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Insulin effects on methotrexate polyglutamate synthesis and enzyme binding in cultured human breast cancer cells

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Summary. Previous studies have demonstrated that insulin augments methotrexate transport and enhances its cytotoxicity to human breast cancer cells. We therefore investigated the effects of insulin on methotrexate polyglutamate synthesis and binding to dihydrofolate reductase (DHFR) in two human breast cancer cell lines, MCF-7 and MDA-MB-231. Cells were exposed to 2 μ*M* [^{3}H]MTX and varying insulin concentrations for the desired time before determination of the polyglutamate content by high-performance liquid chromatography (HPLC). DHFR-bound drug was separated from free intracellular drug by chromatography on DEAE-Sephacel minicolumns prior to HPLC analysis. Incubation of MCF-7 cells with 2.5 n*M* insulin for 48 h before exposure to 2 μ*M* [^{3}H]MTX for a further 24 h resulted in a significant increase in both total drug and total polyglutamates compared with control cells. Increasing the insulin concentration in the medium yielded further increases in polyglutamylation so that at 250 n*M* insulin and above total polyglutamates were increased by 64% compared with control cells. Further evaluation of the effects of physiologic insulin levels on polyglutamate synthesis revealed that 2.5 n*M* insulin caused an increase in the net glutamylation rate for each polyglutamate derivative during the final 12 h of a 24 h exposure to MTX. Analysis of the effects of insulin on polyglutamate binding to DHFR revealed that exposure to 2.5 n*M* insulin resulted in the preferential binding of higher polyglutamates to DHFR. In MDA-231 cells, a breast cancer cell line with a poor capacity for polyglutamate synthesis, insulin exposure resulted in an increase in the cellular accumulation of each polyglutamate derivative, with the greatest proportionate increases occurring in the cellular levels of higher polyglutamates. These data suggest that insulin augmentation of MTX polyglutamate synthesis may account for its previously observed ability to enhance MTX cytotoxicity.

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Abbreviations: DHFR, dihydrofolate reductase; FCS, fetal calf serum; HPLC, high performance liquid chromatography, MTX, 4-amino-4-deoxy-N-10-methylpteroyl glu 1; PBS, phosphate buffered saline; TCA, trichloroacetic acid

Introduction

In recent years, the intracellular conversion of methotrexate (4-amino-4-deoxy-*N*-10 methyl PteGlu1, MTX) to polyglutamate derivatives has been demonstrated in a number of normal and neoplastic human [13, 21, 22] and animal tissues [7, 10, 26]. In human breast cancer cells, methotrexate polyglutamates containing up to five glutamyl residues have been identified as biologically active drug metabolites that bind to dihydrofolate reductase (DHFR), are retained intracellularly, and prolong the cytotoxic effects of the drug long after extracellular MTX has been removed [16, 24].

Variation in the rate and extent of MTX polyglutamylation among different tissues has been demonstrated and may impart some selectivity to the action of this drug [4, 5]. Recent studies have thus begun to focus on those factors which regulate or enhance polyglutamylation in malignant cells. Insulin has previously been shown to augment MTX uptake by cultured human breast cancer cells [23] and to enhance the cytotoxicity of MTX to these cells up to 10 000-fold [1], though the mechanism of this enhanced cell kill has not been elucidated. Recently, however, Galivan has reported that insulin and dexamethasone stimulate MTX polyglutamylation in cultured rat hepatoma cells [9], thus raising the possibility that insulin's augmentation of MTX cytotoxicity in breast cancer cells could be due to increased accumulation of MTX polyglutamates. In the present study, then, we report the effects of insulin on MTX polyglutamylation in cultured human breast cancer cells.

Materials and methods

Chemicals. [3',5',9-^{3}H]MTX (20 Ci/mmol sp.act.) was purchased from Amersham Corporation (Arlington Heights, Ill) and was further purified by DEAE-cellulose chromatography with elution along a linear gradient of 0.1–0.4 *M* NH_4HCO_3, pH 8.3 [12]. The specific activity of MTX used in each experiment was approximately $2–4 \times 10^{10}$ dpm/μmol. Unlabeled MTX was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, Md) and was purified by the same procedure. Purified synthetic MTX polyglutamates (4-NH_2-10-CH_3 PteGlu 2–4) were provided by Dr C. M. Baugh (Dept of Biochemistry, University of South Alabama, Mobile, Ala) and were used as column markers. Aquasol-2 liquid scin-

tillation counting fluid was purchased from the New England Nuclear Corp. (Boston, Mass), tetrabutyl ammonium phosphate from Kodak Chemicals (Rochester, NY), and high-performance liquid chromatography (HPLC)-grade acetonitrile from Fisher Scientific Co. (Pittsburgh, Pa). All other chemicals were of reagent grade and were purchased either from Fisher Scientific Co. or from Sigma Chemical Co. (St. Louis, Mo). Regular porcine insulin was obtained from Eli Lilly Co. (Indianapolis, Ind) and fetal bovine serum from KC Biologicals, Inc. (Lenexa, Kan). Serum was treated with dextran-coated charcoal as previously described [18].

