Effect of Methotrexate on 5-Phosphoribosyl 1-Pyrophosphate Levels in L1210 Leukemia Cells in Vitro

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ABSTRACT

The cellular content of 5-phosphoribosyl 1-pyrophosphate (PRPP) and PRPP synthetase activity levels was examined in cultured L1210 mouse leukemia cells after exposure to methotrexate (MTX). PRPP was determined in cold perchloric acid extracts of cells using a radiochemical method measuring the conversion of [14C]adenine to [14C]adenosine 5'-monophosphate in the presence of added adenine phosphoribosyltransferase activity. PRPP synthetase activity was measured in dialyzed cell-free extracts by a coupled enzyme assay determining PRPP production using the adenine phosphoribosyltransferase reaction. The intracellular levels of PRPP in untreated cells during logarithmic growth varied over 24 hr with a mean of 145 ± 65 (S.D.) pmol/10⁶ cells. Treatment of cells with 1 μM MTX caused an increase in PRPP levels by 1.5 to 3 hr with maximal levels of 5- to 10-fold higher than controls. Elevated PRPP levels persisted for up to 12 hr and declined to control values after 24 hr. Similar results were obtained with 0.1 μM MTX, while exposure of cells to 0.01 μM MTX caused a more gradual increase in cellular PRPP content which was maximal at 12 hr and was maintained for at least an additional 12 hr. The addition of 0.1 mm hypoxanthine led to recovery of PRPP to elevated levels in cells exposed to 0.1 μM MTX for 1.5 hr but not for 10 hr. No large difference in PRPP synthetase activity levels between MTX-treated cells and untreated cells was observed.

The results indicate that in L1210 cells exposure to MTX leads to increased levels of PRPP, more probably due to a decreased utilization of PRPP in de novo purine synthesis than as a result of increased PRPP synthesis. Enhanced levels of PRPP are available for the reutilization of preformed purines; however, PRPP levels are afterwards only partially replenished with prolonged drug exposure because of decreased cellular capacity for PRPP synthesis despite the continued presence of PRPP synthetase activity.

INTRODUCTION

The mechanism of action of MTX has been well studied, and the interaction between cells and the drug was recently reviewed (2, 9). MTX binds to the enzyme dihydrofolate reductase and inhibits its activity of depleting the cells of reduced folates. Reduced folates act as one-carbon donors for the formation of the purine ring in the de novo purine-biosynthetic pathway and for the de novo synthesis of dTMP from dUMP catalyzed by dTMP synthetase. The inhibition of dihydrofolate reductase by MTX leads to a block in nucleic acid synthesis. The inhibitory effect of MTX can be reversed by the addition of FA, dThd alone, or dThd plus a purine (3, 4, 10, 15–17, 25). FA allows the bypass of the initial depletion of reduced folates caused by MTX and prevents the inhibition of de novo nucleotide synthesis, whereas the latter two forms of drug reversal depend on the ability of the cell to use these metabolites for nucleotide synthesis via salvage pathways.

