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Evidence for the Cytotoxic Activity of Polyglutamate Derivatives of Methotrexate

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SUMMARY

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The potential role of the polyglutamate derivatives of methotrexate in the cytotoxicity of methotrexate has been examined in H-35 hepatoma cells in culture. Pulse doses of methotrexate result in the accumulation of a nonexchangeable fraction of methotrexate that is toxic to the cells and consists almost entirely of polyglutamates. The toxicity of the polyglutamates appears to correlate with their effects on *de novo* thymidine synthesis, which was measured in intact cells by the release of tritium from [5-^{3}H]deoxyuridine. Evidence for this comes from the observation that the dose–inhibition response following pulses of methotrexate is nearly identical for cell growth and tritium release. Protection studies demonstrated that both thymidine and hypoxanthine are needed to prevent methotrexate toxicity, offering evidence that inhibition of cell growth is due to a depletion of reduced folate coenzymes. Cells treated with methotrexate were analyzed for the composition of the species bound to the target enzyme dihydrofolate reductase (EC 1.5.1.3). With 0.03 and 10 μM methotrexate in the medium the dihydrofolate reductase bound material consisted of 82 and 95% polyglutamates, respectively, compared with 78 and 88% in the total cell pool. These results demonstrate that the polyglutamates have at least an equivalent affinity for the enzyme in the intact cell when compared to methotrexate and, as such, can be chiefly responsible for the toxicity of methotrexate in those cells that have sufficient capacity to convert methotrexate to its γ-linked glutamate derivatives.

INTRODUCTION

Most of the experimental evidence to date indicates that the primary site of action of the antifolate, MTX,[1] is dihydrofolate reductase (1). Until recently it was felt that MTX itself was the sole active form of the drug. The existence of other forms that had potential antifolate activity was first demonstrated by Baugh *et al.* (2), who showed that poly-γ-glutamate derivatives of the parent compound could be detected in human erythrocytes and rat liver and viscera. Several laboratories extended these observations to show that this reaction occurred in many cells *in vitro* and *in vivo*, and the MTX may exist with one to four additional glutamate residues (3–8).

Evidence has also accumulated suggesting that the MTX polyglutamates may possess cytotoxic acitivity. These derivatives would be expected to have a longer tissue retention time since the polyglutamates do not appear to be readily released from the cell (8, 9). However, a recent study has indicated that a portion of the cellular MTX polyglutamates in human fibroblasts is exchangeable with the medium (10). Their potential for activity was suggested by the observation that they inhibit dihydrofolate reductase as well as or better than MTX itself (6, 8, 11) and that a nonexchangeable fraction of MTX polyglutamate in human fibroblasts impaired deoxyuridine incorporation into DNA (10). While the evidence concerning the effects of MTX polyglutamates on isolated dihydrofolate reductase is convincing, no studies have been conducted on the effects of these derivatives on the enzyme in intact cells. The purpose of this report is to examine this question and to establish the cytotoxic role of MTX polyglutamates in a stable transformed hepatic cell line.

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[1] Abbreviations used are: MTX, methotrexate, 4-amino-10-methylpteroylglutamic acid; MTX(G_2), 4-amino-10-methylpteroylglutamyl-γ-glutamyl-γ-glutamic acid; MTX(G_3), 4-amino-10-methylpteroylglutamyl-γ-glutamyl-γ-glutamyl-γ-glutamic acid; PteGlu, pteroylglutamic acid; H_2PteGlu, dihydropteroylglutamic acid; PBS, isotonic saline buffered with 0.01 M potassium phosphate, pH 7.4.

