Folate antimetabolites are among the most widely used cancer chemotherapeutic agents. Methotrexate (MTX), an inhibitor of dihydrofolate reductase (DHFR), has played a significant role in the overall survival and improved central nervous system (CNS) chemoprophylaxis of children with acute lymphoblastic leukemia (ALL). More recently, a new generation of potent inhibitors of thymidylate synthase (TS; ie, ZD1694 [tomudex] and LY231514) and of de novo purine synthesis via inhibition of glycaminide ribonucleotide formyltransferase (ie, DDATF [lometrexol]) has been developed. These new classes of cytotoxic antifolates, and a wide variety of “classical” antifolates (ie, those with a fused heterocyclic ring linked to p-aminobenzoylglutamic acid), are substrates for the enzyme folylpolyglutamate synthetase (FPGS). Several lines of evidence indicate that polyglutamation plays a central role in the therapeutic action of cytotoxic antifolates. After entering mammalian cells, MTX, tomudex, and lometrexol are extensively converted to polyglutamate (PG) forms and as such are better retained inside the cell. For MTX, this prolonged intracellular entrapment results in the extended inhibition of DHFR by MTX-PG. For the new generation of antifolates, aimed at TS (tomudex) and glycaminide ribonucleotide formyltransferase (lometrexol), polyglutamation not only results in prolonged intracellular retention but also increases their inhibitory activity against their target enzymes by more than 100-fold. This effect is sufficiently pronounced that these compounds have been considered produgs by some investigators, with FPGS being essential for their conversion to the active moiety. Furthermore, recent reports indicate low or altered FPGS activity as a mechanism of resistance to cytotoxic antifolates and suggest a role in the selectivity of antifolates by showing different kinetic behaviors of FPGS extracted from normal murine intestinal epithelium and from two antifolate sensitive mouse tumors.

In the present study, we have determined the levels of FPGS activity in blasts from children with newly diagnosed ALL treated with high-dose or low-dose MTX administered as a single agent before conventional remission induction therapy. We found higher FPGS activity in ALL blasts when compared with acute nonlymphoblastic leukemia (ANLL) blasts, and this lineage difference in FPGS activity was also found in normal lymphoid versus nonlymphoid hematopoietic progenitors. Furthermore, our data indicate that ALL blast FPGS activity increases after in vivo exposure to MTX, and that this increase is greater in B-lineage than in T-lineage ALL.

MATERIALS AND METHODS

Materials. Sephadex G-50 (30 to 80 μm), formaldehyde, ATP, FdUMP, sucrose, HEPES, magnesium chloride, EGTA, benzamidine, soybean trypsin inhibitor, type II ovomucoid trypsin inhibitor, monothioglycerol, and L-glutamic acid were purchased from Sigma Chemical Co Inc (St Louis, MO). α-Toluene-sulfonyl fluoride was from Eastman Kodak Co (Rochester, NY). One-milliliter syringes were from Becton Dickinson (Rutherford, NJ). [3,4-3H] Glutamic acid was purchased from New England Nuclear (Wilmington, DE) and was used without further purification. The scintillation cocktail used was Ready Protein- from Beckman Instruments Inc (Fullerton, CA). All media and serum were obtained from GIBCO BRL (Bethesda, MD). Anti-CD34 (HPCA-1) and anti-CD10 (anti- CALLA) antibodies were purchased from Becton Dickinson (San Jose, CA). Immunomagnetic beads coated with sheep antirabbit IgG (Dynabeads M-450) were from Dynal Inc (Great Neck, NY).

Differences in Constitutive and Post-Methotrexate Folylpolyglutamate Synthetase Activity in B-Lineage and T-Lineage Leukemia

By Julio C. Barreto, Timothy W. Synold, Joseph Laver, Mary V. Relling, Ching-Hon Pui, David G. Priest, and William E. Evans