Propagation of cells in culture. MCF-7 and MDA-MB-231 human breast cancer cells in continuous monolayer culture were provided by Dr Marc Lippman (National Cancer Institute). The human derivation, hormonal responsiveness, and growth characteristics of these cells have been described elsewhere [3]. Both cell lines contain insulin receptors, and the MCF-7 cells respond to insulin with an increase in metabolic activity and growth rate. Cells were maintained in minimum essential medium (MEM, KC Biologicals, Inc.) supplemented with 10% FCS, 4 m*M* *L*-glutamine, penicillin at 124 μg/ml, and streptomycin at 270 μg/ml under 5% CO_2 at 37 °C. Prior to each experiment, cells were passaged three times (9 days) in MEM + 10% charcoal-treated FCS. During the period of incubation with [^{3}H]MTX the medium was changed to MEM without folate or serum but containing 4 m*M* *L*-glutamine, 10 μ*M* thymidine, and 10 μ*M* deoxyinosine plus the desired insulin concentration. This medium allowed cell growth to proceed unperturbed in the presence of MTX.

Assay of intracellular MTX polyglutamates. Following incubation with [^{3}H]MTX, the medium was aspirated from the flasks and the cells were washed twice with ice-cold phosphate-buffered saline. The cells were then scraped from the flask surface with a rubber policeman and lysed by addition of 4.5 ml ice-cold water. The cell lysate was transferred to a test tube containing 0.5 ml 100% trichloroacetic acid (TCA) to give a final TCA concentration of 10%. Cellular debris was pelleted by centrifugation at 10 000 *g* for 15 min, and the supernatant obtained was injected into a Sep-Pak C 18 cartridge (Waters Associates, Milford, Mass) that had been prepared by prior injection with 5 ml 100% CH_3CN followed by 10 ml water. The cartridge was then washed by injecting 10 ml water, after which MTX and its polyglutamyl metabolites were eluted with 3 mlCH_3CN. The sample was evaporated to dryness under N_2 and resuspended in the initial HPLC mobile phase. The total amount of intracellular drug was calculated from the radioactivity in the TCA supernatant, the specific activity of the [^{3}H]MTX used, and the amount of protein in the TCA precipitate measured by the Lowry protein assay [19].

MTX and its polyglutamate derivatives were separated by means of a recently described ion-pairing HPLC assay [15]. The mobile phase was prepared with a model 660 solvent programmer (Waters Associates), which mixed the effluents of two model M6000A pumps (Waters Associates). Mobile phase A was 10 m*M* KH_2PO_4 containing 5 m*M* tetrabutyl ammonium phosphate pH 5.0, and mobile phase B was 100% CH_3CN. Cell extracts containing approximately 5000 dpm were injected into a C18 μ Bondapak column and eluted at 2 ml/min along a gradient of 23%–34% CH_3CN for the first 15 min of analysis, followed by 34% CH_3CN for an additional 8 min. The retention times of authentic 4-NH_2-10-CH_3-PteGlu1–4 were determined by monitoring UV absorbance at 254 nm. Column fractions were collected directly into scintillation vials every 30 s and assayed for radioactivity by liquid scintillation counting in a Beckman LS 6800 liquid scintillation counter.

Separation of DHFR-bound and free intracellular drug. Following drug exposure, cells were washed twice with ice-cold PBS, scraped off the flask surface with a rubber policeman, and lysed by addition of 1 ml KH_2PO_4 0.15 *M*, pH 6.2. Cytosol was obtained by centrifugation at 100 000 *g* for 30 min at 4 °C and was applied to a 0.8 × 2.5 cm DEAE-Sephacel minicolumn equilibrated with 0.15 *M* KH_2PO_4, pH 6.2 at 4 °C. Protein-bound drug was first eluted with 4 ml of the same buffer, after which unbound drug was recovered with 5 ml 1 *M* NH_4HCO_3. Column fractions were collected directly into TCA then prepared for injection onto the HPLC column as described above. Standardization of this minicolumn procedure has been reported previously [16].

