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RESCUE EFFECT OF EXOGENOUS REDUCED FOLATES ON METHOTREXATE POLYGLUTAMYLATION AND DIHYDROFOLATE REDUCTASE ACTIVITY IN L1210 CELLS*

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L1210 can be rescued from exposure to an inhibitory effect of methotrexate (MTX) by subsequent addition of 5-formyltetrahydrofolate, 5-methyltetrahydrofolate or dihydrofolate. All folates listed caused marked reduction of long-chain MTX polyglutamates content and increased the activity of dihydrofolate reductase. This indicates that the rescue is a result of interaction of the reduced folates with two processes-polyglutamylation of MTX and generation of dihydrofolate.

Reduced folates such as 5-formyltetrahydrofolate, 5-methyltetrahydrofolate and dihydrofolate can rescue cells from deleterious effect of the anticancer drug methotrexate (2,4-diamino,N^{10}-methyl-pteroylglutamic acid, MTX) [1 - 3]. Rescue from MTX toxicity by 5-formyltetrahydrofolate has been shown in both experimental and clinical studies [3, 4]. Recent studies were amined to explain the mechanism by which 5-formyltetrahydrofolate prevents MTX-toxicity [5, 6]. Much less attention has been paid to the role of other reduced folates especially dihydrofolate in the rescue of cells after MTX treatment.

Cellular conversion of MTX to γ-glutamyl derivatives was demonstrated in many cell lines and tissues [7 - 12]. The reaction is probably catalyzed by the same enzyme that converts the reduced folates to their polyglutamates [13]. The potential importance of MTX polyglutamates was established

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when it was found that they are selectively retained by cells and that they inhibit dihydrofolate reductase [(EC 1.5.1.3), $K_D < 10^{-9}$] [13], as effectively as MTX itself [14]. Selective retention of polyglutamates endows the drug with much greater cytotoxic potential, since concentration of long-chain polyglutamates must exceed inhibitory concentration for dihydrofolate reductase in order to inhibit cell growth effectively [15, 16].

This paper demonstrates the relationship between potential of some reduced folates to rescue cells from cytotoxic effects of MTX and their effect on cellular synthesis of MTX polyglutamates and dihydrofolate reductase activity in the MTX polyglutamate containing cells.

MATERIALS AND METHODS

MTX (Lederle Laboratories, Pearl River N.Y., U.S.A.) and [3'5'7'-^{3}H]MTX (Amersham/Searle, Arlington Hights 111., U.S.A.) were purified by DEAE-dellulose (Sigma Chemicals Co., St. Louis Mo., U.S.A.) column chromatography as described previously [17, 18].

MTX polyglutamate synthetic standards were synthesized and kindly provided by Dr. John Montgomery from Southern Research Institute (Birmingham Ala., U.S.A.). Dihydrofolate and 5-methyltetrahydrofolate were synthesized according to Blakley [19] and Sakami [20], respectively. 5-Formyltetrahydrofolate was purchased from INC Pharmaceuticals (Plain View N.Y., U.S.A.). All others chemicals were reagent grade and obtained from Sigma Chemicals Co. (St. Louis Mo., U.S.A.) or POCh (Gliwice, Poland). Media for tissue culture were purchased from Serum and Vaccine Producer (Lublin, Poland).

Cell culture. L1210 cells were maintained in DBA-2×CFW F1 mice by weekly intraperitoneal inoculations. The cells were harvested 5 - 6 days after inoculation and used the same day for experiments.

Experimental procedures. Cells after harvesting, were washed in 0.85% NaCl solution then resuspended in Hanks medium and incubated with 2 μM [^{3}H]MTX (5×10^9 dpm/nmol) for the 2 h treatment. After this time the cells were washed, placed in the serum enriched Hanks medium at 37°C in the absence or presence of either dihydrofolate, 5-formyltetrahydrofolate or 5-methyltetrahydrofolate (dissolved in 100 μM sodium ascorbate) and sampled at various times. The termination of the ihcubation and measurement of the intracellular concentration of MTX and its polyglutamates were the same as described previously [7, 17].

