Effect of Methotrexate on Intracellular Folate Pools in Purified Myeloid Precursor Cells from Normal Human Bone Marrow

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Abstract

We investigated the effects of the antifolate methotrexate on intracellular folate pools of human myeloid precursor cells (MPCs). Immature MPCs, representing 3.2% of the original marrow population, were selected from normal human bone marrow by immune rosetting. The intracellular folate pools were labeled by incubation with 5×10^{-8} M [^{3}H]5-formyl-FH$_4$ and were quantitated by high performance liquid chromatography. The predominant folates were 5-methyl-tetrahydrofolate (5-methyl-FH$_4$) (36%), 10-formyl-FH$_4$ (41.4%), 5-formyl-FH$_4$ (12.3%), and FH$_4$ (10.3%). A 12-h exposure to 1 μM methotrexate (MTX) resulted in a 34% reduction in the intracellular concentration of 10-formyl-FH$_4$, a 61% decrease in 5-formyl-FH$_4$, and a 62% decrease in 5-methyl-FH$_4$, as well as the appearance and progressive expansion of the FH$_2$ and 10-formyl-FH$_2$ pools. These changes were maximal after 4 h of incubation with MTX. Paralleling the changes in folates, particularly the increase in FH$_2$, were a 64% reduction in myeloid colony formation and a 77% depression of de novo purine synthesis after 4 h of MTX. We conclude that MTX does not produce quantitative depletion of 10-formyl-FH$_4$ and that its antipurine effect may be mediated by direct inhibition of de novo purine synthesis by FH$_2$ and, at later time points, by MTX polyglutamates.

Introduction

Methotrexate (2,4-diamino,10-methylpteroyl glutamic acid; MTX)[1] is a tight-binding inhibitor of dihydrofolate reductase (DHFR) and has shown potent antitumor activity against human leukemia, non-Hodgkin's lymphoma, choriocarcinoma, and other human tumors (1). Its primary toxic effects are myelosuppression and mucositis. Its antineoplastic effect as well as toxicity have been ascribed to depletion of reduced folates with consequent arrest in the synthesis of purines, thymidylate, and proteins. However, significant depletion of reduced folate pools after MTX treatment has not been demonstrated in tissue culture (2) or in vivo.

An alternative explanation for inhibition of purine and pyrimidine biosynthesis has been forthcoming from studies of the properties of the polyglutamated forms of MTX and dihydrofolate (FH$_2$), which accumulate intracellularly during MTX exposure. Polyglutamated MTX as well as dihydrofolate pentaglutamate have a direct inhibitory effect on thymidylate synthase (3, 4) and aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase (5), the key folate-dependent enzymes in the synthesis of thymidylate and purines, respectively.

Evidence in support of this alternative explanation of MTX action has been forthcoming from direct measurement of the folate pools in MCF-7 breast cancer cells, accomplished by labeling the intracellular folates with [^{3}H]folic acid, followed by separation of individual folates by high performance liquid chromatography (HPLC). Exposure of these cells to 1 μM MTX resulted in marked inhibition of de novo purine synthesis despite only 20% depletion of 10-formyl-tetrahydrofolate (10-formyl-FH$_4$), the substrate required for purine biosynthesis. The authors suggested that the purine biosynthetic pathway was directly inhibited by MTX and/or FH$_2$ polyglutamates.

To determine the effects of MTX on intracellular folates in these cells, we have isolated an immature fraction of marrow cells by immune rosetting. The immature fraction thus obtained was composed of 80% myeloblasts, promyelocytes, and myelocytes (the dividing population of marrow myeloid lineage). The following study demonstrates relative preservation of the 10-formyl-FH$_4$ pool in the presence of concentrations of MTX that were cytotoxic to myeloid colony formation. At the same time, MTX exposure led to significant expansion of pools of FH$_2$, and the appearance of a new folate compound, 10-formyl-FH$_2$.

