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Inhibition of Methotrexate Polyglutamate Accumulation in Cultured Human Cells

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SUMMARY

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Incubation of confluent monolayers of human diploid fibroblasts in 1 μM [^{3}H]methotrexate results in the accumulation of increasing levels of nonexchangeable intracellular methotrexate (MTX). This accumulation is due to metabolism of MTX to poly(γ-glutamyl) derivatives of progressively longer polyglutamate chain length and retention of the latter in a nonexchangeable state. This metabolism is unaffected by the addition of 10 μM folic acid (pteroylglutamic acid) to the incubation medium. In contrast, the addition of 10 μM 5-formyltetrahydrofolate or 5-methyltetrahydrofolate results in cessation of synthesis of MTX polyglutamates and therefore cessation of accumulation of high intracellular total MTX levels. Direct evidence of depletion of the exchangeable intracellular pool of MTX derivatives suggests that the reduced folates act by competing with MTX for transport into cells. We cannot rule out that inhibition of polyglutamate accumulation may be due in part to competition of these reduced folates for the polyglutamate synthetase enzyme. Inhibition of polyglutamate synthesis by folinic acid and other reduced folates may play a role in the "rescue" of tissues from MTX toxicity.

INTRODUCTION

High-dose MTX[3] therapy has been found to induce clinical responses in tumors previously considered to be resistant to MTX (1). The achievement of intracellular concentrations of MTX in excess of those required to bind to dihydrofolate reductase (EC 1.5.1.3), the principal target enzyme, has been advanced as a mechanism for the increased efficiency of high-dose MTX therapy (2, 3). Toxicity of MTX has been reversed through the use of folinic acid (5-formyltetrahydrofolic acid, citrovorum factor), in the interval following MTX treatment (4). Folinic acid, a stable, reduced folate, is capable of supplying cells with a product which theoretically can bypass the MTX-induced block and restore one-carbon atom metabolism. In addition, folinic acid competes with MTX for entry into cells (3). Therefore, folinic acid may act by depleting cells of MTX and by preventing uptake in the face of continuing high circulating levels of the drug.

Recently it was demonstrated that MTX undergoes metabolism in cells, being converted to poly-γ-glutamyl derivatives (5–18). Synthesis of MTX polyglutamates has been found in rat liver and kidney; in rat hepatoma; in mouse liver, kidney, and small intestine; in both MTX-sensitive and MTX-resistant L1210 leukemia cells; and in cultured Chinese hamster ovary and baby hamster kidney cells. Formation of these derivatives has also been shown in human liver, in cultured human lymphocytes and fibroblasts, and in human bone marrow and leukemic cells in short-term culture. Polyglutamate formation was found not to occur in rat thymus and small intestine and in mutant Chinese hamster ovary cells which lack the enzyme pteroylpolyglutamate synthetase (14). Studies have shown that MTX polyglutamates are as good or better inhibitors of dihydrofolate reductase (8) and thymidylate synthetase (19) (EC 2.1.1.45) and that DNA

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[3] The abbreviations used are: MTX, methotrexate, 4-amino-10-methylpteroylglutamic acid; MTX(+G_1), methotrexate monoglutamate, 4-amino-10-methylpteroylglutamyl-γ-glutamic acid; MTX(+Gn), methotrexate polyglutamates longer than MTX(+G_1).

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synthesis is inhibited more profoundly and longer in human fibroblasts containing MTX polyglutamates than in cells contained only MTX (11).

We now report evidence that MTX polyglutamate synthesis is inhibited by folinic acid and by the natural circulating coenzyme form of the vitamin 5-methyltetrahydrofolate. No inhibition was seen with folic acid. A preliminary report of a portion of this work has been published (15).

MATERIALS AND METHODS

Human diploid fibroblasts were maintained in Petri dishes under an atmosphere of 5% CO_2-95% air in medium (Eagle's minimal essential medium and 10% fetal calf serum) containing 2.26 μM folic acid (20). For individual experiments, folate-free medium containing 10% dialyzed fetal calf serum was used as indicated.