The activity of dihydrofolate reductase was assayed spectrofotometrically [21] in cell extracts prepared as described previously [22]. The enzyme activity was always a linear function of the amount of cell extract added to the reaction mixture.

For each biochemical experiment in this study the toxicity assay was performed. The cells were treated with MTX and folates as in a corresponding biochemical experiment then cultured in the serum enriched Eagle's medium for 72 h and counted as described previously [17].

RESULTS

Effects of reduced folates added after 2 h treatment of 2 μM MTX on growth of L1210 cells are shown in Table 1. Addition to the medium of 5-formyltetrahydrofolate at a concentration below 5 μM caused only partial recovery of cell growth while at 100 μM concentration of 5-formyltetrahydrofolate the recovery was as high as 70 - 90%, but was not full upon further increase in concentration up to 500 μM. 5-Methyltetrahydrofolate or dihydrofolate at a concentration of 100 M or higher caused only 40 - 50% recovery of cell growth. A prolonged incubation with dihydrofolate or 5-methylterahydrofolate resulted in 50 and 65% recovery, respectively. The same concentrations of dihydrofolate or 5-methyltetrahydrofolate could not support cell growth after 10 μM MTX pulse (data not shown).

Table 1

Effect of folates on growth of L1210 cells pulsed with 2 μM MTX

Suspensions of cells in Hanks medium (6×10^4/ml) were incubated in 2 μM MTX for 2 h, then the cells were transferred to Eagle medium enriched with reduced folates and were cultured for the time indicated. Each result is a mean from 5-6 independent experiments ± SD

Addition	Number of cells $\times 10^{-4} \times ml^{-1}$ following treatment after		
	24 h	48 h	72 h
none	16±2	32±3	40±5
MTX	5±1	3±0.5	0.5±0.1
MTX + 5 μM 5-formyltetrahydrofolate	8±1	15±0.5	20±2
MTX + 100 μM 5-formyltetrahydrofolate	10±1	30±4	36±1
MTX + 500 μM 5-formyltetrahydrofolate	12±2	32±1	38±5
MTX + 100 μM 5-methyltetrahydrofolate	8±0.5	20±1	26±1
MTX + 500 μM 5-methyltetrahydrofolate	10±2	22±1	25±1
MTX + 100 μM dihydrofolate	6±0.5	15±2	20±2
MTX + 500 μM dihydrofolate	6±0.5	16±1	21±1

MTX is extensively converted by L1210 cells to γ-glutamyl derivatives which contain primarily 3 to 5 glutamate residues (Table 2, [3, 17]). A 2 h treatment with the drug resulted in approximately 70% of it being converted to polyglutamates. The intracellular concentration of total MTX polyglutamates was 8 μM. In the cells treated with the drug and placed in MTX-free

Table 2

Intracellular composition of MTX polyglutamates after 2 h pulse of MTX and after 6 h of efflux in the presence of reduced folates

L1210 cells were incubated with 2 μM [^{3}H]MTX for 2 h, then were placed for 6 h in the medium lacking MTX (efflux) or lacking MTX but containing reduced folates at concentration of 100 μM. Cells were sampled and MTX polyglutamates were identified and quantitated as described in Materials and Methods. Means of two determinations of two separate experiments are given. ND-not detected.

Sample	Methotrexate species pmol/10^8 cells					
	Glu_1	Glu_2	Glu_3	Glu_4	Glu_5	$Glu_{>5}$
2 h pulse only	2.0	0.6	3.4	3.0	0.4	ND
2 h pulse + 6 h efflux						
without additions	ND	ND	0.8	4.2	1.8	0.3
+ 5-formyltetrahydrofolate	ND	ND	0.6	0.6	0.3	ND
+ 5-methyltetrahydrofolate	ND	ND	0.2	2.4	0.8	ND
+ dihydrofolate	ND	ND	0.1	2.0	0.6	ND

