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Synthesis of Methotrexate Polyglutamates in Cultured Human Cells

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SUMMARY

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We describe the biosynthesis of poly-γ-glutamyl metabolites of methotrexate in cultured human fibroblasts. The accumulation of polyglutamates by these cells is dependent on the concentration of methotrexate in the culture medium, duration of incubation, and stage of the culture cycle. When [^{3}H]methotrexate is replaced with equimolar unlabeled methotrexate, label is found in successively longer polyglutamates. Synthesis of the polyglutamates is dependent on the continued presence of methotrexate in the culture medium.

INTRODUCTION

Methotrexate is an important antineoplastic agent whose cytotoxic action has been attributed to the inhibition of dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) (1). Recent studies have identified a role for a component of methotrexate in excess of that presumably bound to this enzyme (2-5). A number of investigators have reported the synthesis of poly-γ-glutamyl derivatives of MTX[1] (6-11). In L1210 cells, Jacobs *et al.* (12) found that MTX(+G_1) and MTX are equally potent as inhibitors both of dihydrofolate reductase activity and of cell replication. Whitehead (13) showed similar affinities of MTX, MTX(+G_1), MTX(+G_2), and MTX(+G_6) for the dihydrofolate reductase contained in lysates of L1210 cells. The present study reports some kinetics of synthesis of MTX polyglutamates by cultured human cells.

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[1] The abbreviations used are: MTX, methotrexate, 4-amino-10-methylpteroylglutamic acid; MTX(+G_1), 4-amino-10-methylpteroylglutamyl-γ-glutamic acid; MTX(+G_2), 4-amino-10-methylpteroylglutamyl-γ-glutamyl-γ-glutamic acid; MTX(+G_6), 4-amino-10-methylpteroylglutamyl-γ-glutamyl-γ-glutamyl-γ-glutamyl-γ-glutamyl-γ-glutamyl-γ-glutamic acid.

MATERIALS AND METHODS

Fibroblasts were derived from punch biopsy specimens of skin from normal individuals and grown in Petri dishes of 100-cm^2 surface area (P100) containing 10 ml of medium (85% Eagle's minimal essential medium containing 2.26 μM folic acid and 15% fetal calf serum) under an atmosphere of 5% CO_2-95% air. All cell lines were determined to be free of mycoplasma contamination (14). Cultures were refed twice weekly. Methotrexate was added to fresh medium of confluent cultures where

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indicated. The plates were harvested with 0.25% trypsin at the indicated times, and the cells were counted (15) and then washed twice with phosphate-buffered NaCl (pH 7.4). The twice-washed cell pellet was collected by centrifugation and resuspended in 2 ml of 0.1 M sodium phosphate, pH 7. Methotrexate derivatives were extracted by heating for 10 min in boiling water, followed by centrifugation and storage of the supernatant at −20°. Separation of methotrexate polyglutamates was performed by chromatography on 0.9 × 55 cm columns of Sephadex G-15, and the polyglutamates were identified by co-chromatography with authentic standards (7) and by anion-exchange chromatography after permanganate oxidation (16). MTX(+G_1) and MTX(+G_2) were kindly supplied by Drs. C. M. Baugh and M. G. Nair of the Department of Biochemistry, University of South Alabama (6). Results were expressed as picograms of MTX or equivalents of MTX polyglutamate per milligram of cell protein or as nanograms per 10^9 cells. Protein concentrations were determined by the method of Lowry *et al.* (17).

All chemicals were of reagent grade. [3′,5′,9-^{3}H]Methotrexate (Amersham/Searle) was mixed with MTX (Lederle), and the mixture was purified by Sephadex G-15 gel chromatography (7).

RESULTS

The result of co-chromatography of a heat extract of fibroblasts with synthetic standards is shown in Fig. 1. Methotrexate was eluted at tube 33; MTX(+G_1), at tube 18; and MTX(+G_2), at tube 12.

Diploid human fibroblasts synthesize MTX polyglutamates during the entire culture cycle (Fig. 2). The level of polyglutamates in the cell increases during the early logarithmic phase of growth, decreases through the late logarithmic phase, and remains low at confluence. All subsequent experiments were performed at confluence in order to minimize any inhibitory effect of MTX on cell growth.

Confluent cultures refed with 0.1 μM [^{3}H]MTX readily accumulate polyglutamates, so that by 48 hr they represent

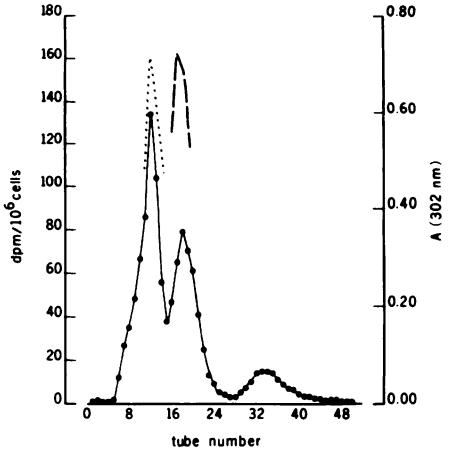

FIG. 1. *Separation of MTX polyglutamates on Sephadex G-15*

A heat extract prepared from fibroblasts that had been incubated in 0.1 μM [^{3}H]MTX (●——●) for 42 hr was co-chromatographed with 0.85 μmole of synthetic MTX(+G_1) (- - -) and 0.39 μmole of MTX(+G_2) (····) as described in MATERIALS AND METHODS.

