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Regulation of methotrexate polyglutamate accumulation *in vitro*: effects of cellular folate content

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Recent studies by a number of investigators have indicated that conversion of the folate analog methotrexate to γ-glutamyl derivatives is widespread and probably plays an important role in the antitumor activity of this compound [1–6]. Investigations into the nature of the γ-glutamylation reaction are still in their initial phases. It seems fairly certain from inhibition studies with partially purified enzyme preparations [7] and cultured cell systems [8, 9] and from genetic and biochemical analyses [10, 11] that the same enzyme in mammalian cells that catalyzes the γ-glutamylation of the folate coenzymes also catalyzes the γ-glutamylation of analogs, such as methotrexate.

Little is known about the metabolic control of polyglutamate formation and accumulation but some evidence indicates that these processes may be self-regulating. The proportion of longer-chain folypolyglutamates was highest in the livers of folate-deficient rats and lowest in the livers of folate-supplemented rats [12]. Similar observations have been made with cultured mouse hepatoma cells (D. M. Priest, personal communication; [13]). The synthesis of longer-chain length polyglutamates in the presence of limiting substrate was also obtained with isolated rat liver folypolyglutamate synthetase [7] and with methotrexate polyglutamate formation in cultured hepatocytes [9].

Further evidence for regulation of polyglutamate accumulation came from the observation of saturable net synthesis of methotrexate polyglutamates in hepatocytes and H35 hepatoma cells in culture [9]. In the presence of high extracellular concentrations of methotrexate, the formation of methotrexate polyglutamates was restricted despite the relatively large amounts of unreacted methotrexate in the cell.

Several studies have indicated that addition of methotrexate to cells or tissues reduces the amounts of cellular folates [14–16]. These results imply a relationship between folyl and methotrexate polyglutamates in regulating their own and each other's synthesis. To examine this possibility, we have investigated the effect of depletion of folate pools on the synthesis of methotrexate polyglutamate in rat H35 hepatoma cells *in vitro*.

Methods and results

Insulin was obtained from Eli Lilly & Co., lysolecithin (Type I) from the Sigma Chemical Co., and folinic acid from ICN Phamaceuticals. [3′,5′,7-^{3}H]Methotrexate and [3′,5′,7-^{3}H]folic acid were from Amersham and [G-^{3}H]folinic acid was from Moravek Chemicals. Published procedures were utilized for the purification of radiolabeled folic acid [17] and folinic acid [18].

H35 hepatoma cells were cultured as described previously [2,9] and utilized at confluency, which occurs after 96 hr of growth. The cells were then incubated under the conditions specified, with purified [3′,5′,7-^{3}H]methotrexate ($5–50 \times 10^4$ dpm/nmole) [2,9]. The cellular contents were extracted, and the intracellular concentration of the methotrexate pool was analyzed as described previously [2,9]. The results are expressed as molar concentration based upon 1 mg cell protein being equivalent to 3.15 μl of cell water [2]. The composition of the methotrexate and methotrexate polyglutamates with the contents of two 60 mm culture dishes was established by the high-pressure liquid chromatography described by Fry *et al.* [5] and DEAE cellulose chromatography [9].

Permeabilized H35 cells were prepared by a modification of the lysolecithin technique [19]. This procedure was altered by including 1 mM $CaCl_2$ in the lysolecithin solution and reducing the incubation time to 60 sec, which resulted in a 33% increase in cell yields over that previously reported 24 hr after lysolecithin treatment [19]. Following lysolecithin treatment, the cultures were allowed to seal in folate-free Swims S-77 medium with 10 mU insulin/ml for 24 hr before adding [3′,5′,7-^{3}H]methotrexate. As before, the viability of the sealed cells was 100% when analyzed with Trypan blue [19].

Previous studies have demonstrated [9] that the net formation of methotrexate polyglutamates in folate-supplemented H35 hepatoma cells is saturable with respect to time and to the extracellular concentration of methotrexate. In that study and the present one, saturation was reached at 10 μM methotrexate, and little further synthesis of polyglutamates was observed after a 24-hr incubation. The cellular concentration of methotrexate polyglutamates under control conditions is shown in Table 1. Parallel experiments were performed with cells whose folate contents had been restricted by omission of folates from the medium or by lysolecithin treatment [17] followed by folate omission.