Methods

Chemicals. MTX was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD) and was purified by DEAE-Sephacel chromatography as previously described (6). [3',5',7,9-^{3}H]-*l*-5-Formyl-H$_4$-PteGlu (specific activity, 1 Ci/mmol) was synthesized from [3',5',7,9-^{3}H]PteGlu according to the methods of Moran et al. (7). Hog kidney polyglutamate hydrolase (13–15 mg protein/ml) was prepared according to the method of McMartin et al. (8). Folate-free RPMI-1640 medium was obtained from the National Institutes of Health Media Unit (Bethesda, MD). Eagle's minimal essential medium (MEM) and phosphate-buffered saline (PBS) (pH 7.4) were purchased from M. A. Bioproducts (Walkersville, MD). Agar (BactoAgar) and fetal calf serum (FCS) were purchased from Difco Laboratories Inc. (Detroit, MI). FCS was heat-inactivated at 56°C for 30 min, followed by extensive dialysis to remove nucleosides and folates. Colony-stimulating factor prepared from conditioned medium of the MO cell line, a T-cell variant of hairy cell leukemia (9, 10), was provided by Dr. D. W. Golde, UCLA School of Medicine (Los Angeles, CA). Pooled human AB serum was obtained from the National Institutes of Health Blood Bank (Bethesda, MD), and sheep erythrocytes (SRBCs) were obtained from the National Institutes of Health Animal Center (Rockville, MD). Monoclonal antibodies OKT3, B1, and M3 were purchased from Ortho Pharmaceutical Corp. (Raritan, NJ), Coulter Immunology (Hialeah, FL), and Becton Dickinson Monoclonal Center Inc. (Mountain View, CA), respectively. MY8 (11, 12) was provided by Dr. J. Griffin, Dana-Farber Cancer Institute (Boston, MA) and EP1 (13) by Dr. T. Papayannopoulou, University of Washington

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1. *Abbreviations used in this paper:* AICAR, aminoimidazolecarboxamide ribonucleotide; CFU-C, colony-forming units in culture; DHFR, dihydrofolate reductase; FH$_2$, dihydrofolate; FH$_4$, tetrahydrofolate; MEM, minimal essential medium; MNC, mononuclear cell; MPCs, myeloid precursor cells; MTX, methotrexate; SRBC, sheep erythrocyte.

(Seattle, WA). Affinity-purified goat anti–rabbit IgG [F(ab)$_2$ fragment–specific] antibody was obtained from Cappel Laboratories (Malvern, PA) and extensively dialyzed against 0.15 M borate-buffered saline (pH 7.4). The F(ab)$_2$ fragment of rabbit anti–mouse Ig (IgG + IgA + IgM) was purchased from Zymal Laboratory Inc. (San Francisco, CA).

Purification of myeloid precursor cells (MPCs). After obtaining informed consent, heparinized bone marrow samples were collected by aspiration from the posterior iliac crest of normal volunteers. The mononuclear cell (MNC) population was separated by Ficoll-Hypaque gradient centrifugation. A fraction enriched in myeloid precursor cells was obtained by an affinity rosetting technique, as described previously (12, 14, 15). Briefly, SRBCs were coupled with goat anti–rabbit IgG [F(ab)$_2$ fragment–specific] by the chromium chloride technique and reacted with the F(ab)$_2$ fragment of rabbit anti–mouse Ig (IgG + IgA + IgM)–specific antibody. Bone marrow MNCs (0.5–1.0 × 10^8/ml) were incubated in MEM containing 2.5% human AB serum (MEM-AB) with a mixture of monoclonal antibodies—OKT3, B1, and M3 at a final dilution of 1:200, MY8 (1:100), and EP1 (1:500)—and allowed to rosette with a 10% suspension of Ig-coated SRBCs. The nonrosetted cells (antibody negative) were separated from the rosetted cells by Ficoll-Hypaque gradient centrifugation, collected from the interface, washed, and red cells were lysed by incubation in 1 ml ice-cold hypotonic buffer (0.17 M NH$_4$Cl, 0.01 M·KHCO$_3$, and 0.0001 M EDTA) for 5 min. The cells were counted with a hemacytometer and their viability assessed by trypan blue exclusion. 2–6 × 10^6 cells, i.e., 3.2±1.1% of the original mononuclear cell population of the marrow were recovered in the final purified fraction and > 95% were viable as assessed by trypan blue exclusion.