MATERIALS AND METHODS

Materials. Swims S-77 medium, horse serum, and fetal calf serum were obtained from Grand Island Biological

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Company, Grand Island, New York. MTX (Lederle Laboratories, Pearl River, N. Y.), [3′,5′,7-^{3}H]MTX (Amersham Searle, Arlington Heights, Ill.), and PteGlu (Sigma Chemical Company, St. Louis, Mo.) were purified by DEAE-cellulose column chromatography prior to use (12). The radiochemical purity of [3′,5′,7-^{3}H]MTX was not less than 98% when measured by thin layer chromatography. H_2PteGlu was synthesized by the procedure of Blakely (13) and the concentrations of the folate derivatives and analogs were determined by their absorption spectra (14). MTX(G_2) and MTX(G_3) were synthesized and provided by Dr. Charles M. Baugh of the University of South Alabama (15). Amino acid analysis in this laboratory has indicated that MTX(G_2) and MTX(G_3) have 2.8 and 3.9 mol of glutamic acid per mol of MTX, respectively.

Methods. H-II-E-C3 cells derived from the Reuber hepatoma were grown as described (8) and are referred to as H-35 cells. The cells were counted with a ZB1 Coulter counter following release from 60-mm dishes with 0.05% trypsin. Dihydrofolate reductase was measured spectrophotometrically by a modification (16) of the method of Mathews (17).

Intracellular levels of MTX were measured by a procedure similar to that previously described (8). The cells were incubated with the indicated concentration of [3′,5′,7-^{3}H]MTX (1 to 300 × 10^4 dpm/nmol) at 37° in a 5% CO_2 incubator. To terminate the reaction the cells were cooled to 0°, washed four times with 4-ml successive washes of ice-cold PBS, and removed with two 1-ml successive washes of 1 N NaOH. An aliquot of this was used to measure protein and the radioactivity was measured in 15 ml of Aquasol (New England Nuclear, Boston, Mass.) following neutralization with an equal volume of 1 N HCl. All results are the average of duplicate plates. Cell MTX is expressed as nanomoles per gram of cell protein.

When the distribution of MTX among its polyglutamate derivatives was evaluated, the same procedure was followed except that the indicated number of plates were scraped with a rubber policeman into two 1-ml aliquots of ice-cold PBS. Following addition of 0.5 μmol of carrier MTX, the sample was boiled for 15 min, centrifuged at 10,000*g* for 10 min, and lyophilized. The residue was dissolved in a volume of 0.5 ml PBS and chromatographed on an 0.9 × 50-cm Sephadex G-15 column with 0.02 M NH_4HCO_3 as the solvent (4, 9). MTX and its derivatives were located by monitoring radioactivity. The column was standardized with MTX, MTX(G_2), and MTX(G_3), which can be identified by their absorbance at 302 nm. These three standards eluted at Fractions 25, 11, and 8, respectively; the volume of each fraction was 2.1 ml.

RESULTS

Several approaches have been developed in this study to determine whether the MTX polyglutamates play a significant role in the toxicity of MTX. Because of the rapid conversion of MTX to its polyglutamates in H-35 cells (8), a short-term exposure to MTX followed by removal of MTX from the medium should result in an intracellular population that consists almost entirely of MTX polyglutamates. If present in sufficient amounts, these should be toxic to the cells. The MTX that is bound to the dihydrofolate reductase should consist of at least the same percentage of the polyglutamates as is present in the intracellular pool. If these criteria are met, it would supply strong evidence that the polyglutamate derivatives of MTX are the active species in this cell line, and suggest the possibility of MTX being the active species in other cell systems and tissues.

