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Fluoropyrimidine-Induced Antagonism to Free and Tightly Bound Methotrexate: Suppression of [^{14}C] Formate Incorporation into RNA and Protein*

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Abstract—*The inhibitory effect of methotrexate (MTX) on* [^{14}C] *formate incorporation into RNA and protein in Ehrlich ascites tumor cells is decreased by pretreatment with fluoropyrimidines. When the basal rate of* [^{3}H] *deoxyuridine (UdR) incorporation into DNA is decreased to* 26, 64 *and* 95% *by 5-fluorodeoxyuridine (FUdR), the inhibitory effect of MTX on* [^{14}C] *formate incorporation into (a) RNA is decreased to* 40, 19 *and* 3%, *and (b) protein is decreased to* 52, 25 *and* 7%. *MTX alone, however, suppressed* [^{14}C] *formate incorporation into RNA and protein* 56 *and* 67%, *respectively.*

Free intracellular MTX is required for maximal suppression of [^{14}C] *formate incorporation into RNA and protein after pretreatment of cells with FUdR. When high-affinity binding sites for MTX are* >95% *saturated with MTX, but free or exchangeable MTX is not present,* [^{14}C] *formate incorporation into RNA and protein is stimulated in the presence of FUdR. However, as the intracellular levels of MTX in excess of the tightly bound fraction is increased with* 1 μM *and* 10 μM *MTX, there is progressive inhibition of* [^{14}C] *formate incorporation into RNA and protein in cells exposed to FUdR. Theses data support the concept that fluoropyrimidine-induced antagonism to MTX is related, in part, to levels of reduced folates available for purine and protein synthesis.*

INTRODUCTION

PREVIOUS studies from this laboratory characterized aspects of the interaction between fluoropyrimidines and methotrexate (MTX), and indicated the importance of the basal rate of thymidylate (dTMP) synthesis in the inhibition of tetrahydrofolate (THF)-dependent nucleic acids and protein synthesis by these agents [1, 2]. These studies clarified the role of fluoropyrimidine antagonism on intracellular levels of MTX beyond that which is necessary for saturation of high-affinity dihydrofolate reductase (DHFR) binding sites.

Goldman *et al.* [3, 4] observed that when cells are loaded with MTX to a level which should saturate more than 99% high-affinity DHFR sites, and then suspended into a MTX-free medium, there is a fraction of intracellular drug that rapidly leaves the cells (free intracellular MTX) and a component which remains tightly bound within the cell (presumably bound to DHFR). Sirotnak *et al.* [5] and Jackson *et al.* [6] reported that efflux of MTX from cells resuspended into a MTX-free medium continues to a point where the cellular level of tightly bound MTX is equivalent to the cellular DHFR concentration. Recently, Cohen and co-workers [7] demonstrated that this tightly bound fraction is MTX complexed with DHFR.

In this study, we have (a) examined the effect of intracellular MTX on THF-dependent RNA and protein synthesis when the rate of deoxyuridine (UdR) incorporation

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into DNA is decreased by fluoropyrimidines, and (b) clarified the role of fluoropyrimidine-induced antagonism to free intracellular MTX and tightly bound MTX on THF-dependent RNA and protein synthesis.

MATERIALS AND METHODS

Chemicals

[^{14}C] Formate, sodium salt, (sp. act. 52mCi/mmole) and [6−^{3}H] UdR (sp. act. 21.9 Ci/mmole) were supplied by New England Nuclear Corporation (Boston, Mass., U.S.A.). MTX was obtained from Lederle Laboratories, and was purified on DEAE-cellulose as previously described [3]. Fluorodeoxyuridine (FUdR) was obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A.

Cells, media and incubation techniques

Ehrlich ascites tumor cells were harvested from CF-1 mice 6–12 days after i.p. inoculation of 0.2 ml undiluted ascitic fluid and washed at least twice in a bicarbonate-buffered electrolyte solution to remove erythrocytes as previously described [3, 8]. For experimentation, cells were suspended into a modified Eagle's medium [4], free of folates, serum or methionine with the following electrolyte composition: 135mM NaCl, 17mM $NaHCO_3$, 1mM Na_2HPO_4, 4mM KCl, 1.9mM $CaCl_2$, and 1mM $MgCl_2$. The cell suspension was continuously stirred by gentle mixing with a Teflon paddle as previously reported [3]. The pH of the suspension was maintained at 7.2–7.4 by passing warmed and humidified 95% O_2–5% CO_2 over the suspension at 37°C.