All cells were determined to be free of *Mycoplasma* contamination (21). Folic acid and methyltetrahydrofolate (Sigma Chemical Company, St. Louis, Mo.), and folinic acid (Lederle Products Department, Cyanamid of Canada Ltd., Montreal, Quebec) were added to the incubation medium as indicated. After incubation at 37° the fibroblast monolayer was rinsed rapidly three times with 0° phosphate-buffered saline and reincubated for 60 min in 37° phosphate-buffered saline.[4] Methotrexate derivatives found in the buffer following this 60-min reincubation were designated as the exchangeable fraction. The zero time value for this exchangeable fraction obtained as indicated above for cells incubated in 1 μM MTX was 1.15 ± 0.30 nmoles/g of protein (n = 12). No attempt was made to differentiate MTX adsorbed to the cell membrane from MTX in the exchangeable fraction.

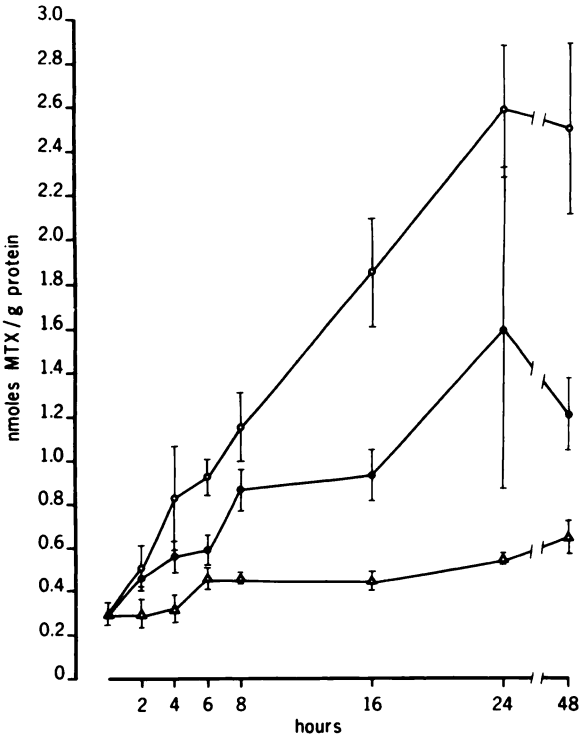

FIG. 1. *The effect of folinic acid on the accumulation of nonexchangeable MTX by cultured human fibroblasts*

Fibroblasts were incubated for 1 hr in medium containing 1 μM MTX. After 1 hr the incubation was continued in 1 μM MTX alone (n = 6) (○——○), in 1 μM MTX + 1 μM folinic acid (n = 3) (●——●), or in 1 μM MTX + 10 μM folinic acid (n = 3) (△——△) for an additional 48 hr. The cells were washed and reincubated for 60 min in phosphate-buffered saline as indicated under Materials and Methods to remove the exchangeable MTX derivatives. In this experiment, all media contained 2.26 μM folic acid. Results are expressed as means ± standard deviations.