Recent studies from this laboratory examined the inhibitory effect of MTX on colony formation (in semisolid medium) of normal mouse bone marrow cells and of L1210 mouse leukemia cells and its reversal (19, 22). FA was found to block the action of MTX in a competitive manner, whereas dThd and purines caused a noncompetitive reversal of drug toxicity. FA-mediated reversal was qualitatively and quantitatively similar in both the normal and leukemia cells. Conversely, dThd and dinosine at concentrations as low as 10 μM completely protect bone marrow cells from MTX toxicity independent of drug concentration. However, these nucleosides at similar or higher concentrations provide only partial reversal of MTX toxicity in L1210 cells. These studies and those of others (3, 4, 10, 15–17, 25), examining the potential of dThd and purines to circumvent the MTX-mediated block of nucleotide biosynthesis, have not been concerned with factors which may influence the cellular capacity for reutilization of preformed pyrimidines and purines. Such factors include enzymes, other required substrates, essential cofactors, and environmental conditions. These factors have been grouped together and are regarded as the cellular capacity for salvage pathway synthesis of nucleotides, which may differ among different cell types or change following treatment with MTX alone. For the salvage pathway utilization of dThd and purines in nucleotide biosynthesis, other substrates are required. The phosphorylation of dThd to dTMP by dThd kinase requires ATP, and in the conversion of purine bases to nucleotides by phosphoribosyltransferases PRPP serves as the high-energy phosphate substrate. MTX has been demonstrated to cause a rapid decrease of intracellular ATP levels, which could restrict the cellular utilization of dThd (16). However, little is known about the effect of MTX on cellular PRPP content, which may be critical for purine salvage (7, 21). PRPP, synthesized from ATP and ribose 5-phosphate by the action of PRPP synthetase, plays an important role in cellular metabolism (7). Not only is PRPP used in the purine salvage pathway but it also serves as an essential substrate in the de novo synthesis of both purines and pyrimidines.
The present study deals with the aspect of essential substrate availability and, more specifically, the availability of PRPP for the conversion of preformed purines to nucleotides. The results of experiments with L1210 mouse leukemia cells in culture indicate that PRPP is markedly elevated following exposure to MTX. These increased cellular levels of PRPP are depleted during hypoxanthine utilization and recovery after removal of hypoxanthine. However, recovery of PRPP levels is limited following prolonged MTX treatment.

MATERIALS AND METHODS

Materials. dThd, adenine, other purine compounds, PRPP (sodium salt), and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. MTX and FA (calcium salt) were obtained from Lederle Laboratories, Pearl River, N. Y. [8-14C]Adenine (24.6 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. All other chemicals used were of analytical grade and were obtained from commercial sources.

Cell Culture. L1210 mouse leukemia cells were cultured in Roswell Park Memorial Institute Medium 1640 supplemented with 10% dialyzed fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) and 60 µM 2-mercaptoethanol. Cells were grown in plastic disposable culture flasks (Nunc A/S, Roskilde, Denmark) which were loosely capped and placed in a 7.5% CO2-92.5% air, humidified incubator at 37°C. In these experiments, cells in logarithmic growth, 12 to 24 hr after subculture at 5 x 10⁶ cells/ml, were transferred to 25-ml flasks for testing of different experimental conditions. Initial cell concentration was adjusted to 3 x 10⁵ cells/ml based on cell counting performed with a hemocytometer. Cell viability determined by the trypan blue dye exclusion test was initially >95%.

Following exposure to MTX or hypoxanthine, cells were again counted; cell suspensions containing 3 to 5 x 10⁶ cells were first cooled in ice; and the cells were collected by centrifugation at 600 x g for 5 min at 4°C.

PRPP Assay. Intracellular PRPP content was determined by incubating cell extracts with [14C]adenine in the presence of excess APRT activity and measuring the amount of AMP produced. The assay is a modification of a previously reported method (14). The following procedure for extraction of cells was performed at 4°C. Cell pellets were directly resuspended in 200 µl 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA. The cell suspension was extracted with 50 µl 2 N perchloric acid for 20 sec followed by neutralization with 100 µl 1 N KOH. The pH was maintained at 7 by the addition of 50 µl 0.1 M ammonium carbonate. The supernatant obtained after centrifugation at 5000 x g for 30 min was stored at -20°C and assayed within 12 hr (stable under these conditions). Fifty µl of perchloric acid extract were added to an enzyme reaction mixture yielding a final volume of 100 µl. The reaction mixture contained, at final concentrations: 0.1 mM [14C]adenine; 5 mM MgCl₂; bovine serum albumin, 0.5 mg/ml; 10 mM Tris-HCl, pH 7.4; and sufficient APRT to provide an activity of at least 100 nmol/hr. APRT was partially purified from human erythrocytes up to the DEAE-cellulose step in the procedure described by Thomas et al. (26). The APRT preparation was extensively dialyzed in the absence of phosphate, heat treated at 70°C in the presence of 0.1 mM PRPP, and again dialyzed. This latter procedure caused inactivation of PRPP synthetase activity. The reaction mixture was incubated at 37°C for 10 min, and the reaction was stopped in ice water by the addition of 20 µl 0.2 M EDTA. A 40-µl aliquot of the reaction mixture was spotted on Whatman No. 3MM chromatography paper along with inosine, IMP, and AMP, as markers. High-voltage electrophoresis was carried out in 25 mM sodium borate-1 mM EDTA buffer, pH 9, at 3000 V for 30 min. The area including the above purines, located under UV, was cut out and counted by liquid scintillation counting in toluene containing PPO, 4 g/liter, and POPP, 0.1 g/liter, at 70% efficiency. Counting of IMP and inosine in addition to AMP was necessary due to the apparent partial metabolism of AMP by residual enzyme activities in the perchloric acid extract. Recovery of PRPP added to a cell pellet before extraction was the same (60%) as that from acid-extracted standard solutions of 1 to 50 µM. PRPP values were determined based on the specific activity of [14C]adenine, corrected for recovery and expressed as pmol/10⁶ viable cells. The assay conditions provided a stoichiometric reaction between [14C]adenine and PRPP (25 to 500 pmol). The lack of interference with the APRT reaction by the presence of nucleotides in the cell extract was evident by the directly proportional relationship between measured PRPP content and volume of cell extract used in the assay. In the reaction mixture, the concentration of cellular metabolites was reduced approximately 40 times and APRT activity was more than 30 times in excess of that required for the conversion of 500 pmol PRPP to nucleotides in 10 min.