Quantitation of intracellular folate pools. The purified MPCs (1–2 × 10^6 cells/ml) were incubated at 37°C in folate-free RPMI-1640 with 10% dialyzed FCS, 2 mM L-glutamine, and [3',5',7,9-^{3}H]-*l*-5-formyl-FH$_4$-PteGlu (specific activity, 1 Ci/mmol and final concentration 5 × 10^{-8} M) for 24 h. Equilibration of radiolabeled 5-formyl-FH$_4$ with intracellular folate pools was reached at 12–16 h as determined by a constant level of ^{3}H-label in each pool and a constant proportional distribution of the ^{3}H-label among the various peaks of the intracellular folate pools thereafter (data not shown). Neither breakdown of the tritiated 5-formyl-FH$_4$ in the medium nor a decrease in the cell viability, as determined by trypan blue exclusion, was noted during the incubation period. In a preliminary study, equilibration was not achieved when the immature myeloid cells were incubated with [^{3}H]folic acid for up to 36 h under similar conditions. The total uptake of [^{3}H]folic acid was low and the total intracellular ^{3}H-label continued to increase after 36 h, although the relative distribution of ^{3}H-label among the various intracellular pools was comparable to that found after incubation of cells with [^{3}H]5-formyl-FH$_4$, with the exception of the intracellular [^{3}H]folic acid pool, which increased constantly over this time period. Although it may have been possible to reach a plateau by longer incubation (beyond 36 h) with [^{3}H]folic acid, extended periods of incubation led to a decrease in viability of the myeloid precursor population. Therefore, [^{3}H]5-formyl-FH$_4$ was used to label the intracellular folates.

After a 24-h preincubation of purified myeloid precursors with [^{3}H]5-formyl-FH$_4$, MTX was added at various concentrations and for various durations according to the particular experiment. After incubation with the drug, the MPCs were washed three times in ice-cold PBS and resuspended in 1 ml PBS. A 50-µl portion was diluted in 750 µl PBS, sonicated, and the protein quantitated by the Biorad microassay method (16). 2 ml of a solution containing 2% 2-mercaptoethanol and 2% ascorbate and adjusted to pH 6 was added to the remaining part of the sample (950 µl) and boiled for 90 s. The supernate was then treated with partially purified hog kidney polyglutamate hydrolase (1 ml) for 30 min at 37°C and was subjected to an additional 90-s boil after the further addition of 2 ml of the 2-mercaptoethanol, ascorbate solution. The denatured protein was pelleted by centrifugation at 2,000 *g* for 10 min and the folates contained in the supernate were extracted into a methanol phase, using a C$_{18}$ Sep-Pak cartridge. The labeled folates were separated by reverse-phase HPLC on a C$_{18}$ Bondapak column, as previously described (2), and quantitated by liquid scintillation counting of the fractions collected from the HPLC. The mobile phase for HPLC separation consisted

of 85% Pic Reagent A (at either pH 7.4, 5.5) and 15% methanol, and compounds were eluted using isocratic conditions and a flow of 2 ml/min.

The proportion of intracellular folate found as a monoglutamate or as a polyglutamate was determined by dividing a cell extract, after the initial boiling step, into two samples. In one sample polyglutamates were subjected to hydrolysis by hog kidney conjugase, as described above, while buffer lacking conjugase was added to the second sample. Both samples were then subjected to centrifugation, Sep-Pak fractionation, and HPLC, and the profile of monoglutamate folates was compared for the two samples. Polyglutamated folates are not eluted in the HPLC systems used in these experiments and cannot be quantitated.