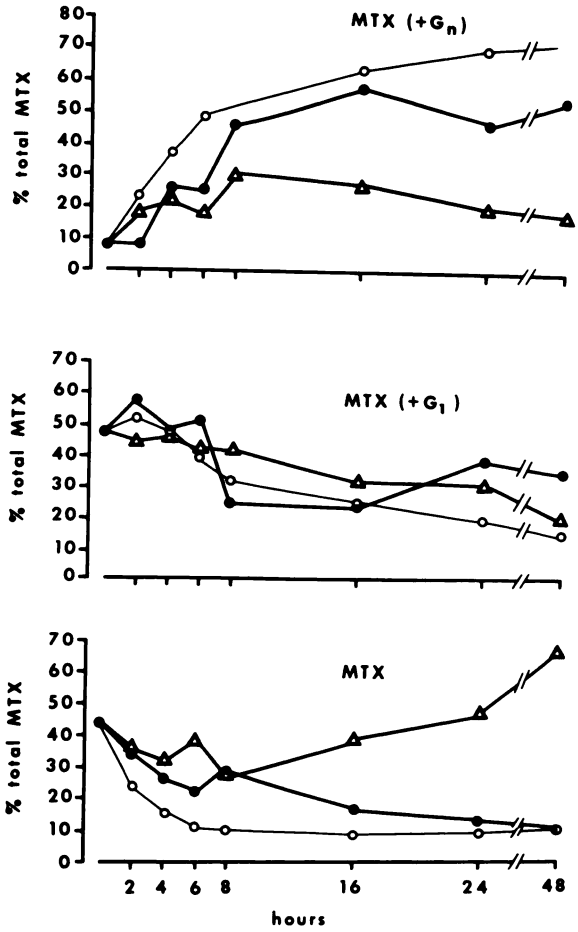

FIG. 2. *The distribution of nonexchangeable MTX metabolites in fibroblasts incubated in MTX plus folinic acid*

Fibroblasts were incubated for 1 hr in medium containing 1 μM MTX. After 1 hr, the incubation was continued in 1 μM MTX alone (○——○), in 1 μM MTX + 1 μM folinic acid (●——●), or in 1 μM MTX + 10 μM folinic acid (△——△). The results are expressed as percentage of total nonexchangeable MTX as each form.

The derivatives remaining in the cell after the 60 min in phosphate-buffered saline were designated as the nonexchangeable fraction. Separation of polyglutamates was achieved by Sephadex G-15 chromatography as previously described (10–12, 14). All chemicals were of reagent grade. 3′,5′,7-[^{3}H]Methotrexate (Amersham/Searle Company, Don Mills, Ontario) with an initial specific activity of either 22 Ci/mmole or 35 Ci/mmole was purified as previously described (12). MTX polyglutamates were identified by chromatography with authentic standards kindly supplied by Dr. M. G. Nair and Dr. C. M. Baugh (22). Hog kidney conjugase was prepared as described by

[4] Composed of 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 0.62 mM KH_2PO_4, and 5 mM glucose, pH 7.4.

Iwai *et al.* (23). Treatment of an extract of human fibroblasts incubated in 1 μM [^{3}H]MTX for 24 hr with hog kidney conjugase caused the material eluting in early peaks to elute as methotrexate. The MTX binding capacity of confluent fibroblasts, measured by incubating cell homogenates with [^{3}H]MTX and then absorbing unbound [^{3}H]MTX with dextran T-10-coated charcoal (24) was 0.421 ± 0.116 n/mole per gram of protein (n = 9).

RESULTS

Inhibition of accumulation of nonexchangeable MTX derivatives by fibroblasts after addition of folinic acid to cultures preincubated with MTX. Figure 1 shows the inhibitory effect of folinic acid at two different concentrations on the accumulation of total intracellular nonexchangeable methotrexate derivatives over 2 days. When fibroblasts were preincubated in fully supplemented medium containing 1 μM [^{3}H]MTX for 1 hr and 1 or 10 μM folinic acid was then added to the medium, there was a striking dose-dependent reduction in the accumulation of nonexchangeable label over the next 48 hr. In untreated fibroblasts the level of nonexchangeable MTX rose for the 1st day and plateaued at about 24 hr. Folinic acid caused an inhibition of MTX accumulation which was greater than 50% at 24 hr for 1 μM folinic acid and greater than 75% for 10 μM folinic acid.

Figure 2 shows the distribution of nonexchangeable drug in untreated and folinic acid-treated cells. In the absence of folinic acid, approximately 70% of the accumulated MTX was in the form of MTX(+Gn) and 20% was in the form of MTX(+G_1) after 1 day. In the presence of 1 μM folinic acid, approximately 45% was in the form of MTX(+Gn) and 40% was in the form of MTX(+G_1). In the presence of 10 μM folinic acid only approximately 20% of MTX was in the form of MTX(+Gn) and 30% was in the form of MTX(+G_1). Thus in the presence of 10 μM folinic acid, almost 50% of the nonexchangeable MTX was unmetabolized after 24 hr.

