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Differential Effects of Folinic Acid and Glycine, Adenosine, and Thymidine as Rescue Agents in Methotrexate-Treated Human Cells in Relation to the Accumulation of Methotrexate Polyglutamates

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SUMMARY

By converting methotrexate (MTX) into poly-γ-glutamyl derivatives, cultured human fibroblasts accumulated high intracellular levels of drug. Once polyglutamates had been formed, DNA synthesis and cell growth remained suppressed even after MTX had been removed from the culture medium. Co-cultivation of cells with MTX and folinic acid reversed the effect of MTX on polyglutamate formation, DNA synthesis, and cell growth. However, if folinic acid was added to the culture medium following a preincubation in methotrexate, DNA synthesis initially remained inhibited and cell growth was only gradually restored. Co-cultivation of cells with 0.67 mM glycine, 37.5 μM adenosine, and 41.3 μM thymidine (GAT) and MTX did not prevent polyglutamate formation but allowed cells to grow. If GAT was removed from the culture medium along with MTX, cell growth and DNA synthesis were inhibited. If GAT was added to the culture medium following growth in MTX, cell growth recovered. These studies differentiate the effects of GAT and folinic acid treatment. Folinic acid prevented MTX polyglutamate accumulation and reversed the effects of MTX on cell growth when present along with MTX in the cultures. Folinic acid was only partially effective in circumventing the MTX-induced block in folate metabolism when added after pretreatment with MTX. In contrast, GAT allowed growth of cells both in the presence of MTX and after a preincubation in MTX. However, co-incubation in MTX plus GAT resulted in the accumulation of polyglutamates and a sustained inhibition of cell growth and DNA synthesis upon removal of both MTX and GAT from the culture medium.

INTRODUCTION

MTX[1] is converted by cultured human fibroblasts (1–4), L1210 cells (5), cultured rat hepatoma cells (6, 7), rat hepatocytes (8, 9) cultured human lymphoma and leukemia cells (10), and cultured human breast cancer cells (11) to poly-γ-glutamyl derivatives. Cultured human fibroblasts which have been incubated for short times in 1 μM MTX rapidly regain the ability to incorporate dUrd into DNA when transferred into MTX-free medium. With longer incubation times associated with the formation of polyglutamates, dUrd incorporation remains inhibited even after transfer of the cells into MTX-free medium (2). Folinic acid or 5-methyltetrahydrofolic acid prevents the accumulation of MTX polyglutamates by cultured human fibroblasts. Direct evidence of depletion of the exchangeable intracellular pool of MTX derivatives suggests that the two reduced folates act primarily by competing with MTX for transport into cells (4). The present study describes the effect of folinic acid on dUrd incorporation. The present study also examines the effect of thymidine, in combination with glycine and adenosine, on both MTX polyglutamate accumulation and dUrd incorporation.

METHODS

Human diploid fibroblasts were derived from punch biopsy specimens of skin from normal individuals and grown in Petri dishes under an atmosphere of 5% CO_2-95% air in Eagle's minimal essential medium (12) containing 2.26 μM folic acid and either 10% fetal calf serum

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[1] The abbreviations used are: MTX, methotrexate (4-amino-10-methylpteroylglutamic acid); folinic acid, 5-formyl-tetrahydrofolic acid (citrovorum factor); GAT, 0.67 mM glycine + 37.5 μM adenosine + 41.3 μM thymidine; MEM, minimal essential medium.

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TABLE 1

Effect of preincubation with MTX ± GAT on the accumulation of MTX polyglutamates in logarithmic-phase fibroblasts

Cultured human fibroblasts were incubated at a density of 10^5 cells in Petri dishes in Eagle's MEM containing 6% fetal calf serum and 6% newborn calf serum and 2.26 μM folic acid. After 3 days, the cells were incubated for 48 hr in medium containing either 1 μM [^{3}H]MTX alone or 1 μM [^{3}H]MTX + GAT. After 48 hr the medium was removed and the cells were washed with 4° phosphate-buffered saline (PBS) three times and then reincubated in 37° PBS for 1 hr. The PBS was removed and the cells were harvested by scraping with a rubber policeman. The levels of non-exchangeable MTX derivatives remaining in the cells were determined as described under Methods. Cells in replicate dishes were refed normal medium without MTX or GAT and harvested as above after an additional 48 hr. The results are the average of either four or five samples ± standard deviation.