Assay of colony-forming units in culture (CFU-C) for MTX cytotoxicity. Aliquots of 2 × 10^5 purified MPCs were incubated for up to 24 h in folate-free RPMI-1640 and 10% dialyzed FCS with 0–10 µM MTX, washed twice in cold PBS, and resuspended in 1 ml of RPMI-1640. The CFU-C assay was performed using a semi-solid agar method (14, 17). 2 × 10^4 cells/plate were cultured in 0.3% agar in drug-free RPMI-1640 medium (containing 2.2 µM folic acid) supplemented by 15% dialyzed FCS and 15% MO colony-stimulating factor. After 10–14 d of incubation at 37°C in a humidified atmosphere of 5% CO$_2$ in air, colonies (≥ 40 cells) were counted using an inverted microscope.

Measurements of purine metabolism. The rate of synthesis of purines by the de novo pathway was measured according to the following method: MPCs, 2 × 10^6 cells in a total volume of 1 ml, were incubated at 37°C in folate-free RPMI-1640, 10% dialyzed FCS, and 10 µM thymidine, with or without 1 µM MTX. [2-^{14}C]Glycine was added 2 h before the end of incubation to final concentration of 0.17 mM and final specific activity of 12 mCi/mmol. The cells were washed three times with 2 ml of cold PBS, followed by final pelleting by centrifugation at 2000 *g* for 10 min. Perchloric acid, 0.5 ml of 1 N, was added to the cell pellet and the precipitated macromolecules were depurinated by boiling for 1 h. After depurination, the supernate was neutralized with 1 N KOH and the adenine and guanine bases separated by reverse-phase HPLC and quantitated by liquid scintillation counting of the separated fractions as previously described (18).

Results

Myeloid precursor cell enrichment. Mononuclear cells were exposed to mouse monoclonal antibodies specific for each cell subpopulation except immature myeloid precursors and the antibody-coated cells were reacted with rabbit anti–mouse Ig-coated SRBCs. The final cell population collected after removal of the immune rosettes consisted of 2–6 × 10^6 cells, which constituted an average of 3.2±1.1% of the mononuclear cells in the original marrow sample. The purified population included 61±17% blasts, 6±5% promyelocytes, and 13±10% myelocytes. Additional cells were metamyelocytes and granulocytes (14%), monocytes (2%), lymphocytes (2%), and normoblasts (2%). In three representative experiments, this procedure led to an increase in cloning efficiency in the CFU-C assay from 43.7±11.8 to 414±73 colonies per 10^5 plated cells, i.e., a 9.5-fold increase. Folate pools were measured in this enriched cell population.

Intracellular folate pool measurements. The MPCs were incubated in folate-free RPMI supplemented with 10% dialyzed FCS and 0.05 µM [^{3}H]5-formyl-FH$_4$ for 24 h. The uptake of radiolabeled folate reached an equilibrium at 12–16 h; at this time, total intracellular folate pools were 20.3±2.1 pmol/mg cytosol protein. After removal of cells from medium containing [^{3}H]5-formyl-FH$_4$ and their resuspension in RPMI containing no folate, 92% of the labeled intracellular folate pool remained at 8 h, and its proportional distribution among the specific cofactors remained unchanged. In control cells, 20% of the intracellular folates were in a monoglutamate form. Only reduced

folates were identified in measurable quantities in cells not exposed to MTX. The major cofactors were 10-formyl-FH$_4$ and 5-methyl-FH$_4$, in approximately equal proportions, 41.4 and 36%, respectively (Table I). The remainder of the folates were FH$_4$ and 5-formyl-FH$_4$. 5,10-methylene-FH$_4$ was consistently identified in the bone marrow samples, although its pool size was < 5% of the total folate pool, and as such its precise quantitation was not possible by these methods.