PRPP Synthetase Assay. PRPP synthetase activity was determined using a coupled enzyme assay, a modification of the method described earlier by Fox and Kelley (6) and similar to that described by Roth et al. (23). The cell pellet was washed twice with 0.15 M NaCl-1 mM potassium phosphate, pH 7.4, and resuspended in 50 mM potassium phosphate-1 mM dithiothreitol, pH 7.4 (100 µl/10⁶ cells). The suspension was frozen (-70°C) and thawed 3 times and centrifuged at 10,000 x g for 60 min. The supernatant was dialyzed overnight against 500 volumes of fresh extraction buffer. The dialyzed cell extract was assayed for PRPP synthetase activity using different dilutions and in duplicate. The enzyme reaction mixture (total volume, 50 µl) consisted of 25 µl of cell extract, 5 mM MgCl₂, 0.3 mM ATP, 0.5 mM ribose 5-phosphate, 0.3 mM [14C]adenine (5.8 mCi/mmol), and APRT activity of at least 100 nmol/hr. The reaction mixture was incubated for 20 min at 37°C, and the reaction was terminated in ice water by the addition of 10 µl 0.2 M EDTA. Separation and measurement of the reaction product was performed as described in the PRPP assay. Enzyme activity values were corrected for nonspecific activity measured in the absence of ribose 5-phosphate. The assay was linear with time for at least 60 min and, with the amount of added extract provided, less than 30% of the total [14C]adenine was consumed. The lower limit of linearity and detection was 3% consumption of total [14C]adenine. Only enzyme dilutions giving activity values in this range were used. Protein was determined according to the method of Lowry et al. (20) using bovine serum albumin as a standard, and enzyme activity was expressed as nmol per mg protein per hr.

RESULTS

Treatment of L1210 cells with MTX at concentrations of 0.01 to 1 µM under the culture conditions described above led to inhibition of cell proliferation at all drug concentrations tested.
Cell viability was also reduced after exposure to MTX. The presence of 0.01, 0.1, and 1 μM MTX for 6 hr resulted in viable cell percentages of 94, 92, and 84%, respectively. These values were further decreased to 85, 67, and 60%, respectively, after 12 hr and 60, 38, and 34%, respectively, after 24 hr. After continuous exposure of cells to MTX for 48 hr, less than 7% of the cells excluded trypan blue dye.

**Cellular PRPP Content.** In untreated control cultures during 24 hr logarithmic growth, PRPP levels varied with a mean (±S.D.) value of 145 ± 65 pmol/10^6 cells (Chart 1, solid line). In the various experiments described in this study, the initial PRPP level was 195 ± 45 (S.E.). The addition of MTX, 0.1 or 1 μM, to cells caused a rapid increase in cellular PRPP content, maximal levels being reached by 1.5 to 3 hr (Chart 1). The mean values during 12 hr of MTX exposure ranged from 902 to 1350 pmol/10^6 cells with 0.1 μM MTX and from 569 to 1200 pmol/10^6 cells with 1 μM MTX. A large variation in PRPP levels was observed in the treated cells, and the values obtained were not significantly different between these 2 MTX concentrations. After 24 hr, PRPP levels in cells treated with MTX at 0.1 and 1 μM declined to control levels. In cells treated with 0.01 μM MTX, increased PRPP levels were observed only after 3 hr of drug exposure. Maximal values were attained after 9 hr MTX and persisted for up to 24 hr following the addition of MTX.