Incorporation of [^{3}H] UdR into DNA, and [^{14}C] formate into RNA and protein was determined after dilute acid and alkaline extractions of a perchlorate precipitate as reported previously [9]. Data are expressed as means ± S.E.

RESULTS

Figure 1 is a representative experiment which illustrates the effect of intracellular MTX on the rate of [^{14}C] formate incorporation into RNA and protein as the basal rate of [^{3}H] UdR incorporation into DNA is suppressed by FUdR. Ehrlich ascites tumor cells were exposed to FUdR for 15 min to suppress [^{3}H] UdR incorporation into DNA (Fig. 1A). The cells were then separated by centrifugation, washed twice and resuspended into fresh medium without FUdR but in the presence and absence of 10 μM MTX. Thirty min later, when intracellular MTX was at a steady-state with extracellular MTX, [^{14}C] formate incorporation into RNA and protein was monitored over an interval when the rate of incorporation was constant (Figs. 1B and 1C). Cells exposed neither to FUdR or MTX were treated similarly. When the rate of [^{3}H] UdR incorporation into DNA is suppressed by 64% with FUdR, [^{14}C] formate incorporation into RNA and protein in the presence of FUdR plus MTX was inhibited by 19 and 25%, respectively; whereas MTX alone suppressed [^{14}C] incorporation into RNA and protein 38 and 64% (Figs. 1B and 1C). The insets to Figs. 1B and 1C show the rate of [^{14}C] formate incorporation into RNA and protein, respectively, in control cells and in the presence of FUdR.

Figure 2 is a composite of three experiments in which cells were pulsed with 0.05 μM to 5 μM FUdR to achieve a range of [^{3}H] UdR incorporation rates into DNA. The data of Fig. 2 indicate as the rate of [^{3}H] UdR incorporation into DNA is decreased to 26, 64 and 95% by FUdR, the inhibitory effect of 10 μM MTX on [^{14}C] formate incorporation into (a) RNA is decreased to 40, 19 and 3%, and (b) protein is decreased to 52, 25 and 7%; whereas the mean MTX effect on [^{14}C] formate incorporation into RNA and protein is 56.0 ± 10.2% and 67.4 ± 8.9%, respectively.

Figure 3 illustrates an experiment in which Ehrlich ascites tumor cells were loaded with MTX to a level which exceeded the capacity of the intracellular high-affinity binding sites. The cells were then separated and resuspended into a MTX-free medium, and the time course of efflux monitored. Two intracellular components can be distinguished by this technique. One component rapidly leaves the cell (free MTX); the other component appears to be tightly bound within the cell and has been identified as MTX bound to DHFR [7].

To evaluate the role that fluoropyrimidines plus tightly bound and free MTX would have upon the rate of [^{14}C] formate incorporation into RNA and protein, a portion of cells, which had been exposed previously to FUdR, was suspended in 10 μM MTX to saturate high-affinity binding sites.

These cells were divided and incubated in the absence and presence of MTX. After free MTX left cells suspended in a medium containing no MTX and intracellular MTX