Effect of MTX on intracellular folate pools. We examined the influence of increasing MTX concentrations on the intracellular folate pools. After equilibration with 5×10^{-8} M [^{3}H]5-formyl-FH$_4$ for 24 h, MPCs were exposed to drug for 2 h. Changes were seen after 2 h at drug concentrations as low as 0.1 μM. At this MTX concentration, the 5-methyl-FH$_4$ pool decreased by 35%, while two oxidized cofactors, FH$_2$ and 10-formyl-FH$_2$, not present in control samples were clearly identified at this drug level and increased at higher MTX concentrations. After 1 μM MTX for 2 h, the FH$_2$ pool constituted 32% and 10-formyl-FH$_2$ reached 6% of the total intracellular folates, while at 10 μM MTX, FH$_2$ represented 27% and 10-formyl-FH$_2$ represented 11% of total folates (Fig. 1). The identity of the 10-formyl-FH$_2$ peak on HPLC was verified by its quantitative conversion to 10-formyl-FH$_4$ in the presence of DHFR (2) and by its catalytic activity as a substrate for AICAR transformylase.

Increasing the MTX concentration from 0.1 to 1 μM for 2 h caused further changes in the reduced folate pools, particularly an additional decrease in 5-methyl-FH$_4$ and a complete disappearance of FH$_4$. In contrast, the pool of 10-formyl-FH$_4$ was relatively preserved, the maximal decrease to 65% of the pretreatment level occurring at 1 μM MTX. A further increase in MTX concentration to 10 μM had little additional effect, except for a small increase in the pool of 10-formyl-FH$_2$ (Fig. 1).

In addition to the changes in intracellular distribution of folates, MTX caused a dose-related decrease in the total intracellular folate content (Table II) to a maximum of 65% of control 4 h after exposure to 10 μM MTX. The monoglutamated fraction, which constituted 20% of intracellular folates in control
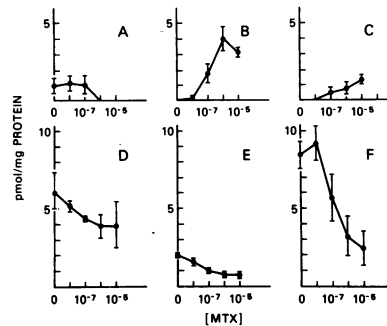

Figure 1. Effect of MTX on intracellular folate pools in MPCs from human marrow. MPCs of normal human marrow were preincubated with 5×10^{-8} M [^{3}H]5-formyl-FH$_4$ for 24 h. MTX was then added to achieve a concentration of 10^{-8}–10^{-5} M and incubation was continued for 2 h. The folates were extracted and analyzed on HPLC as described in Methods. (*A*) FH$_4$; (*B*) FH$_2$; (*C*) 10-formyl-FH$_2$; (*D*) 10-formyl-FH$_4$; (*E*) 5-formyl-FH$_4$; (*F*) 5-methyl-FH$_4$. Each point represents mean±SEM of three separate experiments.

cells, decreased to 14% of the total folates in the presence of 1 μM MTX for 4 h.

We next examined the time course of the intracellular folate changes after exposure to 1 μM MTX (Fig. 2). The main effects, a rapid decrease in the 5-methyl-FH$_4$ (Fig. 2 *F*) and a reciprocal increase in FH$_2$ (Fig. 2 *B*) and 10-formyl-FH$_2$ (Fig. 2 *C*), were measurable after 2 h of exposure, with little further change after 12 h of incubation with MTX. There was a 38% decrease in 10-formyl-FH$_4$ at 2 h, but no further reduction at later time points.