Effect of reduced folate and folic acid on the accumulation of exchangeable and nonexchangeable MTX and derivatives. The effect of the incubation of fibroblasts in medium containing 10% dialyzed fetal calf serum, 1 μM [^{3}H]MTX, and 10 μM folinic acid, methyltetrahydrofolic acid, or folic acid is shown in Table 1. In this experiment, folates and labeled MTX were present to-

TABLE 1

Nonexchangeable and exchangeable MTX derivatives in cultured fibroblasts

Confluent fibroblasts were incubated in triplicate in medium containing 1 μM [^{3}H]MTX and in the indicated folate. The nonexchangeable and exchangeable MTX derivatives were determined as described under Materials and Methods. The zero time value for exchangeable MTX, 1.15 ± 0.30 nmoles/g of protein (n = 12), has not not been subtracted.

	Nonexchangeable MTX			Exchangeable MTX	
	MTX	MTX(+G_1)	MTX(+Gn)	MTX	MTX polyglutamates (G_1 + Gn)
			nmoles/g protein		
MTX (10^{-6} M)					
1 hr	0.312 ± 0.063[a]	0.246 ± 0.055	0.038 ± 0.002	3.137 ± 1.246[a]	0.102 ± 0.031
2 hr	0.370 ± 0.063	0.521 ± 0.036	0.220 ± 0.042	6.125 ± 0.806	0.209 ± 0.031
4 hr	0.238 ± 0.046	0.749 ± 0.136	0.604 ± 0.070	3.547 ± 0.403	0.314 ± 0.052
8 hr	0.347 ± 0.140	0.909 ± 0.157	1.447 ± 0.225	2.394 ± 0.951	0.392 ± 0.027
16 hr	0.585 ± 0.050	1.188 ± 0.176	2.803 ± 0.750	2.736 ± 0.253	0.655 ± 0.144
MTX (10^{-6} M) + folinic Acid (10^{-5} M)					
1 hr	0.400 ± 0.073[b]	0.023 ± 0.004[c]	0.009 ± 0.002[c]	2.562 ± 0.532[b]	0.050 ± 0.030[d]
2 hr	0.428 ± 0.112[b]	0.066 ± 0.018[c]	0.014 ± 0.003[c]	4.005 ± 1.452[d]	0.094 ± 0.040[e]
4 hr	0.370 ± 0.132[b]	0.081 ± 0.009[c]	0.014 ± 0.003[c]	1.304 ± 0.095[c]	0.029 ± 0.018[c]
8 hr	0.213 ± 0.103[b]	0.099 ± 0.031[c]	0.025 ± 0.001[c]	0.933 ± 0.064[d]	0.021 ± 0.005[c]
16 hr	0.273 ± 0.048[c]	0.143 ± 0.036[c]	0.042 ± 0.008[c]	1.595 ± 0.688[d]	0.039 ± 0.025[c]
MTX (10^{-6} M) + 5-methyltetrahydrofolic acid (10^{-5} M)					
1 hr	0.419 ± 0.069[b]	0.025 ± 0.055[c]	0.011 ± 0[c]	3.656 ± 0.781[b]	0.091 ± 0.024[b]
2 hr	0.481 ± 0.051[d]	0.110 ± 0.012[c]	0.018 ± 0.001[c]	2.889 ± 0.544[e]	0.076 ± 0.017[e]
4 hr	0.449 ± 0.063[e]	0.192 ± 0.026[c]	0.026 ± 0.002[c]	2.804 ± 0.510[b]	0.064 ± 0.012[c]
8 hr	0.152 ± 0.027[d]	0.203 ± 0.057[c]	0.060 ± 0.006[c]	1.169 ± 0.368[d]	0.037 ± 0.011[c]
16 hr	0.267 ± 0.062[c]	0.225 ± 0.060[c]	0.086 ± 0.015[c]	2.312 ± 0.473[b]	0.080 ± 0.011[c]
MTX (10^{-6} M) + folic acid (10^{-5} M)					
1 hr	0.328 ± 0.007[b]	0.228 ± 0.028[b]	0.036 ± 0.013[b]	3.447 ± 0.367[b]	0.117 ± 0.057[b]
2 hr	0.356 ± 0.040[b]	0.598 ± 0.032[d]	0.177 ± 0.014[b]	4.296 ± 1.002[d]	0.160 ± 0.078[b]
4 hr	0.295 ± 0.067[b]	0.837 ± 0.110[b]	0.608 ± 0.151[b]	3.945 ± 1.170[b]	0.448 ± 0.149[b]
8 hr	0.171 ± 0.090[b]	0.481 ± 0.197[d]	0.710 ± 0.221[e]	1.190 ± 0.405[b]	0.149 ± 0.058[c]
16 hr	0.275 ± 0.070[e]	0.771 ± 0.149[d]	2.801 ± 0.43[b]	2.378 ± 0.605[b]	0.457 ± 0.134[b]