Incubating medium	Total MTX + polyglutamates	MTX	MTX (+G_1)[a]	MTX (+G_n)[b]
		nmoles/g protein (% total)		
Initial 48 hr				
MTX alone (n = 4)	22.9 ± 1.6	0.59 ± 0.11 (3.6 ± 1.7)	3.95 ± 0.49 (17 ± 1.0)	18.5 ± 1.08 (79.5 ± 1.9)
MTX + GAT (n = 4)	24.7 ± 2.7	1.40 ± 0.30 (5.7 ± 1.2)	4.53 ± 0.91 (18.2 ± 2.2)	18.8 ± 1.82 (76 ± 2.5)
After additional 48 hr in MTX-free medium				
MTX alone (n = 5)	6.8 ± 1.2	0.93 ± 0.17 (13.7 ± 2.3)	0.75 ± 0.10 (11.1 ± 1.5)	5.4 ± 1.34 (78.3 ± 5.9)
MTX + GAT (n = 5)	7.7 ± 1.9	1.17 ± 0.29 (15.4 ± 2.7)	0.70 ± 0.18 (9.0 ± 0.8)	5.9 ± 1.51 (75.6 ± 2.7)

[a] MTX monoglutamate (4-amino-10-methylpteroylglutamyl-γ-glutamic acid).
[b] MTX polyglutamates longer than MTX(+G_1).

or 6% fetal calf serum plus 6% newborn calf serum. GAT medium contained 0.67 mM glycine, 37.5 μM adenosine, and 41.3 μM thymidine.

All cells were determined to be free of mycoplasma contamination (13). MTX and (±)-folinic acid were obtained from Lederle Products Division, Cyanamid of Canada Ltd. (Montreal, Que.). All other chemicals were of reagent grade. After incubation with labeled MTX at 37°, the cells were rapidly rinsed with phosphate-buffered saline (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) on ice and incubated for 60 min in phosphate-buffered saline at 37°. The methotrexate derivatives found in the buffer after 60 min were designated as the exchangeable fraction and were not differentiated from MTX adsorbed to the cell membrane (4). The derivatives remaining in the cell after the 60-min incubation in phosphate-buffered saline were designated as the non-exchangeable fraction. Separation of MTX polyglutamates was achieved by Sephadex G-15 chromatography as previously described (14). [3′,5′,7-^{3}H] MTX (Amersham/Searle Corporation, Don Mills, Ont.), with an initial specific activity of either 22 Ci/mmole or 35 Ci/mmole, was purified by G-15 gel chromatography (14). Incorporation of dUrd into DNA was determined by measurement of the radioactivity in a 4° trichloroacetic acid precipitate after exposure of the cells to 2.1 μM [^{3}H]dUrd (2).

Incorporation of dUrd into DNA varied with the stage of the culture cycle and with the time of refeeding of the cultures. As previously described (2), dUrd incorporation increased over at least 24 hr. In confluent cultures dUrd incorporation varied from 4.8 to 144.3 nmoles/g of protein per 4 hr depending on the length of time at confluence and the time since refeeding. Similarly, for cultures in logarithmic growth, dUrd incorporation varied from 9.9 to 479.9 nmoles/g of protein per 4 hr. Consequently for each experiment all cells were set up and refed in parallel. Values are expressed as nanomoles of dUrd incorporated per gram of protein per time and are comparable within a given experiment.

RESULTS

Effect of incubation in GAT and MTX on accumulation of MTX polyglutamates. Table 1 shows that incubation of logarithmically growing diploid human fibroblasts for 48 hr in 1 μM MTX resulted in the accumulation of greater that 95% of non-exchangeable drug in the form of polyglutamates. GAT did not alter the accumulation