To correlate these changes with the effect of MTX on the rate of de novo purine synthesis, MPCs, 1×10^6 cells per sample, were incubated for 2 and 4 h with 1 μM MTX and labeled with [^{14}C]glycine. The [^{14}C]glycine incorporation decreased to 40±7% of the control after 2 h of MTX and to 23±10% after 4 h of MTX exposure, despite the fact that the folate cofactor for purine biosynthesis, 10-formyl-FH$_4$, decreased by only 38% at 2 h, and remained at this concentration thereafter.

Cytotoxic effect of MTX on CFU-C. To verify that the conditions used in these experiments were cytotoxic to myeloid cells, 2×10^5 MPCs in sample, were incubated with 0.001–10 μM MTX for 12 h and the ability of these cells to form colonies was assessed. A decrease of 24% in CFU-Cs was observed after 0.1 μM MTX for 12 h, 64% after 1 μM MTX, and 84% after 10

Table I. Folate Cofactor Pools in Untreated Myeloid Precursor Cells of Normal Bone Marrow

Folate cofactor	Fraction of total	Pool size
	%	pmol/mg protein
10-Formyl-FH$_4$	41.4	8.4±1.6
FH$_4$	10.3	2.1±0.8
5-Formyl-FH$_4$	12.3	2.5±0.8
5-Methyl-FH$_4$	36	7.3±1.6
Total	100	20.3±2.1

The myeloid precursor cells were preincubated for 24 h in folate-free RPMI-1640 supplemented by 5×10^{-8} M [^{3}H]folinic acid. After extraction and conjugase treatment of the extract, the sample was concentrated with a C$_{18}$ Sep-Pak (see Methods). A 100-μl aliquot of the C$_{18}$ Sep-Pak extract was counted for total folate content, and the remainder of the sample was assayed for individual folates by HPLC. Each value represents the mean±SEM of seven separate experiments. Background counts in the HPLC eluate averaged 50 dpm, while the folate cofactor peaks were 50–300 dpm over background in a typical experiment.

Table II. MTX Concentration Effect on the Total Folate Content in Myeloid Precursor Cells

MTX	Total folates
μM	pmol/mg protein
0	17.5±1.4
0.01	17.2±1.4
0.1	14.4±1.7
1.0	12.5±0.8
10	11.4±1.5

MPCs were preincubated for 24 h in folate-free RPMI-1640 supplemented by 5×10^{-8} M [^{3}H]folinic acid and exposed to indicated concentration of MTX for 2 h. After washing in ice-cold PBS, the cells were processed as per the Methods section. The total radioactivity was measured in 100-μl aliquots of each sample, and total folate content was calculated based on the specific activity of the labeled folates and the cytosol protein concentration. Each value represents the mean±SEM of three separate experiments.

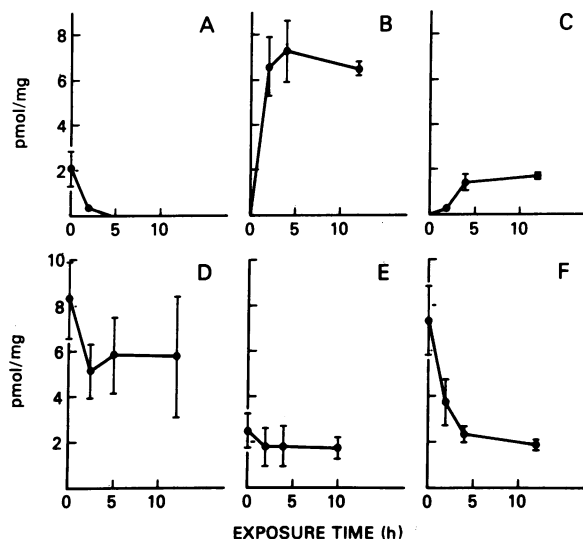*Figure 2.* Effect of 1 μM MTX on intracellular folate pools in MPCs of normal human marrow. MPCs of normal human marrow were preincubated with 5×10^{-8} M [^{3}H]5-formyl-FH$_4$ for 24 h and then exposed to 1 μM MTX for 2, 4, and 12 h. The folates were extracted and analyzed by HPLC. (*A*) FH$_4$; (*B*) FH$_2$; (*C*) 10-formyl-FH$_2$; (*D*) 10-formyl-FH$_4$; (*E*) 5-formyl-FH$_4$; (*F*) 5-methyl-FH$_4$. Each point represents mean±SEM of seven separate experiments.