**PRPP Synthetase Activity.** PRPP synthetase activity levels were determined at different times after MTX addition (Table 1). Enzyme activity in extracts of untreated cells during 24 hr of logarithmic growth varied between 258 and 391 nmol per mg protein per hr. Enzyme activity levels were decreased 10 to 35% after 3 hr MTX and were increased 6 to 24% or not significantly different (with 1 μM MTX) after 6 hr MTX, compared to control cultures at the respective exposure periods. After 24 hr exposure to MTX, PRPP synthetase activity levels were 15 to 38% lower compared to control.

**Reversal of the Stimulatory Effect of MTX on PRPP Levels.** The concurrent addition of 0.1 mM FA and 0.1 μM MTX did not result in blocked cell proliferation or loss of cell viability. Chart 2 shows that FA prevented the MTX-mediated increase in cellular PRPP content. FA alone had no effect on PRPP levels. Hxanthine added to cells at a concentration of 0.1 mM led to a decrease in cellular PRPP levels to 20% of control within 3 hr whether or not MTX was present in the culture medium (Chart 3A). Reduced PRPP levels remained low during hypoxanthine exposure for at least 3 hr. Enhanced PRPP content in MTX-treated cells was reduced by 20% or less of control values also when hypoxanthine was added at either 3, 6, or 9 hr after 0.1 μM MTX (Chart 3B). The effect of a temporary exposure of MTX-treated cells to 0.1 mM hypoxanthine on PRPP levels was examined (Chart 4). When hypoxanthine was added 1.5 hr after MTX, PRPP levels decreased. After subsequent removal of hypoxanthine (1.5 hr later), PRPP levels recovered to the initial, high MTX-induced levels. However, when hypoxanthine was added 10 hr after MTX and subsequently removed 1.5 hr later, recovery of cellular PRPP levels was limited, with levels reaching those of nontreated cultures.

### DISCUSSION

The present study demonstrates that in L1210 mouse leukemia cells exposure to MTX leads to a rapid increase in
Chart 3. Effect of hypoxanthine on PRPP content in L1210 cells exposed to MTX in vitro. In A, 0.1 mM hypoxanthine and 0.1 μM MTX were added at Time 0, the intracellular content of PRPP was determined at 3 and 6 hr (□), and the results were compared to those for untreated controls (○) or cells exposed only to 0.1 μM MTX (◼) or 0.1 mM hypoxanthine (▲). In B, 0.1 mM hypoxanthine was added at 0 (○), 3 (□), 6 (▲), and 9 (◼) hr following the addition of 0.1 μM MTX and PRPP determined 3 and 6 hr after hypoxanthine exposure. ○, 0.1 μM MTX. Bars, S.E.

Chart 4. Recovery of PRPP levels following temporary exposure to hypoxanthine in L1210 cells during the continuous presence of MTX. Hypoxanthine (0.1 mM) was added 1.5 (A) or 10 (B) hr after addition of 0.1 μM MTX and was removed 1.5 hr later. ○, 0.1 μM MTX; □, 0.1 μM MTX plus 0.1 mM hypoxanthine; ▲, untreated controls. Bars, S.E.
cellular PRPP content. This effect is maximal between 0.01 and 0.1 μM MTX, a critical concentration for drug toxicity. The apparent cause of the MTX effect on PRPP is the inhibition of de novo purine synthesis and thus the reduced utilization of PRPP. This is supported by similar findings with 6-methylmercaptopurine ribonucleoside, also an inhibitor of de novo purine synthesis but with a different molecular mechanism of action (12). FA was found to block the stimulatory effect of MTX on PRPP indicating that the latter is not due to a direct effect of MTX. An increase in PRPP synthetase activity levels by MTX, which could result in an increased rate of PRPP synthesis, is also not involved. The cell culture medium with dialyzed fetal calf serum provided purine- and pyrimidine-free conditions preventing PRPP utilization via salvage pathways. Under these experimental conditions, it is likely that MTX allows PRPP to accumulate as a result of decreased consumption. Hypoxanthine added to the cells causes a decrease in PRPP levels indicating that the lack of purine salvage activity contributed to the MTX-induced enhancement of PRPP levels. PRPP degradation in dialyzed human placenta extract has been demonstrated and has been attributed to alkaline phosphatase activity (8). In L1210 cells, PRPP does not appear to be readily catabolized in the absence of utilization but, conversely, accumulated. However, differences in rates of synthesis and catabolism when cells are exposed to different MTX concentrations for extended periods of time could explain why elevated PRPP levels with 0.01 μM but not 0.1 or 1 μM MTX are maintained for up to 24 hr.