medium for 6 h (efflux), marked changes occured in the profile of the MTX species. As expected, MTX rapidly equilibrated with the medium and its intracellular concentration was low. Among polyglutamates dramatic loss of Glu_3 was accompanied by the concomitant increases in Glu_4 and Glu_5 content (Table 2). Total concentration of MTX polyglutamates was 7 μM. Addition of 5-formyltetrahydrofolate after MTX treatment caused very slow conversion of MTX to long-chain polyglutamates (up to Glu_5). Concentration of all polyglutamates species was low and total concentration of MTX polyglutamates was 1.2 μM. Inclusion of dihydrofolate and 5-methyltetrahydrofolate, after 2 h treatment with 2 μM MTX treatment, reduced by half concentration of total polyglutamate pool (3.2 μM), but the presence of $Glu_{>5}$ derivatives still could be detected.

Addition of reduced folates to MTX pulsed L1210 cells could also change the activity of the MTX-target enzyme i.e. dihydrofolate reductase. This was evaluated by measuring dihydrofolate reductase activity in the extracts of L1210 cells treated with MTX and reduced folates (Table 3). MTX pulse reduced the enzyme activity to 25% of the control, but in the presence of 5-formyltetrahydrofolate, 5-methyltetrahydrofolate or dihydrofolate part of dihydrofolate reductase became apparently reactivated.

DISCUSSION

Reduced folates as 5-methyltetrahydrofolate and 5-formyltetrahydrofolate may bypass the MTX-inhibition of dihydrofolate reductase and recover cell

growth by acting as one-carbon donors. On the other hand, at high concentration dihydrofolate can displace MTX from dihydrofolate reductase -MTX complex which leads to partial reduction of dihydrofolate and its re-entering to the folate coenzyme pool [23, 24]. Also it has been postulated that folate coenzymes, i.e. 5-formyltetrahydrofolate and 5-methyltetrahydrofolate can act in the same way as dihydrofolate itself and they can generate in the presence of MTX high levels of dihydrofolate, *via* dissociation of the MTX-dihydrofolate reductase complex [6, 23]. This postulate was confirmed in this study by measuring the activity of dihydrofolate reductase (Table 3). In the cells rescued by reduced folates, the enzymatic activity was detectable and was related to MTX dose, consistently with the suggestion that reduced folates can liberate the enzyme from the enzyme--inhibitor complex.

Table 3

Dihydrofolate reductase activity in L1210 cells pulse after exposure to 2 μM or 5 μM MTX for 2 h and subsequent addition of reduced folates (see legend to Table 2).

Each result is a mean from 4-7 independent experiments ± SD

The activity of non treated cells: 8.2±2.0 nmol/min per mg protein.

Addition	Dihydrofolate reductase (nmol/min per mg protein)	
	2 μM MTX	5 μM MTX
none	0.2±0.1	0
5-formyltetrahydrofolate	2.4±0.5	0.9±0.2
5-methyltetrahydrofolate	1.2±0.2	0.6±0.2
dihydrofolate	2.9±0.6	0.8±0.1

The lag in growth before the onset of cell division at all concentrations tested of reduced folates and the inability to full restoring of cell growth (Table 1) suggest that a more complex rescue mechanism may exist. L1210 cells develop a significant level of long-chain MTX polyglutamates (Glu_{4-6}) particularly after removal of MTX from the medium (Table 2). Reduction in the concentration of MTX polyglutamates by reduced folates could contribute to recovery of cells. When reduced folates are added to the MTX-treated cells both the molecular weight and concentration of polyglutamates are decreased (Table 2). The shorter chain-glutamyl derivatives of MTX are retained by L1210 cells less efficiently than longer ones [18]. The decrease in total cellular pool of MTX could lead to re-appearance of active dihydrofolate reductase and restore one-carbon cycle.

There is ample evidence demonstrating that reduced folates and MTX are glutamated by the same enzyme and therefore compete with one another [25]. Thus any increase in cellular folate pool could effectively decrease the level of available folylpolyglutamate synthetase for MTX polyglutamylation and reduce concentration of intracellular MTX polyglutamates [25, 26].

The present study demonstrated that reduced folates can rescue L1210 cells from toxicity of MTX polyglutamates by interfering with polyglutamylation and generation of dihydrofolate.

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