Absence of folic acid from the medium during the 24 hr prior to methotrexate addition and during the incubation with methotrexate caused a 1.3-fold increase in the methotrexate polyglutamate pools. Greater increase was observed when the cells were permeabilized with lysolecithin and then cultured in folate-lacking medium. This treatment was shown to result in an 85–90% decrease in folate and folylpolyglutamate pools [19] and allowed the cells to accumulate 1.65 times as much methotrexate polyglutamates as did the control cells (Table 1).

The dependence of methotrexate polyglutamate synthesis on the medium concentration of methotrexate is depicted in Fig. 1. The cellular concentrations of methotrexate polyglutamates were elevated in the folate-restricted and folate-depleted cultures at subsaturating and

Table 1. Cellular concentration of methotrexate polyglutamates after a 24-hr incubation of H35 hepatoma cells with 10 μM methotrexate*

Cells	Methotrexate polyglutamates (μM)
Control	62 ± 10.1
Folate-restricted	79.4 ± 13
Folate-depleted	102.2 ± 13.2

* Prior to the 24-hr incubation with [3′,5′,7-^{3}H]methotrexate, control cells were cultured in Swims S77 medium plus 10 mU insulin/ml for 24 hr, folate-restricted cells were incubated with Swims S77 lacking folic acid but with insulin for 24 hr, and folate-depleted cells were treated with lysolecithin (see Methods and Results) and then incubated as the folate-restricted cells were. The methotrexate polyglutamate concentration is the total of all forms. Values are means ± S.D., N = 15.

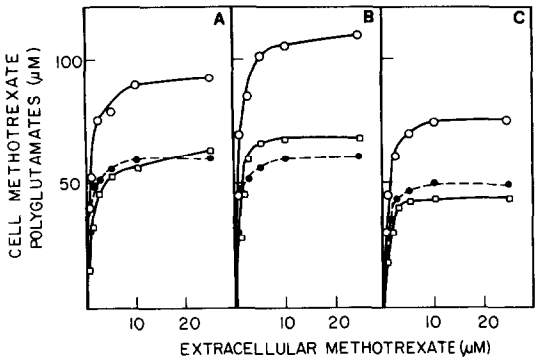

Fig. 1. Formation of methotrexate polyglutamates as a function of extracellular methotrexate. The cell preparation and incubation conditions are identical to those in Table 1. (A) Folate-restricted cells with no additions (○) or with 20 μM folinic acid (□) present during the 24 hr before [3′,5′,7-^{3}H]methotrexate was added. (B) Folate-depleted cells with no additions (○) or with 20 μM folinic acid (□) present for 24 hr before [3′,5′,7-^{3}H]methotrexate was added. In (A) and (B) the dashed line (●---●) represents control cells as described in Table 1. (C) Preparations identical to those in (A), except that insulin was omitted throughout.

saturating medium concentrations of methotrexate. When folinic acid was included during the 24 hr before methotrexate was added to restore the folate pools, The accumulation of methotrexate polyglutamates was reduced to control levels in both folate-restricted and folate-depleted cells. Similar results were observed if 5-CH_3H_4PteGlu* or unlabeled methotrexate was added instead of folinic acid (J. Galivan, unpublished results).

The concentrations of methotrexate polyglutamates observed in control cells (Fig. 1, A and B) were somewhat higher than those reported previously [9]. This appears to have been due to the presence of insulin, which is required for the sealing of lysolecithin-permeabilized cells [19].

* Abbreviations: 4-NH_2-10-CH_3PteGlu$_n$, the glutamyl derivatives of methotrexate (n = 1), where n indicates the total number of glutamate residues; and 5-CH_3H_4PteGlu, 5-methyl-5,6,7,8-tetrahydrdofolic acid.

When the experiment with folate-restricted cells (Fig. 1A) was repeated in the absence of insulin (Fig. 1C), the same effects of folate omission and folinic acid addition were observed, but the absolute concentrations of methotrexate polyglutamates were reduced by 20–30%. The concentration of methotrexate polyglutamate in control cells not exposed to insulin was between 40 and 50 μM, which is compatible with our previous results [9]. Addition of folinic acid to the folate-replete (control) cultures prior to methotrexate incubation (Fig. 1, A–C) caused only a modest reduction in methotrexate polyglutamate concentration, resulting in 35–40 μM methotrexate polyglutamates at 10 μM extracellular methotrexate after a 24-hr incubation.