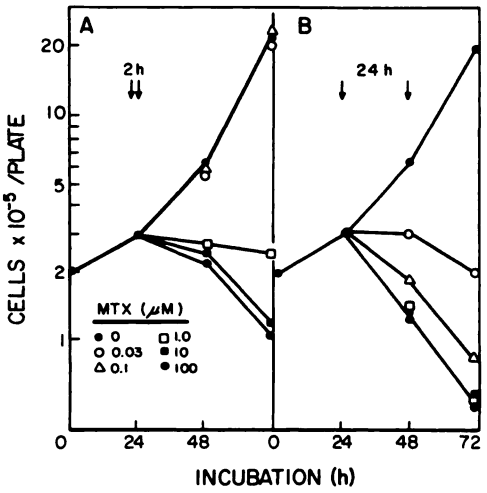

FIG. 1. *The effect of pulse MTX on the growth of H-35 cells*
The cells were plated at 2 × 10^5 cells per 60-mm plate and at 24 hr the indicated concentration of MTX was added. In (A) the methotrexate was removed after 2 hr and in (B) after 24 hr as indicated by the arrows. Cell counting was conducted as described in MATERIAL AND METHODS and the results are the average of duplicate plates.

Exposure of the cells to a pulse of MTX was examined to determine if such a regimen was toxic to the cells, and to evaluate the efflux of MTX and its derivatives from the cells after such treatment. When the cells were exposed to MTX for 2 hr, concentrations of 0.03 and 0.1 μM had no effect on their growth (Fig. 1). By contrast, the cells were unable to grow in the continued presence of 0.03 μM MTX (8). A 2-hr pulse of 1 μM MTX caused partial inhibition, whereas 10 and 100 μM MTX were equally toxic to the cells. When the cells were treated with the same levels of MTX for 24 hr, all concentrations inhibited cell growth, but only partial toxicity was observed with 0.03 and 0.1 μM MTX.

To determine if the toxic effect of MTX was related to the inhibition of *de novo* thymidylate synthesis, the relative rates of tritium release from [5-^{3}H]deoxyuridine (18, 19) were measured. When H-35 cells were treated with a 2-hr pulse of MTX, the inhibition of tritium release correlated well with the cytotoxic effect of MTX (Fig. 2). Concentrations of 0.03 and 0.1 μM had no effect on tritium release, whereas 1 μM caused a 90% inhibition. A comparison of the data in Figs. 1 and 2 indicates that the capacity of MTX to inhibit cell growth is nearly identical to the inhibition of thymidylate biosynthesis. Similar studies with a transport resistant line of H-35 cells (H-35RIII), demonstrated that pulses of 2 and 100 μM MTX had no effect on thymidylate biosynthesis in

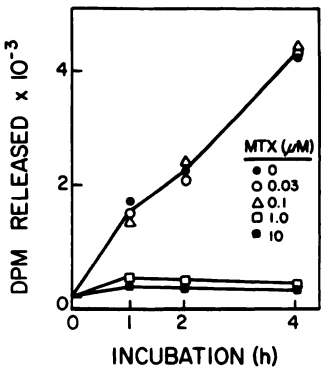

FIG. 2. *The effect of a 2-hr pulse of MTX on de novo thymidylate biosynthesis with H-35 cells*

The cells were treated with MTX as described in Fig. 1A. After a 2-hr pulse the cells were maintained in the absence of MTX for 22 hr at which time the medium was replaced with 2 ml of serum-free Swims S-77. After 1 hr, [5-^{3}H]deoxyuridine (1.5×10^4 dpm/nmol) was added at a concentration of 20 μM. At the indicated times 0.2 ml of medium was removed and added to 0.1 g of activated charcoal and placed in an ice bath. This mixture was passed over an 0.5 x 3.0-cm charcoal column and the radioactivity in the effluent was measured in 15 ml Aquasol in a Beckman LS250 liquid scintillation counter with external standardization. The mean of duplicate samples is presented for each time point and the results are expressed as dpm released per 10^5 cells.