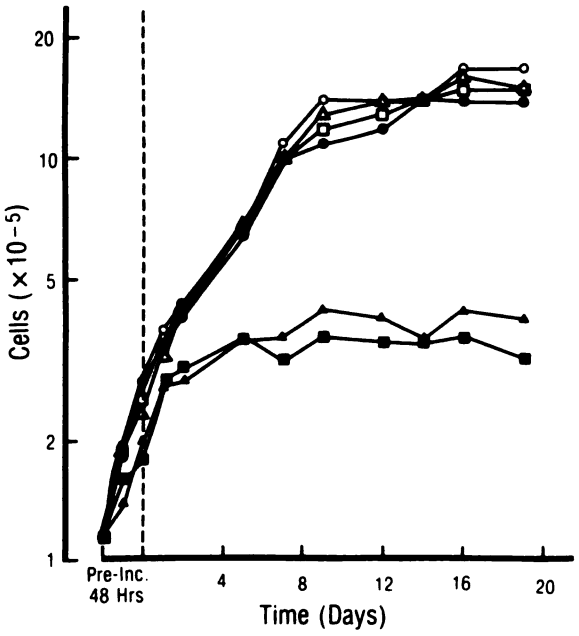

FIG. 1. *Effect of preincubation with (±)-folinic acid (FA) or GAT with or without MTX on growth of cultured fibroblasts*

Cells were plated into Petri dishes containing Eagle's MEM and refed one of the following media for 48 hr: MEM alone (control) (△——△), MEM + 10 μM FA (○——○), MEM + GAT (□——□), MEM + 1 μM MTX (▲——▲), MEM + 1 μM MTX + GAT (■——■), or MEM + 1 μM MTX + 10 μM FA (●——●). After 48 hr as indicated by the *broken line*, the media were removed and all cells were refed MEM alone. The results are the average of triplicate values.

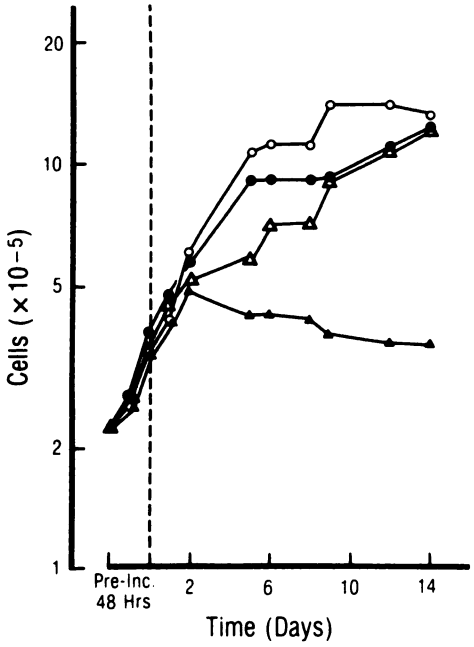

FIG. 2. *Effect of (±)-folinic acid and GAT replacement following preincubation in MTX*

Control fibroblasts (●——●) were incubated in Eagle's MEM for 48 hr and refed Eagle's MEM. The remaining samples were preincubated for 48 hr in Eagle's MEM + 1 μM MTX. The cells were then refed MEM (▲——▲), MEM + GAT (○——○), or MEM + 10 μM (±)-folinic acid (△——△). The results are the average of triplicate values.

of polyglutamates when present in the preincubation medium along with MTX. Following the preincubation in MTX with or without GAT, the cells were refed normal medium lacking both MTX and GAT. There was a dramatic decrease in the level of non-exchangeable MTX derivatives in the cell. The level of nonmetabolized MTX did not alter very much, but the levels of polyglutamates decreased markedly. However, even after 48 hr in MTX-free medium, polyglutamates represented more than 80% of the total non-exchangeable drug in these cells.

Differential effect of folinic acid and GAT preincubation along with MTX on cell growth. Fibroblasts in logarithmic growth were exposed for 48 hr to medium containing either MTX alone, MTX and GAT, or MTX and folinic acid. After the 48 h the cells were refed medium containing no additions. Figure 1 shows that cells preincubated with MTX and folinic acid were able to grow normally. In the presence of both MTX and GAT, cells could grow as long as GAT was maintained in the culture medium. If both GAT and MTX were removed from the medium after the initial preincubation, a sustained inhibition of cell growth was maintained.

Figure 2 shows the effect of supplementation of the culture medium with either GAT or folinic acid following a 48-hr preincubation in 1 μM MTX. If after 48 hr in 1 μM MTX the cells were transferred into MTX-free medium containing neither GAT nor folinic acid, cell growth did not occur. GAT supplementation quickly reversed MTX toxicity, whereas folinic acid allowed return of cell growth but only after a longer delay.

