with a vacuum pump and flushed with nitrogen through a syringe needle. The cell suspension was heated in a boiling water bath for 90 s. After cooling to 0°C, the extract was incubated overnight at 32°C under nitrogen with a conjugate prepared from chicken pancreas (19). This treatment resulted in an essentially complete (greater than 95%) hydrolysis of the polyglutamate folates to the monoglutamyl forms. A nonenzymatic conversion of 10-CHO-FH4 to 5-CHO-FH4 (25%) and 5,10-CHO-FH4 (less than 10%) occurred during the extraction and hydrolytic procedures. While some loss of FH2 was also encountered (approximately 32%) the recoveries of 5-CH3-FH4 and FH2 were essentially quantitative (greater than 90%). Under these conditions, 5,10-CHO-FH4 appears as FH2. The degradation products detected both intracellularly and extracellularly following HPLC analysis (see below) were particularly prevalent following extended incubations with the radiolabeled folate (generally 20 to 30% of total extracellular and intracellular radiolabel after 3 h). These products were attributable primarily to oxidative degradative processes during the incubations; the extent of this nonenzymatic decomposition was not reduced by decreasing the oxygen levels to 20%.

For analysis of total cellular folate polyglutamates, an additional portion of washed cells was boiled in 50 mM sodium phosphate, pH 7.2, and 1% 2-mercaptoethanol for 3 min followed by the chemical cleavage of the folate polyglutamates to the corresponding p-aminobenzoyl polyglutamates using the cleavage procedure of Foo et al. (20).

HPLC Analysis of Folyl Mono- and Polyglutamates. For analysis of the natural folates following the enzymatic hydrolysis of the polyglutamates, the extracts were boiled for 90 s followed by centrifugation. Samples were analyzed on an Altex Model 332 gradient liquid chromatograph equipped with a Model 210 injector using a 5-μm Spherisorb octadeckysilyl column (IBM Instruments, Danbury, CT). The HPLC analysis consisted of a gradient of from 0 to 2.6% acetonitrile over 2 min followed by an increase to 6.75% acetonitrile from 9 to 14 min. The flow rate was 2 ml/min and 0.5-min fractions were collected and measured for radioactivity as described above. The retention times for the radiolabeled cofactors were compared to those for standard folates detected by UV monitoring at 280 nm (Table 1). p-Aminobenzoyl polyglutamates from the reductive cleavage procedure were analyzed on a 10μm Spherisorb octadeckysilyl column (Brownlee Laboratories, Santa Clara, CA) using a linear gradient of from 20 to 35% acetonitrile in 2.5 mM tetrabutylammonium phosphate in 50 mM sodium phosphate, pH 6.5, over 15 min, followed by an isocratic elution

Fig. 1. Pathways of metabolism of (6S)-5-CHO-FH4.
The growth of L1210 cells was monitored after 48 h. The results are expressed as the mean ± SD from 4 separate incubations in 2 separate experiments.

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The standards were detected by UV monitoring at 254 nm.

Described above. Radiolabeled p-aminobenzoyl polyglutamates were identified by comparison of their retention times with authentic standards (Glu, ... provided by Dr. Barry Shane (University of California at Berkeley). The standards were detected by UV monitoring at 254 nm.

RESULTS

Effect of Leucovorin on the Cytotoxicity of MTX to L1210 Cells. Folic acid (5 μM) or leucovorin (50 nm) can meet the anabolic requirements for tetrahydrofolates in L1210 cells previously depleted of endogenous folates. Conversely, pretreatment of these folate-depleted cells with MTX for 3 h followed by their resuspension into drug-free medium limits cell division in the presence of these compounds (Fig. 2). Under these conditions growth inhibition arises from the accumulation of MTX polyglutamyl derivatives during the initial 3-h exposure to drug (at 10 μM MTX, approximately 5- to 8-fold greater than the level of DHFR). In contrast to unconjugated drug, these derivatives are selectively retained intracellularly in the absence of extracellular antifolate (15, 21-25). Hence, this metabolism serves to prolong antifolate activity in the absence of continuous drug exposure (21, 22, 24). In Fig. 2, a 50% inhibitory concentration of 0.59 μM was measured in the presence of 5 μM folic acid following MTX pretreatment; this changes only slightly when 10 μM of leucovorin is provided as a source of reduced cofactors (50% inhibitory concentration, 2.2 μM). At the most inhibitory levels of MTX (greater than 5 μM), only a partial reversal of drug activity was achieved by the addition of leucovorin (20-25%). Only a slightly greater reversal of drug activity (to approximately 35% of control) was observed when the leucovorin concentration was increased to 100 μM (not shown). This incomplete abolition of antifolate activity by leucovorin under these conditions contrasts with the essentially complete protection afforded drug-treated cells by continuous exposure to GAT (96.22 ± 8.68% of control at 10 μM MTX, n = 4; also, see ref. 21).