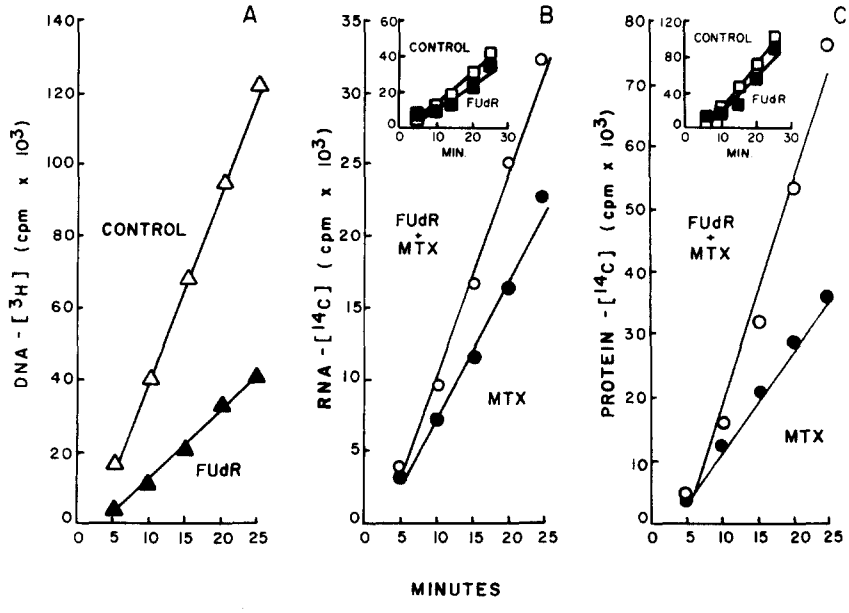

Fig. 1. The effects of MTX on the rate of [^{14}C] *formate incorporation into RNA and protein as the basal rate of* [^{3}H] *UdR incorporation into DNA is decreased by pretreatment with FUdR. Ehrlich ascites tumor cells were incubated with or without* 0.5 μM *FUdR for* 15 min. *The FUdR treated cells were washed twice in a fresh medium at* 37°C, *divided into three portions, and then resuspended into fresh medium in the presence or absence of* 10 μM *MTX. Cells exposed to medium with MTX alone, or without MTX or FUdR were treated similarly. Thirty min later* [^{3}H] *UdR (A) and* [^{14}C] *formate (B and C) were added to achieve a final concentration of* 0.5 μM *and* 100 μM, *respectively. Cell samples were processed as described in Materials and Methods to determine* ^{3}H *associated with DNA and* ^{14}C *associated with RNA and protein.*

reached a steady-state in a medium containing MTX, the cells were centrifuged and suspended in fresh medium of the same composition. [^{14}C] Formate was then added, and radioactivity was determined in RNA (Fig. 4A) and protein (not shown). Control cells and cells exposed only to FUdR or MTX were treated similarly. Figures 4B and C are a composite of nine experiments which summarize the effect of tightly bound and free MTX and FUdR on [^{14}C] formate incorporation into RNA and protein when cells are pretreated with FUdR. With FUdR treatment only, [^{14}C] formate incorporation into RNA and protein is unaffected. The rate of ^{14}C incorporation into RNA in the presence of tightly bound MTX alone is 71% ($P<0.05$) of the control rate (Fig. 4B). In the presence of tightly bound MTX plus FUdR, ^{14}C incorporation into RNA is stimulated by 12% ($P<0.01$) as compared to cells exposed to tightly bound MTX alone. When MTX in excess of the tightly bound fraction is 1μM and 10μM, FUdR induced antagonism to MTX is diminished to 49 and 37% ($P<0.001$) of control rates, respectively. The difference between cells exposed to FUdR plus 1μM free MTX and FUdR plus 10μM free MTX is significant to $P<0.05$. However, 10μM MTX alone produces a small, but significant ($P<0.01$), depression in [^{14}C] formate incorporation when compared to cells exposed to 10μM MTX plus FUdR. The same general pattern is observed for ^{14}C incorporation into protein (Fig. 4C). In the presence of tightly bound MTX alone, cells continue to incorporate [^{14}C] formate into protein at a rate which is 80% ($P<0.1$) of the control rate. Cells exposed to tightly bound MTX plus FUdR increased the rate of ^{14}C incorporation into protein by 13% ($P<0.1$) as compared to tightly bound MTX alone. The difference between the rate of ^{14}C incorporated in cells exposed to FUdR plus 1μM free MTX, FUdR plus 10μM free MTX, and 10μM free MTX is significant to $P<0.1$.

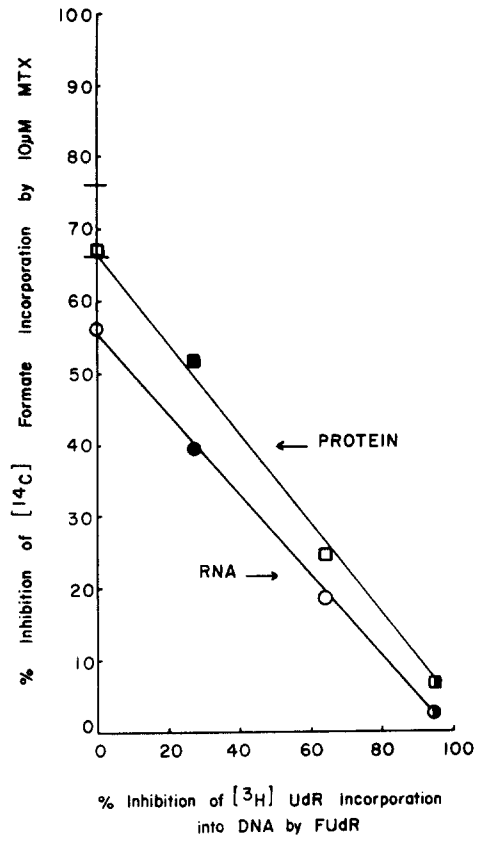

Fig. 2. The inhibitory effect of MTX on the rate of [^{14}C] *formate incorporation into RNA and protein as a function of the percentage of inhibition of the rate of* [^{3}H] *UdR incorporation into DNA by pretreatment with* 0.05 μM, 0.5 μM, *and* 5 μM *FUdR alone. The data are from three experiments, indicated by different symbols, performed as described in the legend to Fig.* 1. *The effect of MTX alone is expressed as the mean* ± S.E. *from three experiments.*