Statistical methods. Statistical analysis of the data was performed according to the Wilcoxon signed-rank test.

Results

We first examined total MTX uptake and polyglutamylation over time in MCF-7 cells incubated with 2 μ*M* [^{3}H]MTX in the absence or presence of 2.5 n*M* insulin. In these experiments, insulin and MTX were added simultaneously to the cell cultures. Figure 1 demonstrates that insulin had little effect on either total drug accumulation or total polyglutamate synthesis during the initial 18 h of drug exposure. By 24 h, however, insulin-treated cells contained significantly more total drug and MTX polyglutamates than control cells. This finding suggested that insulin's influence on MTX metabolism was not immediate and might be enhanced by a period of preincubation with the hormone prior to exposure to MTX. Thus, in all subsequent experiments, insulin (or buffer) was added to flasks of MCF-7 cells 48 h before incubation with 2 μ*M* [^{3}H]MTX for a further 24 h. Figure 2 illustrates the effects of varying insulin concentrations on MTX uptake and polyglutamylation in this cell line. Preincubation with 2.5 n*M* insulin resulted in a significant increase in both total drug (24% above control) and total polyglutamates (27% above control) accumulated during the subsequent MTX exposure ($P < 0.01$ compared with control for all insulin concentrations). Increasing the insulin concentration in the medium yielded further increases in MTX uptake and polyglutamylation, so that at an insulin concentration of 250 n*M*, total drug and total polyglutamates were increased by 46% and 64%, respectively, compared with non-insulin-treated cells.

To evaluate the effects of physiologic levels of insulin on polyglutamate synthesis further, we next examined the influence of preincubation with 2.5 n*M* insulin on the rate and extent of accumulation of each polyglutamate formed by MCF-7 cells. Figure 3 depicts the time course of MTX polyglutamate formation in control cells (Fig. 3 A) and in cells incubated with 2.5 n*M* insulin for 48 h before the ad-

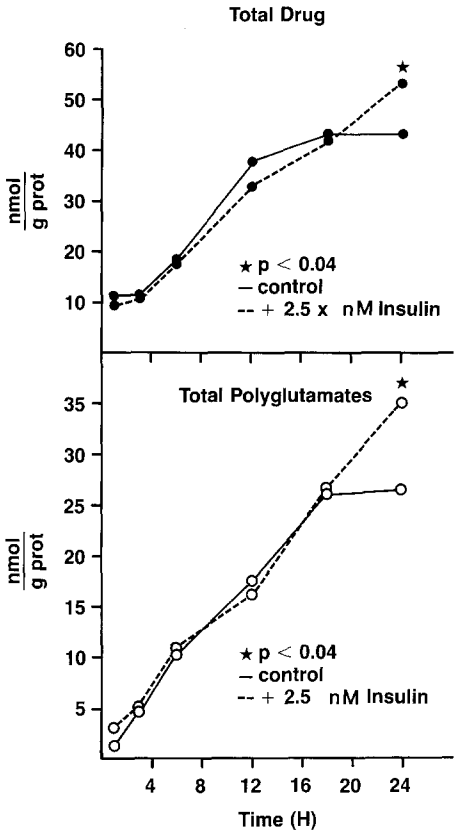

Fig. 1. Time course of insulin effect on accumulation of total drug and total polyglutamates in MCF-7 cells. MCF-7 cells were incubated with 2 μ*M* [^{3}H]MTX for varying periods of time in the absence (–) or presence (– – –) of 2.5 n*M* insulin before HPLC analysis. Insulin and MTX were added simultaneously to the cell cultures. Each *point* represents the mean of four experiments