[a] Values are means ± standard deviation.
[b] According to t-test, comparing treatment with control values at each time, not significant ($p > 0.05$).
[c] $P \leq 0.001$.
[d] $p \leq 0.05$.
[e] $p \leq 0.01$.

gether from the beginning of the experiment. Because of the low values for exchangeable polyglutamates, MTX(+G_1) and MTX(+Gn) values were pooled. The levels of polyglutamates in the exchangeable compartment were not proportional to those found in the nonexchangeable compartment. This fact makes it unlikely that the values represent nonspecific release of drug from dead cells. In addition, trypan blue exclusion studies demonstrated the viability of the fibroblasts under the conditions of these experiments. In the presence of 10 μM folinic acid or 10 μM methyltetrahydrofolic acid, but not in the presence of 10 μM folic acid, the level of total nonexchangeable drug dropped and this drop was due mainly to the failure to accumulate polyglutamates. In general, the level of nonmetabolized, nonexchangeable MTX was not lower in these cells. In the exchangeable compartment there was a marked reduction in MTX polyglutamates in the cells treated with reduced folates but not in those treated with folic acid.

To simulate clinical protocols, fibroblasts were preincubated with labeled MTX for 6 hr and then continued in the labeled drug for an additional 24 h in the presence or absence of folic acid or reduced folates. The results show (Table 2) that in the nonexchangeable pool there was an actual depletion of labeled MTX polyglutamates from cells treated with reduced folates in the continued presence of labeled MTX. The level of nonmetabolized, nonexchangeable MTX remained essentially unaffected by the reduced folates and folic acid did not alter polyglutamate accumulation, as did the reduced folates. Thus, reduced folates, but not folic acid, at concentrations of 10 μM, effectively blocked the further accumulation of 1 μM labeled MTX and actually caused a depletion of labeled polyglutamates.

TABLE 2

Nonexchangeable MTX derivatives in cultured fibroblasts following preincubation in MTX

Confluent fibroblasts were preincubated in triplicate in medium, 1 μM in [^{3}H]MTX, for 6 h. Following this preincubation the cells were incubated in medium, 1 μM in [^{3}H]MTX and in the indicated folate.

	MTX	MTX(+G_1)	MTX(+Gn)
	nmoles/g protein		
Preincubation	0.229 ± 0.086[a]	0.793 ± 0.197	1.418 ± 0.311
MTX (10^{-6} M)			
2 hr	0.396 ± 0.117	0.602 ± 0.075	1.731 ± 0.189
4 hr	0.337 ± 0.031	0.665 ± 0.116	2.339 ± 0.394
8 hr	0.321 ± 0.076	0.409 ± 0.109	2.033 ± 0.564
24 hr	0.306 ± 0.157	0.620 ± 0.039	5.100 ± 0.198
MTX (10^{-6} M) + folinic acid (10^{-5} M)			
2 hr	0.106 ± 0.034[b]	0.287 ± 0.012[c]	0.958 ± 0.178[b]
4 hr	0.172 ± 0.033[b]	0.194 ± 0.048[c]	1.050 ± 0.113[b]
8 hr	0.248 ± 0.101[d]	0.121 ± 0.025[b]	0.948 ± 0.138[c]
24 hr	0.507 ± 0.060[d]	0.125 ± 0.011[c]	0.768 ± 0.055[c]
MTX (10^{-6} M) + 5-methyltetrahydrofolic acid (10^{-5} M)			
2 hr	0.247 ± 0.035[c]	0.378 ± 0.065[b]	1.397 ± 0.143[c]
4 hr	0.217 ± 0.045[b]	0.190 ± 0.041[c]	1.090 ± 0.187[b]
8 hr	0.259 ± 0.034[d]	0.155 ± 0.020[b]	0.808 ± 0.186[c]
24 hr	0.363 ± 0.061[d]	0.265 ± 0.007[c]	1.044 ± 0.046[c]
MTX (10^{-6} M) + folic acid (10^{-5} M)			
2 hr	0.250 ± 0.011[c]	0.589 ± 0.032[d]	1.666 ± 0.157[d]
4 hr	0.353 ± 0.200[d]	0.546 ± 0.100[d]	1.817 ± 0.338[d]
8 hr	0.305 ± 0.020[d]	0.493 ± 0.090[d]	1.979 ± 0.300[d]
24 hr	0.399 ± 0.041[d]	0.613 ± 0.086[d]	4.757 ± 1.077[d]