DISCUSSION

The studies reported here indicate that the basal rate of dTMP synthesis can significantly modify the inhibitory action of MTX on RNA and protein synthesis by affecting the rate at which folate coenzymes are utilized for these processes. This was established by examining the effect of FUdR and MTX on [^{14}C] formate incorporation into RNA and protein. Formate carbon is inserted via tetrahydrofolates into purine nucleotides at C-2 and C-8 as well as into methionine, serine and thymidine [10]. The observation that the inhibition by MTX of formate incorporation into RNA is diminished after FUdR pretreatment is consistent with antagonism to the antipurine effect of MTX. This finding is compatible to those studies in which fluoropyrimidines reduced the MTX-induced depletion of deoxypurine nucleotides required for DNA synthesis [2, 11].

The basal rate of dTMP synthesis can affect the inhibitory action of MTX on RNA and protein synthesis by controlling the availability of reduced folates for purine and amino acid synthesis. As the basal rate of dTMP synthesis is decreased by fluoropyrimidines, the requirement for 5, 10-methylene tetrahydrofolate (5, 10-CH_2THF) in the synthesis of dTMP should be decreased, and that portion of 5, 10-CH_2THF which is not part of the

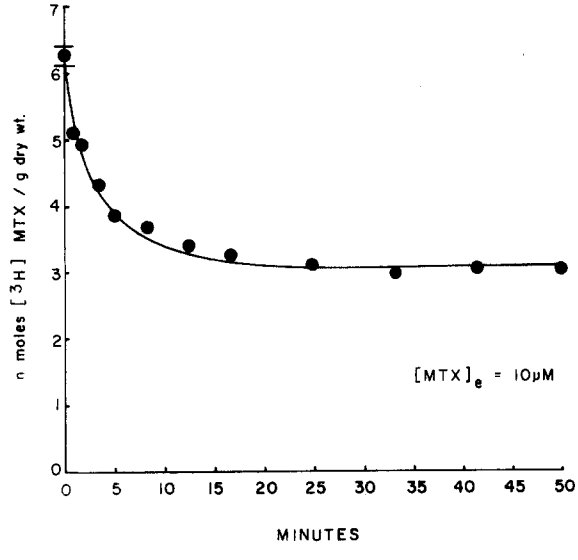

Fig. 3. Exchangeability of intracellular MTX. Cells were incubated with 10μM *MTX for* 30 min *at* 37°C, *then separated by centrifugation, washed twice with* 0°C *buffer, resuspended in a large volume of* 37°C *MTX-free medium, and the fall in the intracellular MTX level was monitored. The intracellular MTX level prior to resuspension (zero-time point) is the mean* ± S.E. *of three determinations.*