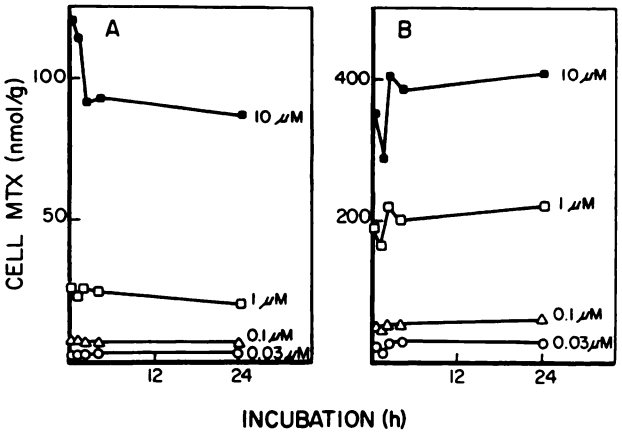

FIG. 3. *Efflux of MTS from H-35 cells*

The cells were cultured at 2×10^5 cells per plate and after 24 hr in culture [3′,5′,7-^{3}H]MTX was added for 2 hr in (A) and 24 hr in (B) at the indicated concentrations. At the end of the incubation the medium was replaced with MTX-free medium and the intracellular levels of MTX were measured as described in MATERIAL AND METHODS.

these cells[2] (8). The growth of the H-35RIII cells was unaffected by these levels of MTX, presumably due to the inability of the resistant cells to accumulate MTX (8).

Efflux of MTX from the H-35 cells treated with pulse doses of MTX as described above indicated that very little MTX was lost after the cells were placed in MTX-free medium (Fig. 3). This observation suggested that a large conversion of MTX to its polyglutamate derivatives had occurred and that these compounds were not able to escape from the cells as readily as unmodified MTX. If this supposition was correct, the polyglutamates would be the agents of cytotoxicity demonstrated in Fig. 1.

[2] Galivan, J. H., unpublished observations.

In order to verify that the MTX polyglutamates predominated, the intracellular contents of the H-35 cells were subjected to Sephadex G-15 chromatography (Fig. 4) following a pulse dose of MTX. With short-term exposure (2 hr) of MTX at toxic levels (1.0 to 10 μM), greater than 95% of the MTX chromatographed as a single peak that ran slightly ahead of MTX(G_3) on Sephadex G-15. At lower, nontoxic levels (0.1 μM) a large quantity of unmodified MTX still existed. We have previously shown that conversion of MTX to its polyglutamates is relatively inefficient at low media concentrations (8). The lack of efflux from the cells treated with 0.1 μM MTX for 2 hr is probably due to the fact that the level of MTX does not greatly exceed that of dihydrofolate reductase. Thus, the unmetabolized MTX retained by these cells may be tightly bound to the enzyme and unable to leave the cell. A 24-hr exposure to MTX (0.1, 1.0, and 10 μM) followed by a 4-hr incubation in the absence of the drug results in an intracellular pool of MTX that consists of 95% or more of the polyglutamates (Fig. 4). Only a single peak is observed which elutes slightly ahead of MTX(G_3) on Sephadex G-15 gel filtration. In order to define the composition of these MTX derivatives DEAE cellulose chromatography was employed. The radioactivity eluted as three distinct peaks, one of which eluted with MTX(G_3) and the other two eluting at a higher salt concentration. This result suggests the presence of higher molecular weight MTX polyglutamates that are not resolved by Sephadex G-15. Additional studies are being carried out to establish the identity of these species.

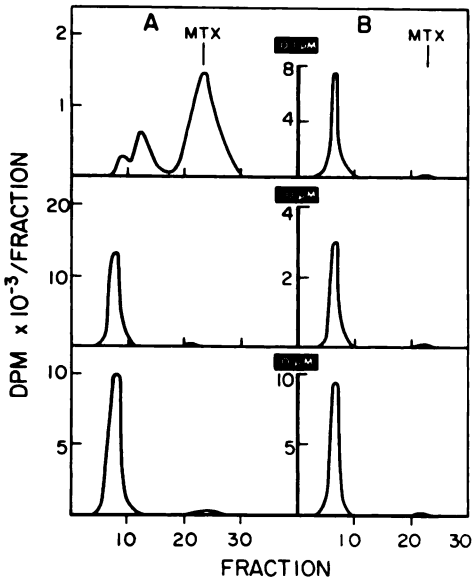

FIG. 4. *Sephadex G-15 gel chromatography of H-35 cell extracts after pulse dosage of MTX*

