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Prolonged Inhibition of DNA Synthesis Associated with the Accumulation of Methotrexate Polyglutamates by Cultured Human Cells

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SUMMARY

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Incubation of cultured human fibroblasts in 1 μM methotrexate results in a progressive accumulation of poly-γ-glutamyl derivatives. This accumulation is not dependent on new protein synthesis. No polyglutamate derivatives are detectable until saturation by nonmetabolized methotrexate of a nonexchangeable intracellular fraction occurs. After saturation occurs both methotrexate and methotrexate polyglutamates are found in a freely-exchangeable and a non-exchangeable fraction. After saturation occurs all of the increase in the size of the nonexchangeable fraction is due to the accumulation of polyglutamates. Cells which have been incubated for up to 2 hr in 1 μM methotrexate rapidly regain the ability to incorporate deoxyuridine into DNA when transferred into methotrexate-free medium. With longer times of incubation resulting in the formation of longer and greater concentrations of polyglutamates, deoxyuridine incorporation is inhibited at least 24 h following transfer into methotrexate-free medium. In contrast, thymidine incorporation into DNA is increased over that seen in cells not incubated in methotrexate. We suggest that the metabolism of methotrexate in cultured human fibroblasts occurs according to the following steps: (i) saturation by nonmetabolized methotrexate of a nonexchangeable pool, (ii) addition of γ-glutamyl residues to methotrexate in a freely-exchangeable pool, (iii) appearance of methotrexate polyglutamates in a nonexchangeable pool. The inhibition of deoxyuridine incorporation in the absence of continued methotrexate may be due to the effects of previous sustained exposure to methotrexate, to the total quantity of methotrexate derivatives, or to some undefined differential effect of methotrexate polyglutamates.

INTRODUCTION

The chemotherapeutic agent and folic acid analogue, methotrexate,[3] is metabolized by cultured human diploid fibroblasts

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[2] Recipient of grants from the Medical Research Council of Canada and the Cancer Research Society.

[3] The abbreviations used are: MTX, methotrexate, 4-amino-10-methylpteroylglutamic acid; MTX (+G_1), 4-amino-10-methylpteroylglutamyl-γ-glutamicacid; MTX (+G_2), 4-amino-10-methylpteroylglutamyl-γ-glutamyl-γ-glutamic acid; MTX polyglutamates, MTX (+G_1), MTX (+G_2) ... MTX (+Gn); PBS, phosphate-buffered NaCl, pH 7.4.

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to γ-glutamyl conjugates (MTX polyglutamates) over a range of extracellular drug concentrations similar to blood levels achieved with clinical use of MTX. The rate of synthesis of MTX polyglutamates is dependent on the stage of the cell culture cycle, the concentration of MTX in the culture medium, and the time of incubation. Synthesis is also dependent on the continued presence of MTX in the culture medium. MTX that is nonexchangeable, presumably because of binding to dihydrofolate reductase, (5,6,7,8-tetrahydrofolate: $NADP^+$-oxidoreductase, EC 1.5.1.3) is not a substrate (1). Recently MTX ($+G_1$) has been found in the livers of patients who had received MTX therapy for neoplastic disease (2). The present study describes the relationship between the synthesis of MTX polyglutamates and the inhibition of DNA synthesis in cultured human fibroblasts.

MATERIALS AND METHODS

Human diploid fibroblasts were grown in Petri dishes under an atmosphere of 5% CO_2-95% air in medium (90% Eagle's minimum essential medium and 10% fetal calf serum) containing 2.26 μM folic acid (3). All cells were determined to be free of mycoplasma contamination (4). For the determination of total MTX derivatives including those adsorbed to the cell membrane, those exchangeable within the cell, and those nonexchangeable within the cell (5), the dishes were rapidly rinsed 4 times in 0° PBS, and the cells scraped with a rubber policeman in 0.1 M sodium phosphate, pH 7. For the separate determinations of nonexchangeable and exchangeable MTX fractions, the cells were rapidly rinsed 4 times in 0° PBS and then incubated for 60 min in 37° PBS. The MTX derivatives found in the PBS after 60 min were designated as the exchangeable fraction. No attempt was made to differentiate MTX adsorbed to the cell membrane from MTX in the exchangeable pool. The MTX derivatives remaining in the cells after 60 min in 37° PBS were designated as the nonexchangeable fraction. Separation of MTX polyglutamates was by Sephadex G-15 chromatography, as previously described (6). Incorporation of deoxyuridine into DNA was determined by the measurement of radioactivity in a 0° trichloracetic acid precipitate after exposure of the cells to 2.1 μM [^{3}H]deoxyuridine (7). For these studies the cells were harvested with 0.25% trypsin and counted by Coulter Counter (3). Protein concentrations were determined by the method of Lowry et al. (8). 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 G-15 gel chromatography (6). 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 (9).

RESULTS

Confluent fibroblasts incubated with 1 μM MTX continue to accumulate nonexchangeable drug for at least 8 h (Fig. 1).