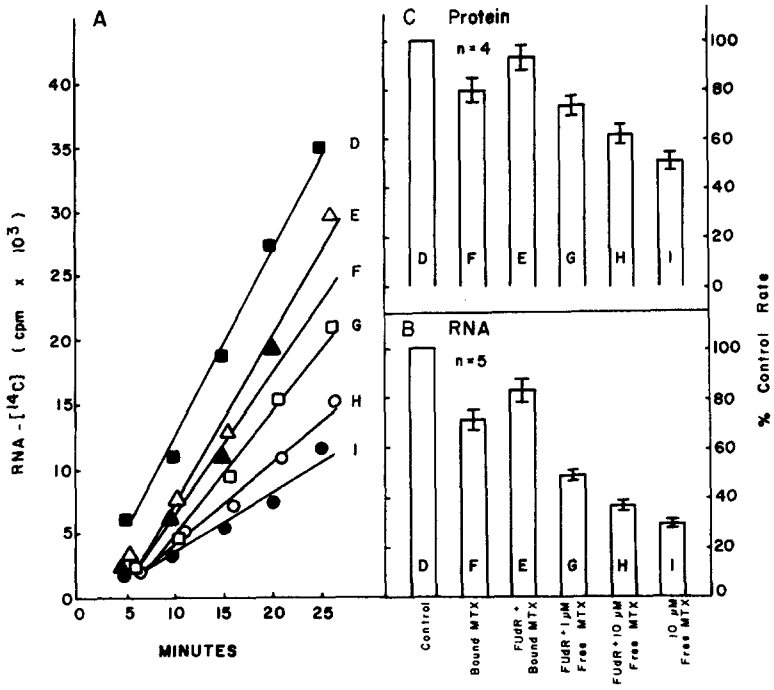

Fig. 4. The effects of tightly bound MTX and exchangeable intracellular MTX on the rate of [^{14}C] *formate incorporation into RNA and protein after pretreatment with FUdR. A cell suspension was divided into two portions. One was exposed to medium containing* 0.05 µM *FUdR for* 15 min, *and the other to a FUdR-MTX-free medium. The cell fractions were separated by centrifugation, washed twice with* 37°C *buffer, further divided into two fractions, and resuspended into fresh medium. The control cells (D) were again suspended in FUdR-MTX-free medium, while the other cells were suspended in medium containing* 10 µM *MTX but no FUdR (F and I), or suspended in medium in the presence of* 10 µM *MTX after FUdR pretreatment (E, G and H). After* 30 min, *the cells were separated, washed with* 0°C *buffer, and resuspended into a large volume of* 37°C *MTX-free medium (D, e and F), or a large volume of medium containing* 1 µM *MTX (G) or* 10 µM *MTX (H and I). The cells were again separated, washed, and resuspended in fresh medium of the same composition. After an additional* 15 min *of incubation, when steady state conditions for intracellular MTX had been achieved,* [^{14}C] *formate was added and the rate of* ^{14}C *incorporation into RNA and protein was monitored. Each value represents the mean of five (B) and four (C) experiments* ± *the* S.E. *determined from an analysis of variance.*

FdUMP-5, 10-CH_2THF–thymidylate synthetase ternary complex will be available in the THF pool for purine and amino acid synthesis. By preventing the oxidation of 5, 10-CH_2THF, FUdR can antagonize the effects of MTX on RNA and protein synthesis. The data in this paper support this formulation and indicate that this interaction is a basis for antagonism between fluoropyrimidines and MTX. Parallel rates of [^{14}C] formate incorporation into RNA and protein (Fig. 2), when UdR incorporation into DNA is depressed by FUdR, suggest the existence of similar rates of THF utilization for purine and amino acid synthesis. Hence, for every molecule of 5, 10-CH_2THF which is converted to 5-methyl THF [12] and then utilized for methionine synthesis (via methyl THF: homocysteine methyl transferase), a molecule of THF is generated and can be utilized for purine biosynthesis. White *et al.* [9] has suggested that the low sensitivity of cells to MTX inhibition of [^{14}C] formate incorporation into protein is compatible with the observation that 5-methyl THF is the major cofactor within the mammalian cell [13] and that

when the regeneration of THF from DHF is inhibited, interconversion enzymes sustain 5-methyl THF at the expense of other reduced folates [12, 14].

These studies further point to the need for increasing the free intracellular MTX concentration in order to deplete cellular stores of reduced folates following fluoropyrimidine treatment (Fig. 4). The rapid decrease of [^{14}C] formate incorporation into RNA and protein by 10μM MTX alone (Figs. 1 and 4) suggest that there are low cellular stores of THF cofactors to sustain these processes and that both reactions are dependent, in part, upon the sustained regeneration of THF from DHF. Under conditions whereby the oxidation of THF cofactors in the synthesis of dTMP has been decreased by fluoropyrimidines, cellular stores of THF cofactors will not be depleted in spite of inhibition of DHFR by MTX. Previous studies by White and Goldman [15] suggested that less than 1% of the total DHFR activity is necessary to maintain normal levels of THF synthesis. Relevant to this were studies by Jackson and Harrap [16] which estimated that only 5% of the total DHFR activity is required to sustain logarithmic growth of L1210 leukemia cells in culture. After FUdR exposure, THF cofactors can be sustained by a smaller fraction of the total DHFR activity. The differences between [^{14}C] formate incorporation in the presence of bound MTX alone and FUdR plus bound MTX indicate (a) the antagonistic nature of these agents when saturation of high-affinity MTX binding sites is at least 95% complete [4, 15] and (b) that sustained metabolism of formate over the interval of these studies is related to regeneration of THF and DHF or utilization of intracellular THF cofactor stores. Since only a small fraction of DHFR is necessary to maintain cellular THF synthesis, and because the rate of THF consumption is slow in fluoropyrimidine-treated cells, a long exposure to high levels of free intracellular MTX would be required to affect THF cofactor stores within the cell.

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