The cells were cultured at a density of 2×10^5 cells per plate. After 24 hr, [3′,5′,7-^{3}H]MTX was added at the indicated concentrations and the incubations conducted for 2 hr in (A) and 24 hr in (B). The medium was replaced with MTX-free medium and after a 4-hr incubation the intracellular contents were analyzed by Sephadex G-15 gel filtration. MTX, MTX(G_2), and MTX(G_3) standards eluted in Fractions 23, 12, and 8, respectively.

Further proof for the involvement of the polyglutamates came from examining the species of MTX bound in the intact cell to the target enzyme, dihydrofolate reductase. When the cells were treated by continual exposure of a minimal toxic dose of [3′,5′,7-^{3}H]MTX (0.03 μM), approximately 80% of the intracellular radioactivity was protein bound (8). Examination of extracts of cells treated with 0.03 μM MTX by Sephadex G-75 column chromatography demonstrated all the bound radioactivity eluted in a position that is nearly identical to that of dihydrofolate reductase from extracts of H-35 cells (Fig. 5A). No radioactivity was observed in Fractions 120–160 where free MTX and MTX polyglutamates elute. A molecular weight of approximately 22,000 was estimated from the elution position of radioactivity, which agrees with a molecular weight for this enzyme from a number of sources, including hepatic tissue (1). This suggests that the major high affinity protein for MTX in the H-35 cell extracts is dihydrofolate reductase. The bound MTX was removed from the enzyme (pooled Fractions 67 to 80) by boiling at pH 8.5 for 15 min. The sample was then lyophilized and chromatographed on Sephadex G-15 to determine the composition of the enzyme bound MTX (Fig. 5A inset). Of the total bound MTX, 82% corresponded to polyglutamates. By applying boiled cell suspensions, prepared identically to those in Fig. 5A, directly to Sephadex G-15 gel filtration, we found that the intracellular pool of MTX was 78% polyglutamates. Thus, the percentage of MTX as polyglutamates was nearly identical whether it was free within the cell or bound to dihydrofolate reductase.

A similar experiment was conducted with 10 μM MTX in the medium. Under these conditions 88% of the intracellular MTX was polyglutamates. Since the concentration of all MTX species within the cell is 78 μM, it means that the unaltered MTX is present at 9.4 μM. Dihydrofolate reductase is present in these cells at approximately 0.8 μM (8). If the dihydrofolate reductase has a greater affinity for MTX than the polyglutamate derivative, a large amount of unmodified MTX should be bound to the enzyme under these conditions. The results of this experiment are depicted in Fig. 5B. The unbound MTX eluting in Fractions 114 to 160 consists of three peaks. The radioactive material eluting at Fraction 150 corresponded to MTX. The two earlier fractions corresponded to MTX(G_3) and MTX(G_2) when isolated and rechromatographed on Sephadex G-15. When the bound MTX was removed from dihydrofolate reductase (Fractions 67 to 80), 95% was found to consist of the polyglutamates (Fig. 5B, inset). The species present on the enzyme elute with MTX(G_2) and MTX(G_3), which is consistent with the observation that these species predominate in extracts of H-35 cells treated with continual doses of MTX (8).

The data shown in Fig. 5 and that previously presented (8) indicate that the MTX polyglutamates are exerting their effect in H-35 cells via the inhibition of dihydrofolate reductase. Protection studies were employed to determine whether thymidine or purine availability was reduced by the presence of MTX (Table 1). The results demonstrate that thymidine offers a slight protection at high levels, but both thymidine and hypoxanthine are needed to completely prevent toxicity.