The distribution of major methotrexate polyglutamates under various conditions of incubation with methotrexate is shown in Fig. 2. In all cases 4-NH_2-10-CH_3PteGlu$_4$ was the most common single species. The cells cultured with folates were similar to folate-restricted and -depleted cells in that longer-chain species were favoured at lower extracellular methotrexate concentrations. The folate-restricted and -depleted cells differed from controls in that they contained higher concentrations of 4-NH_2-10-CH_3PteGlu$_5$ at low extracellular methotrexate concentrations. This was prevented by adding folinic acid for 24 hr prior to the incubation with methotrexate.

The extent of loss of folate pools by the manipulations described above and of restoration by folinic acid was examined (Table 2). Control cells with 10 mU insulin/ml had a cellular folate concentration of just below 100 nmoles/g cellular protein, which was expanded approximately 8-fold by inclusion of 20 μM folinic acid. The presence of the folinic acid caused only a marginal reduction (10–25%) in the accumulation of methotrexate polyglutamates in control cells. In both folate-restricted and folate-depleted cells, there was an extensive loss of the folate pools at the beginning and end of the incubation with methotrexate, and the effect was more pronounced in the lysolecithin-treated cells. Restoration of the folates with folinic acid, which reduced methotrexate polyglutamate accumulation to control levels, was also established. Folinate was a better source of cellular folates than folic acid which can be related to its 4-fold higher concentration in the medium and its greater capacity for accumulation and glutamylation in mammalian systems [7,11,20]. Lysolecithin-permeabilized H35 cells upon sealing generated a cellular folate concentration in the presence of folinic acid similar to control cells which further substantiates the

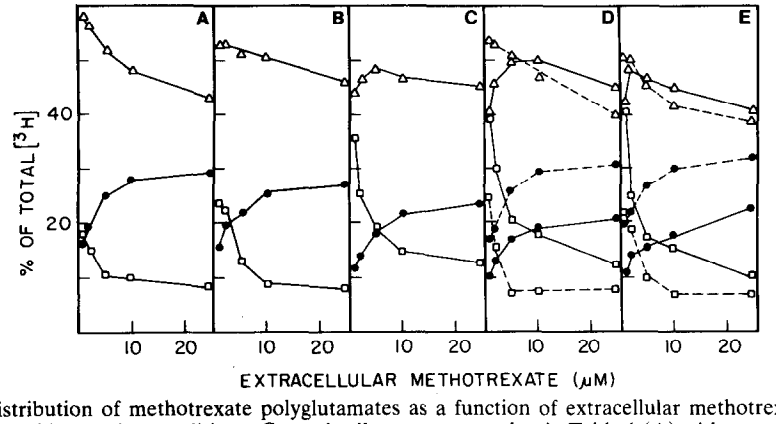

Fig. 2. Distribution of methotrexate polyglutamates as a function of extracellular methotrexate concentration and incubation conditions. Control cells were prepared as in Table 1 (A) without or (B) with 10 mU insulin/ml. Folate-restricted cells (C) without or (D) with 10 mU insulin/ml and (E) folate-depleted cells were cultured as in Table 1. The cultures were incubated with [3′,5′,7-^{3}H]methotrexate for 24 hr, and the methotrexate polyglutamates were identified as described in Methods and Results. Cultures which had been exposed to 20 μM folinic acid for 24 hr prior to methotrexate addition are represented by the dashed line (-----) in (D) and (E). The percentage of the total cellular pool is shown for the major species: 4-NH_2-10-CH_3PteGlu$_3$ (●), -Glu$_4$ (△), and -Glu$_5$ (□).

Table 2. Cellular folate pools as a function of limiting folates and restoration with folinic acid*

Cells	Total cellular folates (nmoles/g cell protein) 0 hr	24 hr
Control	96	88
Folinic acid	750	605
Folate-restricted	62	41
Folinic acid	685	620
Folate-depleted	17	9.8
Folinic acid	866	740

* Cultures were prepared as in Table 1 except that the cells were grown in 4.5 μM [3′,5′,7-^{3}H]folic acid (2.7×10^5 dpm/nmole) for 120 hr (five generations). Where indicated, 20 μM [G-^{3}H]folinic acid (2.7×10^5 dpm/nmoles) was added for 24 hr prior to the addition of unlabeled methotrexate. The total folate content of the cells was measured at the time of addition of methotrexate (0 hr) and after 24 hr in the presence of methotrexate.

similarity of these preparations to untreated cultures [19]. Further studies will be needed to determine the relative capacity of folinic acid and other reduced folates to replenish cellular folate pools and the subsequent effects on methotrexate polyglutamate pools.