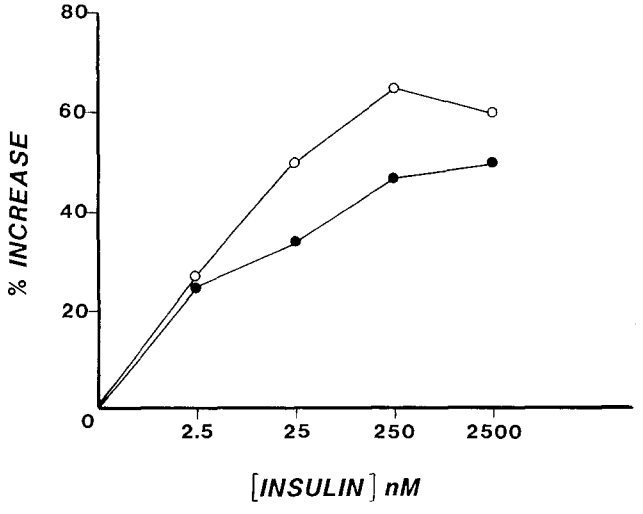

Fig. 2. Dose-response relationship for insulin effect on accumulation of total drug (●) and total polyglutamates (○) in MCF-7 cells. MCF-7 cells were preincubated with varying concentrations of insulin for 48 h before exposure to 2 μ*M* [^{3}H]MTX for a further 24 h. Cells were then harvested and analyzed by HPLC. Each *point* represents the mean of four experiments. For all insulin concentrations, total drug and total polyglutamate levels were significantly increased above control values ($P<0.01$)

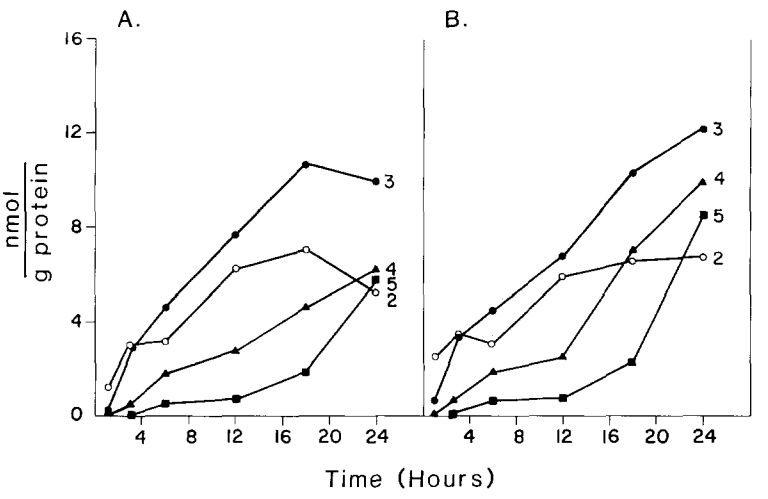

Fig. 3. A, B. Effect of 2.5 n*M* insulin on the time course of MTX polyglutamate synthesis in MCF-7 cells. MCF-7 cells were incubated in the absence **(A)** or presence **(B)** of 2.5 n*M* insulin for 48 h before exposure to 2 μ*M* [^{3}H]MTX for a further 24 h. Cells were then harvested and analyzed by HPLC. Each *point* represents the mean of four experiments. At 24 h, all polyglutamate levels were significantly higher ($P<0.05$) in insulin-treated than in control cells. *2* = 4-NH_2-10-CH_3-PteGlu2; *3* = 4-NH_2-10-CH_3-PteGlu3; *4* = 4-NH_2-10-CH_3-PteGlu4; *5* = 4-NH_2-10-CH_3-PteGlu5

dition of 2 μ*M* [^{3}H]MTX (Fig. 3 B). Insulin enhances the rate of formation and augments the overall accumulation of each polyglutamate derivative, and it appears to be particularly effective in increasing the formation of long-chain derivatives. This becomes quite evident upon examination of the glutamylation rates shown in Table 1, which displays the effects of insulin on the rate of formation of each polyglutamate during the final 12 h of incubation with MTX. Clearly, incubation of breast cancer cells with low concentrations of insulin results in a substantial increase in the rate of formation of polyglutamate metabolites of MTX particularly during the final 12 h of drug incubation, when the synthesis of long-chain derivatives is greatest.

Previous studies from several laboratories have clearly demonstrated that MTX polyglutamates bind to DHFR with an affinity at least equal to that of the parent compound [6, 8, 24]. In human breast cancer cells, long-chain polyglutamates have also been shown to dissociate from DHFR more slowly than mono- and diglutamates, thereby functioning as more efficient enzyme inhibitors than the parent drug [14]. These data led us to examine the influence of insulin on MTX polyglutamate binding to DHFR. MCF-7 cells were incubated for 48 h with 2.5 n*M* insulin before exposure to 2 μ*M* [^{3}H]MTX for a further 24 h. Cells were then harvested, and enzyme-bound and free intracellular drug was determined as described above. As shown in Table 2, MTX polyglutamates are the predominant drug form bound to DHFR, comprising approximately 60% of total enzyme-bound drug in both control and insulin-treated cells. Though insulin produces no significant differences in the total amount of enzyme-bound drug, it does result in a substantial change in the pattern of polyglutamate binding to DHFR. As shown in Fig. 4 A, insulin exposure results in a marked increase in the proportion of