DISCUSSION

In recent years the potential importance of the polyglutamate derivatives of MTX in the toxicity and chemotherapeutic activity of the drug has become apparent (2–11). The basis for this is the greater retention of these derivatives by cells and also the equal or higher affinity for the folate utilizing enzymes. Although the role of these derivatives in the cytotoxicity of MTX has been postulated (6, 8, 10, 11), there have been no experiments reported that directly demonstrate the toxicity of the

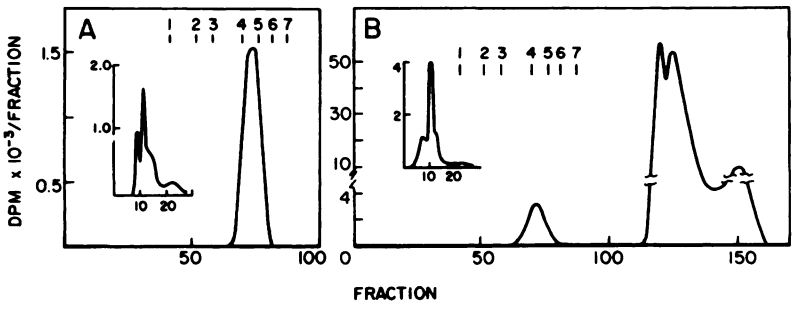

FIG. 5. *Sephadex G-75 gel chromatography of intracellular MTX*

(A) [3′,5′,7-^{3}H]MTX (6×10^5 dpm/nmol) was added to eight plates of H-35 cells at a concentration of 0.03 μM on Day 4 in culture. The cells were incubated for 24 hr, cooled to 0°, and washed four times with 4 ml of ice-cold PBS. The cells were scraped with a rubber policeman into 2 ml of ice-cold PBS and cell extracts made with a Dounce homogenizer. The homogenates were centrifuged at 100,000*g* for 1 hr and the supernatant was applied to a 1.5 x 175-cm Sephadex G-75 column. The elution buffer was 0.02 M potassium phosphate, pH 6.5, and 2.4-ml fractions were collected. The numbers within the figure refer to the molecular weight standards which are: **1**, Blue Dextran (2,000,000); **2**, bovine serum albumin (68,000); **3**, ovalbumin (43,000); **4**, chymotrypsinogen (25,700); **5**, dihydrofolate reductase from H-35 cells (MW not established); **6**, MTX-resistant *Lactobacillus casei* dihydrofolate reductase (17,000); **7**, cytochrome *c* (12,800), 1.4×10^5 dpm were applied to the column and of this 80% was recovered in the fractions containing the bound MTX (67–80). These were pooled, titrated to pH 8.4 with 1 N NaOH, and boiled for 15 min to release the bound MTX. The sample was lyophilized, dissolved in 0.5 ml PBS, and chromatographed by Sephadex G-15 gel filtration as in Fig. 4 to identify the presence of MTX polyglutamates in the bound MTX population (inset). (B) This experiment is identical to that described in (A) except that the concentration of [3′,5′,9-^{3}H]MTX (1.6×10^5 dpm/nmol) in the medium was 10 μM. In addition to the protein standards employed in (A), this column was marked with MTX which eluted at Fraction 150. 3.7×10^5 dpm were applied to the column and a recovery of 85% was achieved.

TABLE 1

Prevention of MTX toxicity with hypoxanthine and thymidine

H-35 cells were plated at 2×10^5 cells in 4 ml of medium in a 60-mm Falcon plastic dish. After 24 hr MTX (0.03 μM) was added along with the indicated additions. At 48 hr the medium was changed and MTX was omitted, but the thymidine and hypoxanthine were included where indicated. The cells were counted as described in MATERIAL AND METHODS at 72 hr. Thymidine at 0.1 mM had no effect on cell growth.