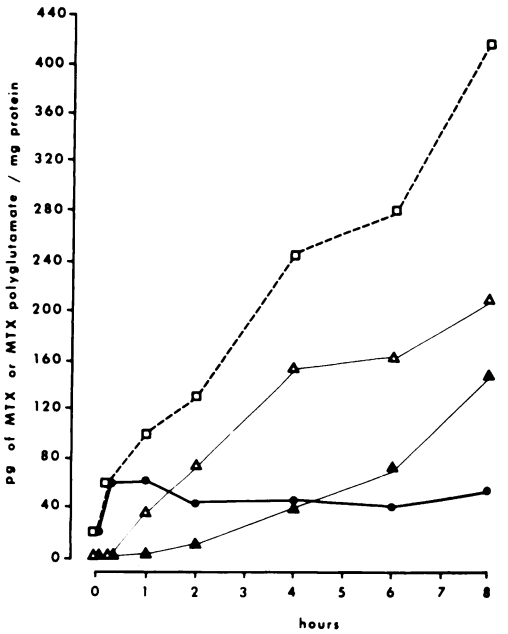

FIG. 1. *Accumulation of nonexchangeable MTX derivatives with time.*

Confluent fibroblasts were incubated in medium containing 1 μM [^{3}H]MTX for the indicated times. The nonexchangeable methotrexate derivatives were determined as described in MATERIALS AND METHODS. (□—□), total nonexchangeable MTX derivatives; (●—●), nonexchangeable MTX; (△—△), nonexchangeable MTX + (G_1); (▲—▲), nonexchangeable MTX + (Gn).

TABLE 1

Exchangeable MTX derivatives in cultured fibroblasts

Confluent fibroblasts were incubated in medium containing 1 μM [^{3}H]MTX for the indicated times. The exchangeable MTX derivatives were determined as described in MATERIALS AND METHODS.

Time	MTX		MTX + (G_1)		MTX + (Gn)	
	pg/mg protein	% of total intracellular nonmetabolized MTX	pg/mg protein	% of total intracellular MTX + (G_1)	pg/mg protein	% of total intracellular MTX + (Gn)
0	361	95.0	0	none present	0	none present
15 min	211	78.4	38	100.0	0	none present
1 h	183	75.0	47	55.8	5	84.6
2 h	231	84.0	56	42.1	9	44.2
4 h	275	85.1	61	28.3	9	16.4
6 h	192	80.8	78	32.5	10	11.4
8 h	181	75.8	78	26.9	13	7.9

Drug accumulation over this period is not inhibited by puromycin levels, which result in 90% inhibition of glycine incorporation into protein.

Figure 1 shows that the level of nonexchangeable but nonmetabolized MTX peaks in these cells within the first hour and then stays constant throughout the experiment. Virtually all of the increase in nonexchangeable cellular MTX after the first hour is due to the formation of MTX polyglutamates.

When cells which have been incubated in 1 μM MTX are transferred into MTX-free PBS at 37° for 1 hr, there is an efflux of both MTX and MTX polyglutamates (Table 1). Initially the majority of the MTX polyglutamates appear in this exchangeable fraction, but with increasing time, most of the polyglutamates are nonexchangeable. Trypan blue exclusion and thymidine incorporation studies demonstrate no loss of viability in these confluent cells.

When fibroblasts that have been incubated for up to 8 h in 1 μM MTX are transferred into MTX-free medium, two patterns of deoxyuridine incorporation are seen (Fig. 2). After short periods of up to 2 hr preincubation with MTX, there is a rapid recovery of deoxyuridine incorporation over the next 24 hr incubation in MTX-free medium. However with longer periods of preincubation with MTX (more than 6 h) there is a prolonged inhibition of deoxyuridine incorporation in the subsequent 24 h, even in the absence of MTX in the second incubation.

Figure 3 demonstrates that with a 16 h preincubation with 1 μM MTX followed by a 24 h recovery incubation in MTX-free medium, there is still marked inhibition of deoxyuridine incorporation into DNA in the subsequent 4 h. In contrast, the greatly increased incorporation of thymidine during the 4 h incubation shows that the MTX effect on deoxyuridine incorporation is not due to cell death.

DISCUSSION

In cultured human fibroblasts the accumulation of MTX occurs first as MTX and then as MTX polyglutamates. Goldman (5) has postulated three states for MTX in L1210 cells. The first is a rapidly-exchanging component thought to represent a low affinity adsorption of MTX to sites on or near the cell membrane. The second is a nonexchangeable fraction believed to be MTX bound to dihydrofolate reductase within the cell. The remaining MTX intracellular is rapidly exchangeable and therefore thought to be present in cell water in an osmotically active state. Goldman went on to show that this last component of MTX is required for maximal inhibition of DNA synthesis in L-cell mouse fibroblasts and Ehrlich ascites cells treated with MTX for short periods of time (7, 10, 11). In addition, he suggested that in chemotherapy protocols which use high dose MTX therapy followed by the reversal of MTX toxicity with 5-CHO-H_4 folate, the "rescue"