Reversal of TMQ Cytotoxicity to L1210 Cells by Leucovorin. While the metabolism of MTX to its polyglutamyl forms inhibits cell division in the presence of folic acid or leucovorin, a greater reversal of the pharmacological effects of equitoxic concentrations of the lipophilic quinazoline, TMQ, can be achieved by exogenous leucovorin (Fig. 3). TMQ binds avidly to DHFR (8); however, this agent neither competes with tetrahydrofolate cofactors for cell entry (26, 27), nor possesses the structural requirements for polyglutamylation by the folylpolyglutamate synthetase. The pharmacological effects of TMQ are reversible following a brief drug exposure (data not shown) consistent with the lack of metabolism of this agent to retained derivatives analogous to MTX polyglutamates. However, continuous treatment with this drug potently inhibits the replication of folate-depleted cells incubated with 5 μM folic acid (Fig. 3). In contrast to the findings with MTX, at concentrations of TMQ (1–10 nm) that inhibit cell growth in the presence of folic acid, drug activity was completely abolished by the addition of 10 μM leucovorin; however, at higher TMQ levels a lesser diminution of the growth inhibition was achieved by the reduced cofactor. At 1 μM TMQ, only 14.16 ± 3.76% (n = 4) of control levels of growth was sustained in the presence of 10 μM leucovorin. As observed in the studies with MTX, essentially complete protection was afforded by the provision of GAT along with the antifolate (91.42 ± 11.42 at 1 μM TMQ, n = 4; not shown).

Metabolism of [3H]-(6S)-5-CHO-FH₄ in the Presence of 2,4-Diaminoantifolates. To better understand the relationships among the extent of reversal of antifolate activities in vitro, extracellular drug concentration, and the effects of MTX polyglutamylation on leucovorin utilization, the radiolabeled (6S)-isomer of 5-CHO-FH₄ was prepared and its metabolism was evaluated under conditions simulating those in the growth inhibition experiments. Previous reports have described extensive metabolism of (6S)-5-CHO-FH₄ in L1210 cells grown in folate replete medium (6, 7). However, in the present studies, as described above, the tumor cells had previously been depleted of their endogenous folate stores prior to exposure to the radiolabeled cofactor. This approach allows a more accurate assessment of tetrahydrofolate...
metabolism without isotope dilution or metabolic perturbations associated with endogenous folate polyglutamates.

Folate-depleted L1210 cells were incubated with 5 μM [3H]-\((6S)-5\text{-CHO-FH}_4\), following which cellular folates were extracted under nitrogen, enzymatically hydrolyzed to their monoglutationyl forms, and analyzed by HPLC as described in "Materials and Methods." Fig. 4 depicts the uptake and metabolism of this tritiated folate in untreated L1210 cells and cells pretreated with MTX (10 μM) for 3 h so as to form high levels of MTX polyglutamates, following which extracellular drug was removed. In the untreated cells, predominantly 10-CHO-FH4 accumulated over this interval (65% of total folates), with lower levels of 5-CHO-FH4 and other tetrahydrofolates (5-CH3-FH4 and FH4). Only very low levels of [3H]FH2 were detected intracellularly under these conditions (less than 0.1 μM). The major effect on the intracellular folate distributions following MTX pretreatment was the high level of FH2 accumulated (approximately 4 μM by 30 min), consistent with the potent inhibition of DHFR by the MTX derivatives. However, even in the presence of these antifolate derivatives, substantial levels of the various tetrahydrofolates were present as well (75% of cellular folates in the experiment shown). Similar findings were obtained in cells continuously exposed to 1 μM TMQ (data not shown).

After 3 h, a similar distribution of intracellular radiolabeled cofactors was found (Fig. 5). Greater than 70% of these intracellular derivatives were present as polyglutamates by 3 h, distributed among cofactor forms containing up to 4 additional glutamates (Table 2). The total intracellular folate levels were not significantly affected by drug treatment. Moreover, the polyglutamyl distribution was only slightly altered, with a small increase in the tetraglutamyl form at the expense of the other derivatives, in the presence of the antifolates.