Medium addition	Concentration	Cell growth	
	(μM)	*(cells/plate) ($\times 10^{-5}$)*	*(% control)*
None (MTX omitted)		23	
None		1.2	5
Thymidine	0.1	1.2	5
Hypoxanthine	0.1	1.4	6
Thymidine plus hypoxanthine	0.1 0.1	12	52
Thymidine (MTX omitted)	1.0	12	
Thymidine	1.0	3.4	28
Thymidine plus hypoxanthine	1.0 0.1	12	100

polyglutamates in intact cells. In the present study we have shown that pulse doses of MTX to H-35 hepatoma cells result in an intracellular pool that is almost entirely polyglutamates, which are retained by the cell when MTX is removed from the medium. The dose–response curves for cytotoxicity and *de novo* thymidine biosynthesis are nearly identical (Figs. 1 and 2), demonstrating that the inability of the cells to synthesize thymidine accompanies the loss of cell replication. This is thought to occur by inhibiting dihydrofolate reductase, which causes a reduction in the folate coenzyme pool. Both thymidine and hypoxanthine are required to completely prevent MTX toxicity, which is consistent with this interpretation since folate coenzymes are involved in the *de novo* synthesis of thymidylate and purines.

Several reports have indicated that MTX polyglutamates are at least as effective as MTX in inhibiting dihydrofolate reductase (6, 8, 11). In order to offer proof that the polyglutamates were involved in MTX cytotoxicity in intact cells, we examined the MTX species bound to dihydrofolate reductase extracted from the H-35 cells. The data reported here demonstrate that the dihydrofolate reductase-bound MTX consists of 82% polyglutamates at 0.03 μM $[MTX]_{Ext}$ and 95% polyglutamates when $[MTX]_{Ext}$ is 10 μM. These percentages are generally equivalent to similar values for the total cell pool of MTX and indicate that the polyglutamates of MTX are the predominant forms of MTX that inhibit dihydrofolate reductase in these cells. There was no evidence of protein other than dihydrofolate reductase binding the MTX species in extracts of H-35 cells. An interaction between MTX or MTX polyglutamates and other enzymes that is not detected by Sephadex gel filtration is still a possibility, especially since these derivatives are known to inhibit other soluble enzymes (20). Although we have not proven the noninvolvement of other enzymes, particularly at high MTX concentrations, the preponderance of data here support dihydrofolate reductase as the primary site of action.

The results of this study lead to the conclusion that the toxicity to the H-35 hepatoma cells is mediated by the polyglutamate derivatives of MTX. Although this point has not been shown with other cell lines, suggestive evidence has been presented to support a role for MTX polyglutamates in L1210 cells (6) and human diploid fibroblasts (10). Recent clinical investigations have shown that the most effective response to MTX therapy occurs with very high doses (21). Studies conducted *in vitro* and *in vivo* have demonstrated that the formation of MTX polyglutamates is more efficient as the concentration of MTX is increased (4, 7, 8). Since the conditions favoring polyglutamate formation are similar to those encountered in clinical chemotherapy, the occurrence of MTX polyglutamates in human normal and neoplastic tissue seems likely. For this reason, the contribution that the polyglutamates make to the destruction of experimental and human tumors should be more thoroughly examined.