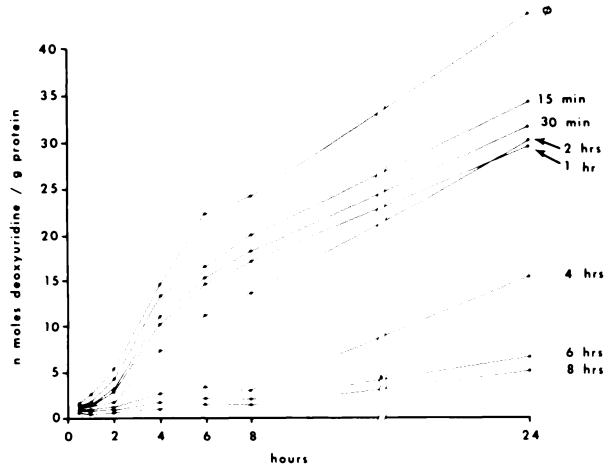

FIG. 2. *Effect of preincubation in MTX on the incorporation of deoxyuridine by fibroblasts.*
Confluent fibroblasts were refed medium containing unlabeled 1 μM MTX for the times indicated on the right margin of the figure. The cells were then refed MTX-free medium containing 2.1 μM [^{3}H]deoxyuridine and the incorporation of radioactivity into a trichloroacetic acid precipitate was determined over the subsequent 24 h.

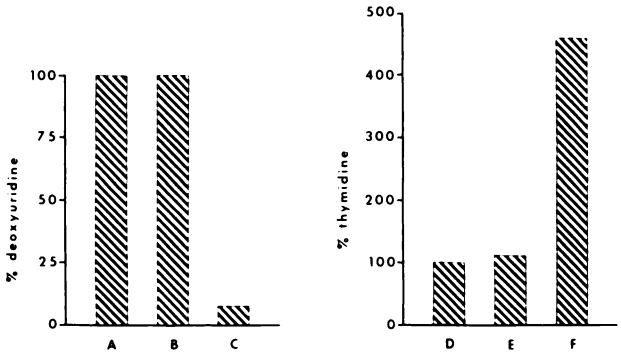

FIG. 3. *Effect of preincubation with MTX on the incorporation of deoxyuridine and thymidine by fibroblasts following a 24 h recovery in MTX-free medium.*
Confluent fibroblasts were incubated in medium containing 1 μM unlabeled MTX for either 0 time (A, D), 1 h (B, E) or 16 h (C, F). The medium was then removed and the cells washed twice with PBS. The cells were next incubated in MTX-free medium for 24 h. The medium was then removed and the cells again washed twice with PBS. Cells were then incubated with 2.1 μM [^{3}H]deoxyuridine (A, B, C) or 2.1 μM [^{3}H]thymidine (D, E, F). The incorporation of radioactivity into a trichloroacetic acid precipitate was determined after 4 h.

may be due in part to the depletion of free intracellular MTX from the cell (12, 13).

Hryniuk (14) studied the effect of prolonged exposure to MTX in L5178Y leukemia cells. He suggested that treatment with 1 μM MTX results in cell death by first producing a purineless and thymineless state through the depletion of cellular pools of reduced folate. He stated that the later effects of the drug were due to the production of a thymineless state as MTX presumably directly blocked dTMP synthesis.

In the present study no MTX polyglutamates are found in cultured human cells until the level of nonexchangeable, nonmetabolized MTX reaches a plateau (Fig. 1). We have already demonstrated that nonexchangeable MTX is not a substrate for polyglutamate synthesis and that polyglutamate synthesis is dependent of the continued presence of MTX in the culture medium (1). The large proportion of the

polyglutamates that is exchangeable early (Table 1) suggests that the later presence of polyglutamates primarily in the nonexchangeable pool is not due exclusively to an inherent inability of polyglutamates to diffuse or to be transported out of the cell. The nature of the polyglutamates in the nonexchangeable pool is as yet undefined. Perhaps the intracellular location of polyglutamates accounts for their retention in the nonexchangeable fraction at later times. Perhaps polyglutamates are bound in the cell to dihydrofolate reductase, to alternate forms of dihydrofolate reductase (14), or even to another folate enzyme such as thymidylate synthetase (15).

Figures 2 and 3 demonstrate that after longer incubations with MTX there is sustained inhibition of deoxyuridine incorporation into DNA. This inhibition is not dependent on the continued presence of MTX in the culture medium and is not dependent on freely diffusible intracellular MTX. This sustained inhibition may be due to the effects of previous exposure to MTX alone, to high levels of total MTX derivatives on the cells, or perhaps to an undefined selective action of the MTX polyglutamates.

These studies have interesting implications with respect to high dose MTX therapy. The relative effectiveness and toxicity of high dose as compared with chronic low dose MTX therapy may be directly related to the kinetics of MTX polyglutamate synthesis in normal and malignant cells. In addition, 5-CHO-H_4 folate "rescue" protocols may be in part explained through depletion of the freely-exchangeable intracellular MTX (12, 13) resulting in a decrease in MTX polyglutamate synthesis (1).

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