μM MTX. These changes parallel the expansion of the intracellular FH$_2$ pool, which was 50% of its maximal value at 0.1×10^{-6} M and was maximal at 1×10^{-6} M (Fig. 3).

The cytotoxic effect of MTX is attributed to its inhibitory effect on both the pyrimidine and purine synthesis. To determine that purine inhibition contributed to the cytotoxic effect of MTX, the cells were incubated for 12 h in the presence of 0.01–10 μM concentrations of MTX and 10 μM thymidine. The cells were washed and resuspended in drug-free and rescue-free medium before CFU-C assays. The addition of thymidine provided minimal protection, increasing the plating efficiency from 36 to 55% of the control in the presence of 1 μM MTX and from 16 to 23% at 10 μM MTX. A complete protection against MTX toxicity in these cells was achieved by incubation of cells with the combination of 10 μM thymidine and 10 μM deoxyinosine (15).
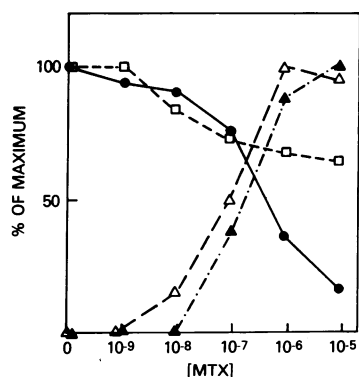

Figure 3. Relationship between MTX concentration, changes in intracellular folates, and cytotoxicity (See legends in Figs. 1 and 2). The quantitation of intracellular folates, as well as assay of myeloid colony formation, were performed as described in Methods after incubation with MTX at indicated concentrations for 12 h. CFU-Cs (*solid circles*) and 10-formyl-FH$_4$ (*open squares*) are shown as percent of control. FH$_2$ (*open triangles*) and 10-formyl-FH$_2$ (*solid triangles*) are expressed as percent of the highest measured level.

Discussion

The present studies provide for the first time an estimation of the intracellular folate distribution in human myeloid precursor cells. The primary intracellular folates in these cells, each comprising $\sim$ 40% of the total folate pool, were 5-methyl-FH$_4$, which is readily converted to one of the active cofactor forms, and 10-formyl-FH$_4$, the required cofactor for de novo purine synthesis. Using these methods, we were unable to precisely measure intracellular levels of 5,10-methylene-FH$_4$, the cofactor for thymidylate synthase and estimate that the concentration of this cofactor must be < 5% of the total folate pool in bone marrow myeloid precursor cells. Our estimate of the total folate pool, 20.3±2.1 pmol/mg cytoplasmic protein, is sevenfold higher than previous determinations of the folate content of unseparated bone marrow cells measured by microbiologic assay (19–21). The difference may be due to the higher folate content of immature cells; previous comparisons of leukemic myeloid cells versus normal leukeocytes revealed fivefold higher folate content in the blast cell population (22–24).