Figures 1 and 2 show clearly a difference in the action of GAT and folinic acid depending on whether these substances were added after treatment with MTX or along with MTX in the preincubation medium.

Effect of GAT and folinic acid on dUrd incorporation. Figure 3 shows the effect of preincubation of logarith-

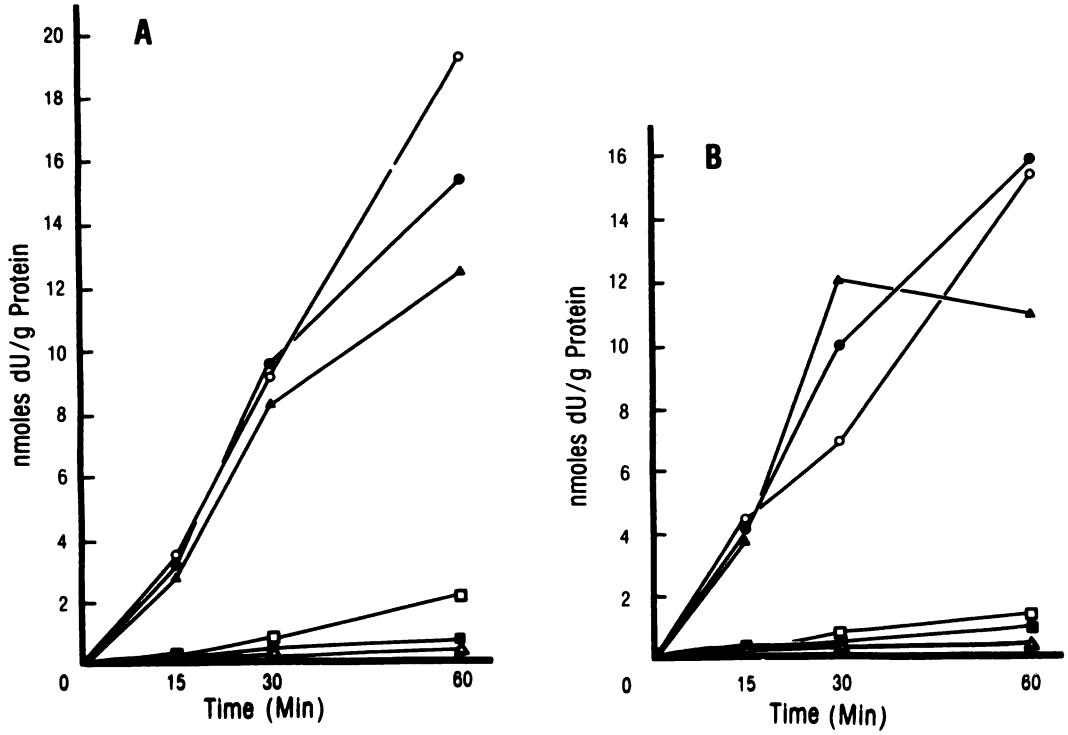

FIG. 3. *Effect of (±)-folinic acid (FA) and GAT on MTX-related inhibition of dUrd incorporation*

Logarithmic-stage cells were preincubated for 16 hr in one of the following media: MEM alone (control) (○——○), MEM + 20 μM FA (●——●), MEM + GAT (□——□), MEM + 1 μM MTX (■——■), MEM + 1 μM MTX + GAT (△——△), or MEM + 1 μM MTX + 20 μM FA (▲——▲). Following preincubation the cells were refed medium containing 2.1 μM dUrd (*dU*) alone (A) or 2.1 μM dUrd + 200 μM FA (B). The results are the average of triplicate values.

TABLE 2

Effect of preincubation with MTX, MTX + GAT, and MTX + folinic acid on the incorporation of dUrd into DNA

Confluent fibroblasts were incubated with the indicated additions for the period of time shown. The cells were then refed MTX-free medium lacking all of the additions (i.e., normal medium) but containing 2.1 μM [^{3}H] dUrd, and the incorporation of radioactivity into a trichloroacetic acid precipitate was determined over the next 24 hr. The values represent the average of triplicate determinations ± standard deviation.