Of particular interest was the finding after 3 h that considerable levels of monoglutationyl metabolites of (6S)-5-CHO-FH4 had accumulated extracellularly (Fig. 5). Indeed, 89% of the total folate metabolites derived from [3H]-\((6S)-5\text{-CHO-FH}_4\) was present extracellularly as 10-CHO-FH4 in untreated cells following a 3-h incubation. For cells pretreated with 10 μM MTX (3 h) and cells continuously exposed to 1 μM TMQ, a small but significant reduction in the level of 10-CHO-FH4 was observed; extracellular 10-CHO-FH4 under these conditions comprised 71 and 63%, respectively, of the total metabolites. While considerable FH2 was detected both intracellularly and extracellularly in the presence of these agents, the total level (extracellular and intracellular) of this radiolabeled oxidized derivative was comparatively small, comprising only 24 and 33%, respectively, of the total folate metabolites in the presence of MTX and TMQ. Hence, while the cellular metabolism of (6S)-5-CHO-FH4 is extensive under these conditions, the utilization of this reduced cofactor for thymidylate biosynthesis is apparently limited.

Relationship between DHFR Activity and the Reduction of Antifolate Activity by Leucovorin. Fig. 6 presents data which suggest that the abolition of 2,4-diaminoantifolate activity by leucovorin is associated with the activation of cellular DHFR as manifested by the maintenance of low levels of FH2. Here the extent of cell growth in the presence of 10 μM leucovorin under various conditions is correlated with the total (sum of intracellular and extracellular) accumulation of FH2 derived from radiolabeled

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### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total folates (μM)</th>
<th>% of polyglutamate distributiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14.71</td>
<td>26.06</td>
</tr>
<tr>
<td>MTX (10 μM)</td>
<td>14.92</td>
<td>17.33</td>
</tr>
<tr>
<td>TMQ (1 μM)</td>
<td>14.74</td>
<td>13.46</td>
</tr>
</tbody>
</table>

a The numbers (1–5) designate the total glutamate residues associated with p-aminobenzoate and, hence, the folate derivatives.

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Fig. 4. Uptake and metabolism of [3H]-\((6S)-5\text{-CHO-FH}_4\). Control cells and cells pretreated with MTX (10 μM) for 3 h were incubated with [3H]-\((6S)-5\text{-CHO-FH}_4\). Total cellular tritium and cofactor distributions were determined as described in "Materials and Methods." The numbers (1–5) designate the total glutamate residues associated with p-aminobenzoate and, hence, the folate derivatives.
cells even when thymidylate synthase is inhibited by treatment with 5-fluoro-2'-deoxyuridine preventing the build-up of FH2 (13). Folate have also been found to displace enzyme-bound MTX in cellular FH2 from the added cofactor and consequent displace placement of the antifolate on the enzyme active site in cells, the DHFR (12).

ment of drug from DHFR by this oxidized derivative, reduced tumor cells (13). While this effect could involve the formation of that leucovorin, if present in sufficient concentrations, promotes the exogenous leucovorin might increase the intracellular FH2 pool, of the intracellular DHFR (28-30) led to the suggestion that further studies from this laboratory demonstrated the accumulation of FH2 was formed in untreated cells (0.24 nmol), considerable levels of this derivative (1.46 nmol) accumulated in the tumor cells pretreated with MTX. Similarly, at a level of TMQ (1 μM) which rendered cells relatively insensitive to exogenous leucovorin, high levels of FH2 were present (2.16 nmol). However, under conditions in which leucovorin appreciably reversed TMQ cytotoxicity (7.5 nm and 0.1 μM), there was a proportional reduction in the level of radiolabeled FH2 detected, suggesting a reactivation to DHFR under these conditions.

DISCUSSION

This report explores the relationship between tetrahydrofolate cofactor metabolism and the pharmacological activities of 2,4-diaminoantifolates under conditions of leucovorin rescue in vitro. While the original basis of the apparent selective reversal of MTX activity by leucovorin in vivo was that an exogenous source of reduced folates, when appropriately administered, would circumvent the drug-induced block at DHFR, considerable experimental evidence now supports alternative mechanisms for rescue.