Table 1. Glutamylation rate during final 12 h of methotrexate incubation

	nmol/g/h				
	Total polyglutamates	Glu 2	Glu 3	Glu 4	Glu 5
Control	0.99 ± 0.24[a]	−0.082 ± 0.04	0.186 ± 0.05	0.278 ± 0.05	0.423 ± 0.06
2.5 n*M* Insulin	1.56 ± 0.20	0.069 ± 0.05	0.458 ± 0.05	0.616 ± 0.05	0.649 ± 0.11

[a] Mean ± SEM for four experiments

Table 2. Insulin effects on MTX polyglutamate binding to DHFR in MCF-7 cells

	DHFR-bound drug (nmol/g protein)		
	MTX	Polyglutamates	Total
Control	2.07 ± 0.54[a]	2.59 ± 0.39	4.66 ± 0.82
2.5 n*M* Insulin	1.81 ± 0.48	2.95 ± 0.50	4.78 ± 0.97

[a] Mean ± SEM for four experiments

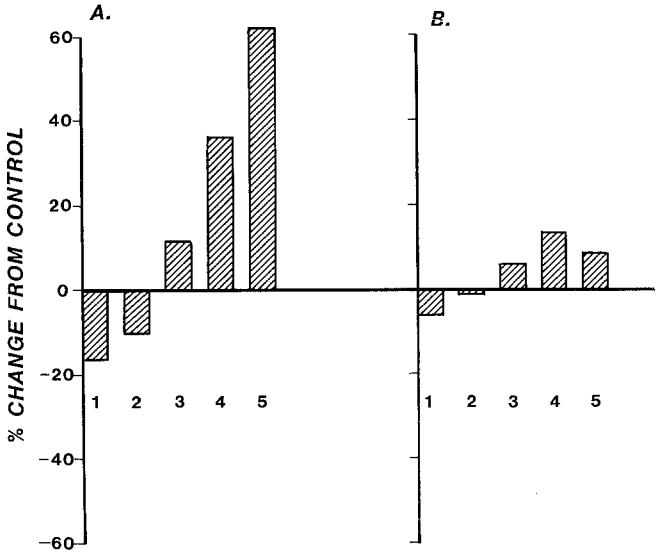

Fig. 4 A, B. Insulin effects on binding of MTX polyglutamates to DHFR in MCF-7 cells. MCF-7 cells were preincubated with 2.5 n*M* insulin for 48 h before exposure to 2 μ*M* [^{3}H]MTX for a further 24 h. Cells were harvested and analyzed for DHFR-bound and free intracellular drug. Results in insulin-treated cells are expressed as percentage change from control for both DHFR-bound **(A)** and free intracellular drug **(B)**. *1* = 4-NH_2-10-CH_3-PteGlu1 (MTX); *2* = 4-NH_2-10-CH_3PteGlu2; *3* = 4-NH_2-10-CH_3-PteGlu3; *4* = 4-NH_2-10-CH_3-PteGlu4; *5* = 4-NH-10-CH_3-PteGlu5

long-chain polyglutamates bound to the enzyme compared with control cells, so that binding of 4-NH_2-10-CH_3-PteGlu4 is increased by 36% and that of Glu5, by 62% compared with control. Though long-chain polyglutamates are also increased in the unbound fraction (Fig. 4B) following insulin exposure, the relative increases are considerably greater in the enzyme-bound-fraction, suggesting an insulin-mediated preferential increase in the binding of higher polyglutamates to DHFR.

Previous studies from our laboratory [16, 24] have demonstrated considerable heterogeneity among human breast cancer cell lines in the capacity for MTX polyglutamate synthesis. Unlike MCF-7 cells, MDA-231 human breast cancer cells have a limited capacity for MTX polyglutamate synthesis, with little or no synthesis of higher polyglutamates occurring until extracellular MTX concentrations are at least 10 μ*M* [16]. In the present study, we examined the influence of preincubation with 2.5 n*M* insulin on polyglutamate synthesis by MDA-231 cells exposed to 2 μ*M* [^{3}H]MTX. As shown in Table 3, insulin exposure results in an increase in the cellular accumulation of each polyglutamate derivative, with the greatest proportionate increases occurring in the higher polyglutamates. 4-NH_2-10-CH_3-PteGlu5 is increased by 93%, for example, whereas 4-NH_2-10-CH_3PteGlu2 is increased by only 19% versus non-insulin-treated cells. While the extent of higher polyglutamate synthesis remains small even in the presence of insulin, the hormonal stimulation results in higher polyglutamate production in amounts similar to those observed in this cell line following exposure to 10 μ*M* MTX without insulin [16] and similar to those previously reported in another MDA human breast cancer cell line (MDA-MB-436) following exposure to supraphysiologic insulin levels [17].