Addition	dUrd incorporation after preincubation for:		
	2 hr	6 hr	16 hr
	nmoles/g protein/24 hr		
None (Eagle's MEM containing 10% fetal calf srum)	91.1 ± 15.6	65.0 ± 7.9	122.9 ± 23.1
MTX (10 μM)	4.4 ± 0.59	1.09 ± 0.16	0.82 ± 0.21
GAT	71.4 ± 6.0	87.8 ± 13.2	326.6 ± 48.5
MTX (10 μM) + GAT	3.2 ± 0.2	1.41 ± 0.62	1.11 ± 0.18
MTX (10 μM) + folinic acid (10 μM)	58.5 ± 16.0	61.6 ± 11.8	210.7 ± 11.6

mically growing cells in MTX with or without GAT or folinic acid on the incorporation of dUrd. Similar results were obtained with confluent cells. Following preincubation for 16 hr, the cells were transferred into control medium or into medium containing folinic acid. Preincubation in MTX and folinic acid resulted in a reversal of the effect of MTX on dUrd incorporation. However, folinic acid concentrations as high as 200 μM did not reverse the effect of a preincubation in 1 μM MTX when added after the preincubation. Since GAT pretreatment for 16 hr suppressed dUrd incorporation over the subsequent 60 min even in the absence of MTX, one cannot evaluate GAT reversal of MTX toxicity using dUrd incorporation. However, Table 2 shows the effect of MTX pretreatment with or without GAT on the incorporation of dUrd in the subsequent 24 hr. Over the 24-hr incubation in dUrd the cells clearly recovered from the effect of GAT alone but not of the effect of MTX or of GAT along with MTX.

DISCUSSION

We have shown previously that the incubation of human fibroblasts in MTX was associated with the formation of polyglutamates and a sustained inhibition of dUrd incorporation even after removal of MTX from the culture medium (1, 2). In order to determine whether the effect of longer exposure to MTX was simply due to the loss of cell viability and not to polyglutamate formation, the cells were preincubated in MTX along with GAT. Because GAT did not inhibit MTX uptake or polyglutamate formation (Table 1), incubation of the cells in GAT allowed MTX to accumulate in viable cells, and the effect of treatment could be compared when both GAT and MTX were removed from the medium. Table 1 shows that removal of GAT and MTX from the medium resulted in a decrease in the level of non-exchangeable MTX polyglutamates over 48 hr but not in the level of non-exchangeable nonmetabolized MTX. Our previous studies (2) in cultured fibroblasts as well as more recent studies in cultured hepatic cells (15) demonstrated that, although MTX polyglutamates could cross the cell membrane, they were preferentially retained by the cell as compared with unmetabolized MTX. It was not clear whether the main result of polyglutamate formation was maintenance of cellular drug levels or whether the polyglutamates had additional sites of action distinct from that of MTX. It has been shown both in cultured H-35 hepatoma cells (7) and in cultured human breast cancer cells (11) that MTX polyglutamates did bind to dihydrofolate reductase, and therefore, like MTX, were involved in inhibition of that target enzyme. The present studies confirm our earlier observation (2) that there was a sustained inhibition of dUrd incorporation and in addition demonstrate an inhibition of cell growth associated with the retention of MTX polyglutamates in the cell.

Folinic acid was able to prevent MTX toxicity if administered in the preincubation medium along with MTX (Figs. 1 and 3; Table 2), presumably because of the effect of folinic acid on MTX uptake and polyglutamate formation (3, 4). Folinic acid was less effective in reversing the toxicity of MTX if added to the medium following a preincubation in MTX. It has been suggested (16, 17) that the competitive relationship between MTX and folinic acid seen under many conditions may have been due to the accumulation of folate pools in the form of dihydrofolate which acted as a competitive substrate versus the inhibitor MTX for dihydrofolate reductase. The present experiments predict that, if the above model is correct, dihydrofolate can compete less well with MTX polyglutamates than with MTX for dihydrofolate reductase under cellular conditions.

The current observations may be relevant to the clinical use of folinic acid and thymidine as rescue agents. Folinic acid, if administered along with MTX, may in fact only be decreasing the effective dose of MTX presented to the cell, and the selective rescue of normal and malignant cells may be attributed to differences in either transport or MTX metabolism. In contrast, the presence of thymidine will not influence the effective dose of MTX presented to the cells, and if the cells remain exposed to MTX, polyglutamate accumulation will continue. Upon removal of thymidine, MTX toxicity should continue in proportion to the residual level of MTX and MTX polyglutamates in the cells.

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