The finding of competition between MTX and FH2 at the level of the intracellular DHFR (28-30) lead to the suggestion that exogenous leucovorin might increase the intracellular FH2 pool, resulting in a competitive displacement of a small amount of bound antifolate sufficient to reverse the pharmacological effects of the drug. Further studies from this laboratory demonstrated that leucovorin, if present in sufficient concentrations, promotes the net dissociation of underderivatized MTX bound to DHFR in tumor cells (13). While this effect could involve the formation of cellular FH2 from the added cofactor and subsequent displacement of drug from DHFR by this oxidized derivative, reduced folates have also been found to displace enzyme-bound MTX in cells even when thymidylate synthase is inhibited by treatment with 5-fluoro-2'-deoxyuridine preventing the build-up of FH2 (13). Further, reduced folates displace drug tightly bound to purified DHFR (12).

Regardless of the actual folate derivative involved in the replacement of the antifolate on the enzyme active site in cells, the critical determinant of the extent of drug displacement is the level of free antifolate in the vicinity of DHFR at the time of provision of the tetrahydrofolate derivative. Indeed, if this free intracellular drug component is elevated, no significant competition at the level of DHFR is observed (13). Similarly, in cells which have accumulated MTX polyglutamates to a concentration exceeding that of the DHFR, intracellular folates derived from leucovorin do not effectively compete with the drug for enzyme binding (13).

Consistent with these findings is the observation that the metabolism of MTX to polyglutamyl derivatives by L1210 cells limits the extent of rescue achieved by leucovorin in vivo. A similar inverse relationship between the accumulation of intracellular MTX polyglutamates and the reversal of drug activity by leucovorin has been suggested (31, 32). Moreover, the present study has demonstrated that an essentially identical effect can be obtained by the continuous exposure of cells to the structurally dissimilar drug, TMQ; however, at lower equitoxic levels of TMQ, a significant diminution of antifolate activity was achieved with leucovorin.

In this report a correlation has been presented between the reduction of antifolate activity in tumor cells by leucovorin and the relative activity of intracellular DHFR. For TMQ, the extent of reduction of cytotoxicity closely paralleled the functional level of DHFR activity, as reflected in the intracellular accumulation of radiolabeled FH2 from exogenous (6S)-5-CHO-FH4. Hence, DHFR reactivation, presumably arising from direct displacement of enzyme-bound TMQ, analogous to unmetabolized MTX, is a key event in the reversal of the pharmacological effects of this antifolate by leucovorin. Antifolate displacement from cellular DHFR is not possible for cells which have accumulated appreciable levels of MTX polyglutamates (13). Under these conditions cytotoxicity is sustained even when leucovorin is supplied because reactivation of the enzyme is not possible. Similarly, in the continued presence of high levels of the TMQ, net displacement of antifolate from DHFR is not possible, resulting in the expression of cytotoxicity even when the rescue agent is provided.

On this basis, it seems that the conversion of MTX to its polyglutamyl forms may be an important determinant of the selectivity of leucovorin rescue in much the same fashion that this differential metabolism appears to be a key element in the therapeutic selectivity of MTX. These polyglutamyl derivatives of MTX are synthesized to a much greater extent in susceptible tumors than in bone marrow (21) and gastrointestinal cells (33, 34), contributing to a sustained intracellular level of antifolate even as the plasma drug concentration falls. In the sensitive host tissue, conversely, the rapid loss of underderivatized MTX parallels the plasma drug clearance (33). The elevated levels of MTX polyglutamates that accumulate in tumor cells would also preclude any significant displacement of antifolate from cellular DHFR by the provision of exogenous tetrahydrofolates. On the other hand, the low levels of MTX polyglutamates formed in bone marrow and gastrointestinal cells should permit a direct displacement of bound antifolate by cellular folates derived from leucovorin, allowing a selective restoration of endogenous tetrahydrofolate biosynthesis in these tissues.

The finding in the present study that (6S)-5-CHO-FH4 is extensively metabolized to 10-CHO-FH4, even in the presence of the antifolates, with incomplete conversion to FH2, raises the possibility that tetrahydrofolate utilization is somehow limited under...
these conditions. This contrasts with previous suggestions of a rapid interconversion of cofactor forms and maximal depletion of reduced cellular folates following drug exposure (28, 30). While no obvious perturbations by the antifolates on either the monoglutamate or polyglutamate cofactor distributions are apparent in these data to account for this discrepancy, this effect could conceivably arise from unfavorable equilibria between cellular folate derivatives (i.e., 10-CHO-FH4) and 5,10-CH2-FH4 which limit thymidylate biosynthesis. Alternatively, these data could reflect an inhibition of intracellular folate-dependent enzymes, including thymidylate synthase or the purine biosynthetic enzymes by antifolate derivatives (35, 36), or by dihydrofolate polyglutamates which accumulate in the presence of these agents (37). Studies are currently in progress to evaluate these possibilities.

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