Discussion

In this paper, we have examined the effects of insulin on MTX polyglutamylation and enzyme binding in human

Table 3. Insulin effects on MTX polygutamate synthesis in MDA-231 human breast cancer cells

	nmol/g protein (% increase)				
	MTX	Glu 2	Glu 3	Glu 4	Glu 5
Control	6.99 ± 0.67[a]	0.95 ± 0.22	1.23 ± 0.08	0.50 ± 0.09	0.15 ± 0.03
2.5 n*M* Insulin	6.38 ± 1.1	1.12 ± 0.27 (19 ± 9.6)	1.70 ± 0.12* (39 ± 12)	0.75 ± 0.12* (52 ± 5)	0.30 ± 0.07* (93 ± 15)

[a] Mean ± SEM for four experiments
* $P < 0.04$ versus control

breast cancer cells. Preincubation of MCF-7 cells with low levels of insulin clearly augments the overall rate of polyglutamate synthesis, leading to the accumulation of increased intracellular levels of each polyglutamate derivative following a 24 h incubation with MTX. Indeed, the results reported here may represent only the minimum effect of insulin on MTX polyglutamylation, as the cells were maintained in medium containing charcoal-treated serum (rather than serum-free medium) during the 48 h period of insulin exposure prior to the addition of MTX. The presence of insulin or insulin-like peptides in the serum could potentially have blunted the effect of additional exogenous insulin. In rat hepatoma cells, for example, physiologic concentrations of insulin do not exert appreciable effects on glutamylation until the cells have been serum-free for at least 48 h [9]. Thus, it seems quite possible that the stimulatory effects of insulin observed in the present study represent a submaximal effect. Nevertheless, these studies demonstrate the important role of insulin in modulating the glutamylation of MTX.

The overall increase in polyglutamate accumulation appears to result both from an increase in the polyglutamate synthetic rate and from the maintenance of this high rate of synthesis for a prolonged period of time. As shown in Fig. 3 and Table 1, preincubation of MCF-7 cells with 2.5 n*M* insulin enhances the rate of long-chain polyglutamate synthesis and prevents the decline in synthesis of short-chain derivatives which tends to occur in control cells following a 24 h incubation with MTX. The primary mechanism whereby insulin exerts these effects has not been defined in the present studies, but stimulation of folylpolyglutamate synthetase activity, inhibition of conjugase activity, or enhanced membrane transport of MTX, as has previously been observed following insulin exposure [23] are certainly possible sites of insulin action.

The binding of MTX polyglutamates to DHFR has been a subject of intense interest in recent years. Previous studies from our laboratory and others have shown that MTX polyglutamates bind to DHFR with an affinity at least equal to that of the parent compound [6, 8, 24]. Recent studies in human breast cancer cells have now demonstrated that long-chain polyglutamates dissociate from DHFR more slowly than do mono- and diglutamates, resulting in more prolonged enzyme inhibition by the longer chain derivatives [14]. These studies lend particular importance to our observation that insulin exposure results in the preferential binding of higher polyglutamates to DHFR. While the DHFR-binding capacity of MCF-7 cells remains essentially unchanged by insulin, there is a shift in the pattern of polyglutamate binding to DHFR, such that the binding of the mono- and diglutamates is diminished while that of the pentaglutamate is considerably enhanced compared with control cells. This shift in the pattern of polyglutamate binding would clearly have the potential to prolong the duration of enzyme saturation and drug effect.

Enhanced tumor cell killing by drugs in the presence of hormones has been demonstrated for MTX [1], cytosine arabinoside [25], cyclophosphamide [20], and other agents [2, 11]. Few studies, however, have been devoted to the biochemical mechanisms of such enhanced cytotoxicity. In the present studies we have begun to gain some insight into the ways in which hormones can modulate the biochemical pharmacology of antineoplastic drugs. Further understanding of drug-hormone interactions at the cellular level may lead to a more rational approach to the use of drug-hormone combinations in clinical practice.

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