This assay was used to examine the changes in intracellular folates induced by MTX, an agent that inhibits DHFR and is believed to exert its effects by inhibition of DHFR and depletion of cellular reduced folates. MTX caused a rapid decrease in 5-methyl-FH$_4$ and depletion of FH$_4$ to unmeasurable levels, but only a partial (30–40%) decrease in the important cofactor 10-formyl-FH$_4$. Thus, the biologically important 10-formyl-FH$_4$ pool is conserved at the expense of 5-methyl-FH$_4$, a depot form of folate. The drug's inhibition of purine synthesis and that component of its toxicity related to purine deficiency thus could not be ascribed to depletion of 10-formyl-FH$_4$. Rather, inhibition of purine synthesis and CFU-C toxicity correlates with the marked expansion of the intracellular pool of FH$_2$, a change that may contribute to MTX toxicity by direct inhibition of AICAR transformylase (5) and thymidylate synthase (3). At later time points, MTX polyglutamates that are formed in human myeloid cells, albeit in limited quantities compared with malignant cells (15), may contribute to the further direct inhibition of these enzymes. The intracellular FH$_2$ concentration after 2 h exposure to 1 or 10 μM MTX reached a plateau level of 7 pmol/mg protein, or $\sim$ 1–2 μM. This concentration is on the order of magnitude required to inhibit AICAR transformylase (K_i = 2.7 μM) when facing pentagluatamated folate substrate (5). The degree of inhibition of the enzyme by FH$_2$ will ultimately depend on the intracellular concentrations of FH$_2$ and the substrate (10-formyl-FH$_4$) and the relative extent of polyglutamation of each.

It should be noted that the final cytotoxic effect of MTX is achieved through a collateral inhibition of purine as well as thymidylate synthesis. Only the purine arm of the MTX toxicity mechanism was examined in the present study.

The folate pool perturbations caused by MTX in normal myeloid precursors closely parallel the changes found in MCF-7 breast cancer cells after MTX exposure (2). In this tumor cell line, the 10-formyl-FH$_4$ pool was only 20% diminished by 21-h incubation with 1 μM MTX, as compared with the 40% reduction in the normal myeloid precursors in the present study. Thus, in neither the normal cells nor the malignant cells does MTX cause quantitative depletion of this cofactor under cytotoxic conditions in tissue culture. Its selective action against malignant cells may relate to the more avid formation of MTX polyglutamates in sensitive malignant cells (25–31), because these forms of MTX markedly extend its intracellular half-life and

contribute to direct inhibition of purine and thymidylate synthesis.

The accumulation of 10-formyl-FH_2 during MTX exposure bears a comment. The occurrence of this folate was previously reported in MCF-7 breast cancer cells treated by MTX, and it was suggested that 10-formyl-FH_2 might be generated through metabolic conversion from FH_2 (2). The physiologic and pharmacologic actions of this folate are currently under investigation.

In addition to the effect of MTX on the intracellular distribution of folates, the drug caused a depletion of total intracellular folate levels, reaching a maximum reduction of 35% at 10 μM MTX. The mechanism of this depletion is uncertain but may be caused by the heteroexchange mechanism described by Goldman (32). At least one of the three cellular efflux pumps for folate is coupled to the influx mechanism. Movement of MTX molecules into the cell by this pump would drive out an intracellular folate molecule or another anion that shares the pump. Consistent with this possibility was the finding of a disproportionate decrease in the intracellular concentration of monoglutamated folates, the most readily transported fraction of the intracellular folate pool, from 20 to 14% of total folate after 4-h exposure to 1 μM MTX.

The distribution of intracellular folates found in myeloid precursor cells resembles that found in intestinal mucosa, but differs from that of nondividing tissue. According to microbial assays, 5-methyl-FH_4 is the primary folate found in nondividing tissues such as liver (8, 33–35), kidney (33), or red blood cells (36). In contrast, the predominant folate in rapidly growing tumor cells is 10-formyl-FH_4 (34, 37, 38), the required cofactor for the AICAR transformylase and glycinamide ribonucleotide (GAR) transformylase reactions in the purine synthetic pathway. These two folate forms (5-methyl-FH_4 and 10-formyl-FH_4) are present in equal concentrations in intestinal mucosa (34) and, according to the present study, in the immature myeloid fraction of normal marrow, indicating the intermediate proliferative potential of the cell fractions used in these experiments. It is possible that a more highly purified myeloblast fraction would contain higher levels of 10-formyl-FH_4.

Acknowledgments

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