Discussion

The polyglutamate derivatives of the folates [20] and methotrexate [3–6,9] have been established as forms which are retained by cells. In attempting to understand the processes that regulate the accumulation of polyglutamate derivatives, we have examined the effect of the reduction of folate pools on methotrexate polyglutamate synthesis in H35 hepatoma cells.

Two techniques have been used to assess the effects of cellular folates on methotrexate polyglutamate accumulation. First, removal of folate from the medium causes cells which have been grown in [^{3}H]folate to lose 35% of the radiolabel after 24 hr in culture and 55% after 48 hr. Such cells, when incubated with 10 μM methotrexate between 24 and 48 hr, still demonstrate saturable methotrexate polyglutamate synthesis [9] but accumulate 1.28 times as much methotrexate as those in control cultures. Second, an even greater increase in methotrexate polyglutamates is observed when cells are permeabilized with lysolecithin prior to culture in folate-lacking medium. The higher concentrations of methotrexate polyglutamates under these conditions parallel the more extensive but not complete loss of folate and folylpolyglutamates caused by lysolecithin (Table 2, [19]). The total folate pool was not lost, presumably due to the fraction of cells (5–15%) which did not permeabilize [19] and the possibility of high affinity intracellular folate or folylpolyglutamate binders. It is conceivable that even higher levels of methotrexate polyglutamates could be accumulated in cells completely devoid of folates. When reduced folates were added to the folate-starved or -depleted cells for 24 hr prior to methotrexate addition, methotrexate polyglutamate concentration and distribution returned to control values.

It is interesting to compare the concentration of methotrexate polyglutamates formed under the most restrictive conditions to the concentration under conditions which allowed the greatest synthesis. Cells cultured in Swims medium contained approximately 45 μM methotrexate polyglutamates when incubated with 10 μM methotrexate and this could be reduced to approximately 37 μM by including 20 μM folinic acid in the medium for 24 hr before methotrexate addition. Reduction of the cellular folates by lysolecithin-dependent permeabilization followed by incubation in folate-lacking medium for 24 hr coupled with the presence of insulin caused a nearly 3-fold increase in cellular methotrexate polyglutamates which were slightly over 100 μM under these conditions. Further investigation may uncover conditions which more dramatically alter the cellular capacity to generate polyglutamate derivatives of methotrexate.

Previous studies have suggested that the introduction of methotrexate into hepatic tissues and erythrocytes *in vivo* causes a reduction in folates [14–16]. The present study demonstrates that a reciprocal relationship may exist between folyl- and methotrexate polyglutamate accumulation *in vitro*.

The mechanism of the interaction between the folate and folylpolyglutamate pools and methotrexate polyglutamate pools is yet to be elaborated. Neither the exact molecular species involved in the proposed regulatory process nor the site(s) of regulation is known. The effect could be accomplished by altering the process of either γ-glutamyl addition or cleavage, each of which is carried out by distinct enzymatic processes [18]. Prior studies have shown that methotrexate and the reduced folate coenzymes compete for the same enzyme for glutamylation [7,9,11,20]. Thus, substrate competition could contribute to the effects observed here.

These data, along with other studies [7,9,13], indicate similarities and regulatory interactions in the γ-glutamylation of the natural folates and analogs, such as methotrexate. The results suggest further that cells which contain less folates are potentially more susceptible to methotrexate cytotoxicity because more methotrexate polyglutamates can accumulate which can lengthen the half-life of the drug in the cell. In addition, the lower levels of folates would cause the cells to be less resistant to the biochemical stress caused by the presence of methotrexate and methotrexate polyglutamates.