[a] Values are means ± standard deviation.
[b] According to *t*-test comparing treatment with control values at each time, $p \leq 0.01$.
[c] $p \leq 0.001$.
[d] Not significant ($p > 0.05$).
[e] $p \leq 0.05$.

DISCUSSION

MTX polyglutamates have now been identified in many mammalian cells. MTX(+G_1) has been found in the livers of patients who had received MTX therapy (9). Bone marrow cells from leukemic children also convert MTX to polyglutamates (16). For some cells, such as cultured human fibroblasts (11) and bone marrow cells (16), polyglutamate synthesis appears to be the mechanism by which they accumulate high levels of MTX. For other cells, such as Chinese hamster ovary, polyglutamate synthesis does not seem to be a mechanism for the accumulation of high levels of drug, since mutant cells which cannot synthesize MTX polyglutamates accumulate approximately the same level of drug (15).

Goldman (2) has stressed the importance of intracellular levels of MTX in excess of those required to bind to dihydrofolate reductase (EC 1.5.1.3). In human fibroblasts the accumulation of MTX polyglutamates is associated with a prolonged inhibition of DNA synthesis even after removal of MTX from the culture medium (1). We have suggested that exchangeable intracellular MTX is required for polyglutamate synthesis (10, 11, 15). Compared with MTX, MTX polyglutamates have equal binding affinity to dihydrofolate reductase (8, 14) and appear to be better inhibitors of thymidylate synthetase (EC 2.1.1.45) (19). The prolonged inhibition of DNA synthesis observed in fibroblasts (11) may be due to a specific effect of MTX polyglutamates, to the higher levels of drug associated with polyglutamate accumulation, or to the prolonged exposure to MTX which is associated with polyglutamate accumulation.

The present study shows that the two reduced folates, 5-formyltetrahydrofolic acid and 5-methyltetrahydrofolic acid, but not folic acid, are effective in preventing the accumulation of MTX polyglutamates by cultured fibroblasts. The reduced folates were able to effect a decrease in nonexchangeable total intracellular drug after a preincubation in 1 μM MTX and a subsequent incubation in 1 μM MTX and 10 μM reduced folate. Both folinic acid and methyltetrahydrofolic acid have been shown to inhibit MTX transport and to lower the levels of exchangeable MTX in L1210 cells (3) but not in freshly isolated hepatocytes (25). Table 1 shows that fibroblasts incubated from time zero in both 1 μM MTX and 10 μM reduced folate are able to accumulate similar levels of unmetabolized, nonexchangeable MTX to cells which have been incubated in 1 μM MTX alone, despite a marked inhibition in the accumulation of polyglutamates. These levels are in the range of those for dihydrofolate reductase as determined by the binding capacity of extracts of confluent fibroblasts. This accumulation may be due to the high affinity of the dihydrofolate reductase relative to the polyglutamate synthetase for the MTX which is transported in the presence of reduced folate. The level

of exchangeable MTX polyglutamates are also greatly decreased in the presence of reduced folates. It is more difficult to comment on the levels of exchangeable, non-metabolized MTX because of the high zero time values, which probably reflect in part adsorption of MTX to the cell membrane (2).