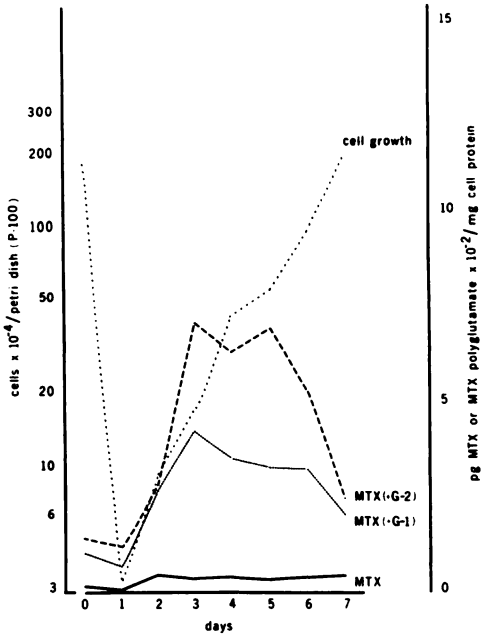

FIG. 2. *Effect of culture cycle on MTX polyglutamate formation*

Confluent fibroblast cultures were refed with medium containing 0.1 μM [^{3}H]MTX and harvested at 24 hr (day 0). Replicate plates were subcultured at low density and refed daily with minimal essential medium. Twenty-four hours prior to harvesting, cells were refed with 0.1 μM [^{3}H]MTX.

fully 80% of the label in these cells (Fig. 3). The formation of polyglutamates is dependent on the concentration of MTX in the culture medium (Table 1), and there is a tendency toward formation of greater amounts of longer derivatives at the same incubation time with increasing MTX concentration.

Incubation of confluent fibroblasts for 24 hr in 0.1 μM [^{3}H]MTX followed by incubation in equimolar unlabeled MTX results in a shift of label into the polyglutamates (Fig. 4). The longer polyglutamates accumulate with increasing time. That synthesis of polyglutamates is dependent on the continued presence of MTX in the culture medium is shown in Fig. 5. Cells that have been incubated for 24 hr in 0.1 μM [^{3}H]MTX and transferred into fresh medium containing neither labeled nor unlabeled MTX maintain the same distribution of MTX derivatives seen after the initial 24-hr incubation. In contrast, cells transferred into equimolar unlabeled MTX show a shift toward the formation of MTX(+G_2).

DISCUSSION

These studies demonstrate that MTX polyglutamates are synthesized by human fibroblasts over a range of extracellular MTX concentrations similar to blood levels achieved with clinical use of this drug, and that synthesis is time-dependent. MTX polyglutamates larger than MTX(+G_2) are poorly resolved on Sephadex G-15, and several of the experiements with long incubation times (Fig. 4) suggest that longer forms are made by these cells. Because the cell-harvesting procedures used in these experiments involve trypsinization at 37° and two washes of a cell pellet with buffer at room temperature, the MTX derivatives examined probably represent those which are not freely diffusible. Thus the high levels of MTX derivatives seen during early logarithmic growth may reflect binding of these derivatives to

TABLE 1

Effect of MTX concentration on polyglutamate synthesis

Confluent fibroblasts were incubated with [^{3}H]MTX at the indicated micromolar concentrations and harvested after 24 hr.

Dose uM	MTX	MTX(+G_1) ng/10^9 cells	MTX(+G_2)
0.03	204	23	2
0.10	147	107	11
0.20	95	151	47