From these studies we conclude that the accumulation of methotrexate polyglutamates in H35 hepatoma cells *in vitro* is saturable and is modified by the cellular folates and by insulin. Reduction in cellular folates or the presence of insulin caused higher concentrations of methotrexate polyglutamates to be synthesized after a 24-hr incubation at extracellular methotrexate concentrations between 1 and 25 μM. At all concentrations the predominant species contained four glutamate residues. Low methotrexate concentrations (1–2 μM) caused the concentration of the pentaglutamate to exceed the triglutamate whereas the reverse occurred at higher methotrexate concentrations. A greater percentage of methotrexate pentaglutamates was formed in folate-depleted cells at low methotrexate concentrations than in folate-replete cells. The elevated levels of methotrexate polyglutamates found in cells containing reduced concentrations of cellular folates were returned to control levels by including folinic acid in the medium for 24 hr before methotrexate was added. The data suggest that folyl and methotrexate polyglutamates exert regulatory effects on their own and each other's synthesis.

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Oxidation of [^{14}C]diethylstilbestrol epoxide by uterine peroxidase: a possible protective mechanism

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Previous studies on the binding of diethylstilbestrol (DES) to DNA [1] and other tissue macromolecules [2], together with the known expoxidation of the stilbene double bond during the metabolism *in vivo* of aminostilbene derivatives [3], have provided indirect evidence for the conversion of DES into an epoxide. The nature of the urinary products of rats treated with DES [4], some of which are also formed after *in vitro* incubation of ^{14}C-labeled DES-epoxide with rat liver preparations [5], also supports the proposal that DES-epoxide may be an intermediate in the breakdown of DES. However, it has also been suggested [6] that the bioactivation of DES may involve peroxidase and that the localization of this enzyme could be an additional factor in the tissue specificity of DES-associated tumors. Oxidation of DES *in vitro* by a preparation of peroxidase from horseradish or the mouse uterus yielded reactive metabolites which combined covalently with salmon sperm DNA and bovine serum albumin [7]. It was therefore decided to study the effect of peroxidase on the further metabolism of the putative epoxide of DES which we have synthesized [5] in order to determine the extent of its conversion to water-soluble products and also to identify ether-soluble metabolites. For this purpose, lactoperoxidase and estrogen-induced rat uterine peroxidase [8] were chosen. Both catalyzed the rapid cleavage of DES-epoxide to 4′-hydroxypropiophenone in the presence of H_2O_2.

Materials and Methods

Reagents. Diethylstilbestrol (DES), lactoperoxidase and GSH were purchased from the Sigma Chemical Co., St. Louis, MO, and guaiacol, 2,4-dichlorophenol and 4′-hydroxypropiophenone from Eastman Organics, Rochester, NY. [^{14}C]Diethylstilbestrol epoxide (specific radioactivity 0.49 mCi/mmole) was prepared from [monoethyl-1-^{14}C]diethylstilbestrol (58 mCi/mmole) (Amersham Corp., Arlington Heights, IL) as described previously [5]. Silica gel pre-coated on aluminum sheets for TLC was purchased from Brinkmann Instruments, Rexdale, Ontario. All chemicals were the purest available commercially, and the solvents were redistilled.

Animals. Mature (190–250 g) female Sprague–Dawley rats (Canadian Breeding Laboratories, St. Constant, Quebec) with free access to food (Purina Labena) and water were injected subcutaneously with estradiol (10 μg in 0.2 ml oil) 18 hr before isolation of uterine peroxidase. Uteri, devoid of peroxidase activity, were obtained from immature (23 to 25-day-old) female rats.

Preparation of uterine extracts and incubation. The tissue was dissected free of any adhering fat, blotted, and weighed. It was then cut into small pieces and homogenized in the appropriate volume of 10 mM Tris–HCl (pH 7.2) to give a 5% (w/v) homogenate using a Polytron homogenizer (10-sec burst at speed setting 4.5). After centrifugation at 40,000 g_{av} for 30 min at 4° the pellet was resuspended in a Potter–Elvehjem homogenizer with a Teflon pestle in 10 mM Tris–HCl (pH 7.2) containing 1.2 M NaCl and centrifuged again at 40,000 g_{av} for 30 min to obtain a supernatant fraction (NaCl extract) containing most of the peroxidase in the tissue.

The NaCl extract (1 ml) from 50 mg of uterine tissue was incubated for various time periods with constant shaking at 37° with [^{14}C]diethylstilbestrol epoxide (4.5 μM) or [^{14}C]DES, together with H_2O_2 (0.33 mM) or GSH (1 mM) in 10 mM Tris–HCl, pH 7.2, in a total volume of 4 ml. The