McGuire *et al.* (26) have demonstrated in studies of the polyglutamate synthetase in rat liver that methyltetrahydrofolate has between 10% and 48% of the activity of tetrahydrofolate, depending on substrate concentration. Interestingly, folic acid showed a similar activity. Hoffbrand *et al.* (7) have shown in phytohemagglutinin stimulated lymphocytes that MTX inhibits the accumulation of folate polyglutamates when folic acid (but not when folinic acid) is used as substrate. It would be of interest to study the effect of reduced folates on MTX polyglutamate accumulation in cells such as isolated hepatocytes in which reduced folates do not inhibit MTX transport or in mutant Chinese hamster ovary cells which have lost the specific transport mechanism for MTX and reduced folates (27).

The present studies suggest that at least part of the effect of protocols which use reduced folates to prevent MTX toxicity may be to reverse the accumulation of MTX polyglutamates. In some cell types this inhibition of polyglutamate accumulation results in a decrease in the net intracellular level of drug. The effect of folinic acid "rescue" on malignant and non-malignant cells may prove to be due in part to the differential metabolism of MTX in different cell types.

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REFERENCES

1. Jaffe, N., Frei, E., III, Traggis, D., and Bishop, Y. Adjuvant methotrexate and citrovovrum-factor treatment of osteogenic sarcoma. *N. Engl. J. Med.* **291:** 994–997 (1974).
2. Goldman, I. D. Analysis of the cytotoxic determinants for methotrexate (NSC-740): a role for "free" intracellular drug. *Cancer Chemother. Rep.* **6:** 51–61 (1975).
3. Goldman, I. D., Lichtenstein, N. S., and Oliverio, V. T. Carrier-mediated transport of the folate acid analogue, methotrexate, in the L1210 leukemia cell. *J. Biol. Chem.* **243:**5007–5017 (1968).
4. Hryniuk, W. M., and Bertino, J. R. Treatment of leukemia with large doses of methotrexate and folinic acid: clinical-biochemical correlates. *J. Clin. Invest.* **48:**2140–2155 (1969).
5. Baugh, C. M., Krumdieck, C. L., and Nair, M. G. Polygammaglutamyl metabolites of methotrexate. *Biochem. Biophys. Res. Commun.* **52:**27–34 (1973).
6. Brown, J. P., Davidson, G. E., Weir, D. C., and Scott, J. M. Specificity of folate-γ-L-glutamate ligase in rat liver and kidney. Biosynthesis of poly-γ-L-glutamates of unreduced methotrexate and the effect of methotrexate on folate polyglutamate synthesis. *Int. J. Biochem.* **5:**727–733 (1974).
7. Hoffbrand, A. V., Tripp, E., and Lavoie, A. Synthesis of folate polyglutamates in human cells. *Clin. Sci. Mol. Med.* **50:**61–68 (1976).
8. Jacobs, S. A., Adamson, R. H., Chabner, B. A., Derr, C. J., and Johns, D. C. Stoichiometric inhibition of mammalian dihydrofolate reductase by the γ-glutamyl metabolite of methotrexate 4-amino-4-deoxy-N^{10}-methylpteroylglutamyl-γ-glutamate. *Biochem. Biophys. Res. Commun.* **63:**692–698 (1975).
9. Jacobs, S. A., Derr, C. J., and Johns, D. G. Accumulation of methotrexate diglutamate in human liver during methotrexate therapy. *Biochem. Pharmacol.* **26:**2310–2313 (1977).
10. Rosenblatt, D. S., Whitehead, V. M., Dupont, M. M., Vuchich, M.-J., and Vera, N. Synthesis of methotrexate polyglutamates in cultured human cells. *Mol. Pharmacol.* **14:**210–214 (1978).