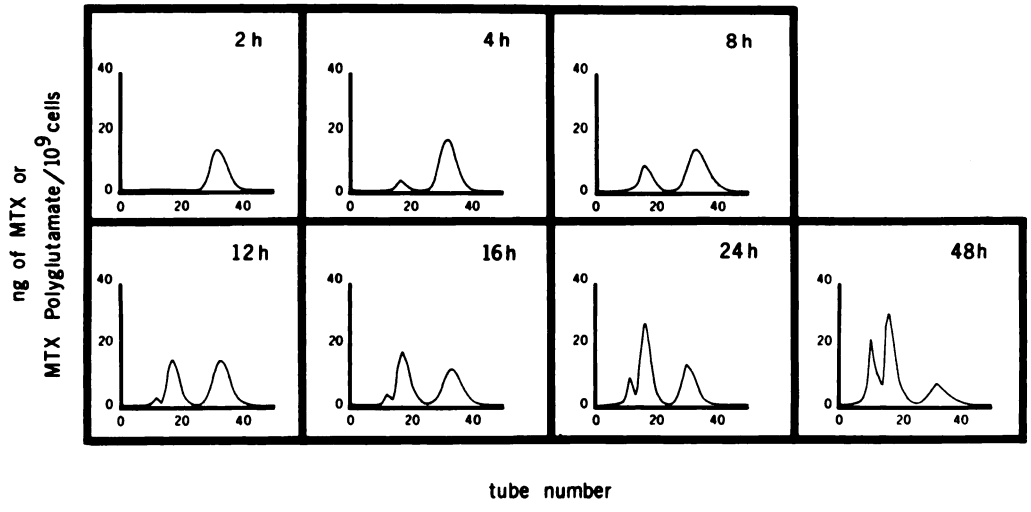

FIG. 3. *Time course of synthesis of MTX polyglutamates*

Confluent fibroblast cultures were incubated with 0.1 μM [^{3}H]MTX and harvested at the indicated times.

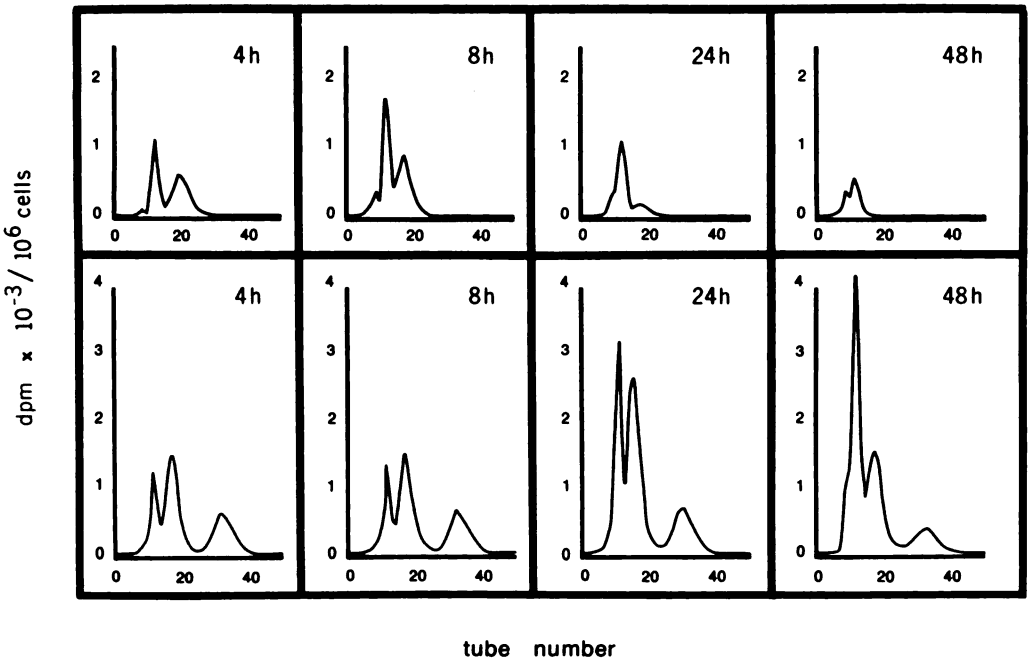

FIG. 4. *Chase of [³H]MTX with labeled and unlabeled MTX*

Confluent fibroblasts were incubated with 0.1 μM [³H]MTX for 24 hr. The cells were then incubated with either 0.1 μM unlabeled MTX (upper graphs) or 0.1 μM [³H]MTX (lower graphs) and harvested at the indicated times.

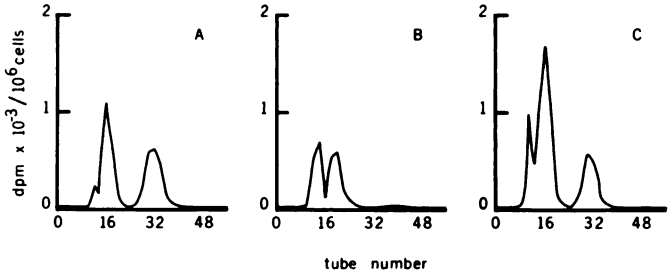

FIG. 5. *Dependence of MTX polyglutamate synthesis on the presence of MTX in the culture medium*

A. Confluent fibroblasts were incubated with 0.1 μM [³H]MTX for 24 hr and then incubated for 24 hr in MTX-free medium.

B. Confluent fibroblasts were incubated with 0.1 μM [³H]MTX for 24 hr and then incubated for 24 hr in 0.1 μM unlabeled MTX.

C. Confluent fibroblasts were incubated with unlabeled 0.1 μM MTX for 24 hr and then incubated for 24 hr in 0.1 μM [³H]MTX.

the high levels of dihydrofolate reductase known to be present in fibroblasts during this stage of growth (15). Synthesis of the polyglutamates is dependent on the continued presence of MTX in the culture medium, suggesting that only free MTX is a substrate for synthesis. The "chase" studies suggest that there is a sequential appearance of longer polyglutamates with time.

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