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REFERENCES

1. Huennekens, F. M., K. S. Vitols, J. M. Whiteley and V. G. Neef. Dihydrofolate reductase, in *Methods in Cancer Research*, Vol. XIII (H. Busch, ed.). Academic Press, New York, 199–225 (1976).
2. Baugh, C. M., C. Krumdieck and M. G. Nair. Polygammaglutamyl metabolites of methotrexate. *Biochem. Biophys. Res. Commun.* **52**: 24–27 (1973).
3. Brown, J. P., G. E. Davidson, D. G. Weir and J. M. Scott. Specificity of folate-γ-glutamate ligase in rat liver and kidney. *Int. J. Biochem.* **5**: 727–733 (1974).
4. Whitehead, V. M., M. M. Perrault and S. Stelcner. Tissue specific synthesis of methotrexate polyglutamates in the rat. *Cancer Res.* **35**: 2985–2990 (1975).
5. Buehring, U., Y. S. Shin and E. Folsch. The influence of folate antagonist on the metabolism of folic acid and its reduced derivatives in rat liver and kidney. *Cancer Res.* **37**: 299–304 (1977).
6. Whitehead, V. M. Synthesis of methotrexate polyglutamates in L1210 murine leukemia cells. *Cancer Res.* **37**: 408–412 (1977).
7. Rosenblatt, D. S., V. M. Whitehead, M. M. Dupont, M.-J. Vuchich and N. Vera. Synthesis of methotrexate polyglutamates in cultured human cells. *Mol. Pharmacol.* **14**: 210–214 (1978).
8. Galivan, J. H. Transport and metabolism of methotrexate in normal and resistant cultured rat hepatoma cells. *Cancer Res.* **39**: 725–733 (1979).
9. McBurney, M. W. and G. F. Whitmore. Isolation and characterization of folate deficient mutants of chinese hamster cells. *Cell* **2**: 173–182 (1978).
10. Rosenblatt, D. S., V. M. Whitehead, N. Vera, A. Pottier, M. Dupont and M. J. Vuchich. Prolonged inhibition of DNA synthesis associated with the accumulation of methotrexate polyglutamates by cultured human cells. *Mol. Pharmacol.* **14**: 1143–1147 (1978).
11. Jacobs, S. A., R. H. Adamson, B. A. Chabner, V. J. Derr and D. G. Johns. Stoichiometric inhibition of mammalian dihydrofolate reductase by thy-γ-glutamyl metabolite of methotrexate, 4-amino-4-deoxy-N-10-methyl-pteroyl-glutamyl-γ-glutamate. *Biochem. Biophys. Res. Commun.* **63**: 692–698.
12. Galivan, J. H., G. F. Maley and F. Maley. Factors affecting substrate binding in Lactobacillus casei thymidylate synthetase studied by equilibrium dialysis. *Biochemistry* **15**: 356–362 (1976).
13. Blakely, R. L. Crystalline dihydropteroylglutamic acid. *Nature* **188**: 231–232 (1960).
14. Blakely, R. L. The biochemistry of folic acid and related pteridines, in *Frontiers of Biology*, Vol. 13 (A. Neuberger and E. L. Tatum, eds.). American Elsevier, New York, 92–94 (1969).
15. Nair, M. G. and C. M. Baugh. Synthesis and biological evaluation of poly-γ-glutamyl derivatives of methotrexate. *Biochemistry* **12**: 3923–3927 (1973).
16. Bonney, R. J. and F. Maley. Effect of methotrexate on thymidylate synthetase in cultured parenchymal cells isolated from regenerating rat liver. *Cancer Res.* **35**: 1950–1956 (1975).
17. Mathews, C. K. Evidence that bacteriophage-induced dihydrofolate reductase is a viral gene product. *J. Biol.Chem.* **242**: 4083–4086 (1967).
18. Tomich, P. K., C.-S. Chui, M. Wovcha and G. R. Greenberg. Evidence for a complex regulating the *in vivo* activities of early enzymes induced by bacteriophage T4. *J. Biol. Chem.* **249**: 7613–7622 (1974).

19. Kalman, T. I. and J. C. Galowich. Studies on the effect of folic acid antagonists on thymidylate synthetase activity in intact mammalian cells, in *Developments in Biochemistry*, Vol. 4: *The Chemistry and Biology of Pteridines* (G. Brown and R. Kisliuk, eds.). Elsevier, New York, 671–676 (1979).
20. Borsa, J. and G. F. Whitmore. Studies relating to the mode of action of methotrexate. III. Inhibition of thymidylate synthetase in tissue culture cells and cell-free systems. *Mol. Pharmacol.* **5**: 318–332 (1969).
21. Frei, E., III, N. Jaffe, M. H. N. Tattersall, S. Pitman and L. Parker. New approaches to cancer chemotherapy with methotrexate. *N. Engl. J. Med.* **292**: 846–851 (1978).

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