11. Rosenblatt, D. S., Whitehead, V. M., Vera, N., Pottier, A., Dupont, M., and Vuchich, M-J. Prolonged inhibition of DNA synthesis associated with the accumulation of methotrexate polyglutamate by cultured human cells. *Mol. Pharmacol.* **14:**1143–1147 (1978).
12. Whitehead, V. M., Perrault, M. M., and Stelcner, S. Tissue-specific synthesis of methotrexate polyglutamates in the rat. *Cancer Res.* **35:**2985–2990 (1975).
13. Whitehead, V. M., Perrault, M. M., and Stelcner, S. Tissue-specific synthesis of methotrexate polyglutamates in the rat, in *Chemistry and Biology of Pteridines* (W. Pfleiderer, ed.) Walter de Gruyter, Berlin, 475, 483 (1976).
14. Whitehead, V. M. Synthesis of methotrexate polyglutamates in L1210 murine leukemic cells. *Cancer Res.* **37:**408–412 (1977).
15. Whitehead, V. M., and Rosenblatt, D. S. Decreased synthesis of methotrexate polyglutamates in mutant hamster cells and in folinic acid-treated human fibroblasts, in *Chemistry and Biology of Pteridines* (R. L. Kisliuk and G. M. Brown, eds.). Elsevier/North Holland, New York, 689–694 (1979).
16. Witte, A., Whitehead, V. M., Rosenblatt, D. S., and Vuchich, M-J. Synthesis of methotrexate polyglutamates by bone marrow cells from patients with leukemia and lymphoma. *Dev. Pharmacol. Ther.* **1:**40–46 (1980).
17. Galivan, J. Transport and metabolism of methotrexate in normal and resistant cultured rat hepatoma cells. *Cancer Res.* **39:**735–743 (1979).
18. Gewirtz, D. A., White, J. C., Randolph, J. K., and Goldman, I. D. Formation of methotrexate polyglutamates in rat hepatocytes. *Cancer Res.* **39:**2914–2918 (1979).
19. Kisliuk, R. A., Gaumont, Y., Baugh, C. M., Galivan, J. H., Maley, G. F., and Maley, F. Inhibition of thymidylate synthetase by poly-γ-glutamyl derivatives of folate and methotrexate, in *Chemistry and Biology of Pteridines* (R. L. Kisliuk and G. M. Brown, eds.). Elsevier/North Holland, New York, 431–436 (1979).
20. Rosenblatt, D. S., and Erbe, R. W. Reciprocal changes in the levels of functionally related folate enzymes during the culture cycle in human fibroblasts. *Biochem. Biophys. Res. Commun.* **54:**1627–1633 (1973).
21. Schneider, E. L., Stanbridge, E. J., and Epstein, C. J. Incorporation of ^{3}H-uracil into DNA. A simple technique for the detection of mycoplasma contamination of cultured cells. *Expt. Cell Res.* **84:**311–318 (1974).
22. Nair, M. G., and Baugh, C. M. Synthesis and biological evaluation of poly-γ-glutamyl derivatives of methotrexate. *Biochemistry* **12:**3923–3927 (1973).
23. Iwai, K., Luttner, P. M., and Toennies, G. Blood folic acid studies. VII. Purification and properties of the folic acid precursors of human erythrocytes. *J. Biol. Chem.* **239:**2365–2369 (1964).
24. Kamen, B. A., Pakach, P. L., Vatev, R., and Caston, J. D. A rapid radiochemical ligand binding assay for methotrexate. *Anal. Biochem.* **70:**54–63 (1976).
25. Horne, D. W., Briggs, W. T., and Wagner, C. A functional, active transport system for methotrexate in freshly isolated hepatocytes. *Biochem. Biophys. Res. Commun.* **68:**70–76 (1976).
26. McGuire, J. J., Kitamoto, Y., Hsieh, P., Coward, J. K., and Bertino, J. R. Characterization of mammalian folypolyglutamate synthetases, in *Chemistry and Biology of Pteridines* (R. L. Kisliuk and G. M. Brown, eds.). Elsevier/North Holland, New York, 471–476 (1979).
27. Flintoff, W., and Saya, L. The selection of wild-type revertants from methotrexate permeability mutants. *Somatic Cell Genet.* **4:**143–156 (1978).

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