Benke et al. (1) have recently reported similar findings of increased cellular PRPP content upon exposure of cultured human fibroblasts to an antifolate. PRPP levels were found to increase 20- to 50-fold after 48 hr of treatment with aminopterin. These investigators also noted a 2- to 3-fold concomitant increase in synthesis was responsible for the enhanced levels of PRPP. Our results with MTX (amethopterin) in mouse leukemia cells differ with those of Benke et al. with respect to the effect of the antifolate on PRPP synthetase activity. Enzyme activity levels were not enhanced in L1210 cells by MTX and were actually decreased slightly after 12 hr of drug exposure. Also, the increase in PRPP levels with MTX occurred more rapidly (1.5 hr) in L1210 cells than in human fibroblasts with aminopterin (48 hr). This latter difference may be due, at least in part, to differences in cell-doubling time and degree of antifolate toxicity; however, differences in purine metabolism, particularly de novo synthesis, are more probably the responsible factors.

Cellular PRPP synthesis in the presence of MTX was examined in L1210 cells by addition of hypoxanthine, which markedly reduced PRPP levels, and measuring PRPP after subsequent removal of hypoxanthine. In these experiments, complete recovery of PRPP levels was observed after 1.5 hr, but not 10 hr, of MTX exposure. These findings suggest that PRPP synthesis decreases after prolonged treatment of cells with MTX despite the continued presence of PRPP synthetase. A limiting potential factor in continued PRPP synthesis is the availability of substrates, ATP and ribose 5-phosphate. Hryniuk et al. (17) have reported a marked decrease in ATP levels in L5178Y lymphoma cells in vitro 2 hr after MTX treatment. In addition, Kaminskas and Nussey (18) observed a block in glucose consumption in Ehrlich ascites cells in vitro as a result of MTX, which was believed to be a secondary effect resulting from ATP depletion. Such inhibition of glucose metabolism could lead to ribose 5-phosphate depletion as well. These latter effects of MTX on intracellular ATP levels and glycolysis indicate that MTX may inhibit PRPP synthesis eventually due to decreased availability of essential substrates. However, changes in the intracellular concentrations of other potentially regulatory factors such as P (13) and 2,3-diphosphoglycerate (7, 13) may also occur during MTX treatment and affect PRPP synthesis. Hryniuk (16) found that reversal of MTX toxicity to L5178Y cells by the addition of hypoxanthine occurred only during the first 8 hr of MTX treatment and suggested that MTX inhibits purine nucleotide synthesis before thymine nucleotide synthesis. An alternative explanation is that a diminished PRPP level or PRPP synthesis after prolonged MTX treatment limits utilization of purines for salvage pathway synthesis of nucleotides.

At least in L1210 leukemia cells, MTX leads to increased intracellular PRPP levels which may be used in salvage pathway utilization of hypoxanthine. PRPP availability is limited after exposure of cells to MTX for extended periods of time apparently due to decreased synthesis. Variation in the reversibility of MTX toxicity in different cell types by dThd and purines may be related to differences in the effect of MTX on PRPP metabolism and availability. These findings have important implications in current investigations in experimental (11, 24) and clinical (5) chemotherapy examining the reversal of MTX cytotoxicity by dThd and purines.

REFERENCES