Folylpolyglutamate synthetase (FPGS) is responsible for the metabolism of natural folates and a broad range of folate antagonists to polyglutamate derivatives. Recent studies indicate increased accumulation of methotrexate (MTX) polyglutamates (MTX-PG) in blast cells as a predictor of favorable treatment outcome in childhood acute lymphoblastic leukemia (ALL). We determined the expression of FPGS activity in blasts from children with ALL at diagnosis and after treatment with MTX as a single agent, before conventional remission induction therapy. The levels of enzyme activity in ALL blasts at diagnosis (median of 689 pmol/h/mg protein) were significantly higher \( (P = .003) \) than those found in acute nonlymphoblastic leukemia (ANLL) blasts (median of 181 pmol/h/mg protein). Comparable lineage differences in normal lymphoid versus nonlymphoid cells suggest a lineage-specific control of FPGS expression. FPGS activity increased in ALL blasts after in vivo exposure to MTX. The median increase in FPGS activity was significantly higher \( (P = .003) \) in B-lineage ALL (188%) than in T-lineage ALL (37%). Likewise, the percentage of intracellular long chain MTX-PG (Glut₄₅) was significantly higher \( (P = .02) \) in B-lineage ALL (92%) than in T-lineage ALL (65%), consistent with higher FPGS activity in B-lineage blasts. This finding could explain, at least in part, the superior outcome in children with B-lineage ALL treated with antimetabolite therapy. © 1994 by The American Society of Hematology.

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Leukemic blasts cytosol preparation. Blasts were isolated from bone marrow aspirates by a standardized technique using a Ficoll-Hypaque centrifugation gradient at 4°C. The mononuclear layer was collected and washed three times in a culture media consisting of 90% RPMI and 10% fetal bovine serum at 4°C. Then, two volumes of homeogenizing buffer, containing protease inhibitors as previously described, were added. Cell suspensions were disrupted using an Ultrasonics W-380 Sonicator (Heat Systems Ultrasonics, Farmingdale, NY). A 160,000g supernatant fraction was then prepared using a Beckman Airfuge driven at 30 psi for 18 minutes. This high-speed supernatant fraction was passed through a Sephadex G-50 spin column (Sigma Chemical Co, St Louis, MO) to remove endogenous glutamic acid that might interfere with the assay.

Hematopoietic cell isolation and cytosol preparation. After we received approval from the Institutional Review Board and informed consent was obtained, heparinized bone marrow was collected from eight normal human volunteer donors. Mononuclear cells were separated using a Ficoll-Hypaque centrifugation gradient at 4°C. Cells were then washed and incubated with mouse antihuman CD34 or CD117 monoclonal antibodies. Selected populations were isolated using immunomagnetic beads coated with sheep antimouse IgGl. Finally, isolated cell populations were released from the immunomagnetic beads by the addition of chymopapain. After washing cells with phosphate-buffered saline (PBS), high-speed supernatants were prepared as described for leukemic blasts. This isolation was performed following a modification of the procedure described for immunomagnetic purging of neuroblastoma cells from human bone marrow. The purity of all isolated cell populations was determined by flow cytometric analysis. In addition, CD34+ cells were plated and cultured for determination of colony-forming unit granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and mixed colonies (CFU-GEMM) using standard clonogenic assays.

FPGS microassay. This assay relies on the formation of a covalently bound ternary complex of TS, 5,10-methylenetetrahydrofolate (or the polyglutamate forms of this compound), and fluorodeoxyuridine (FdUMP). The reaction mixture is then passed through a Sephadex G-50 spin column to isolate macromolecular complex from excess unreacted L-[3H] glutamic acid. The eluate is collected into scintillation vials, and the radioactivity is determined using a liquid scintillation counter. Protein concentrations were determined by the Bradford dye-binding procedure. An HPLC method was used to quantify intracellular long chain MTX-PG in B-versus T-lineage ALL blasts. This two-step methodology consists of an HPLC separation, followed by a radioenzymatic assay using DHFR as the binding protein to quantitate MTX and MTX-PG in each HPLC fraction.

Statistical analysis. Individually comparisons of FPGS activity were made using a Mann-Whitney U test, whereas intra-individual comparisons were made using a Wilcoxon-Signed rank test. The percentage of intracellular long chain MTX-PG in B-versus T-lineage ALL blasts was compared using a Mann-Whitney U test.

RESULTS

Patients studied. FPGS activity was measured in leukemic blasts obtained from 47 patients with ALL (34 B-lineage and 13 T-lineage) and 10 with ANLL. Blasts were obtained from all ANLL patients at diagnosis, before starting therapy. In the 47 ALL patients, 20 had sufficient blasts for measurement of FPGS activity both at diagnosis and at 44 hours after starting MTX therapy, whereas 17 were studied only at diagnosis and 10 patients were studied only at 44 hours after starting MTX therapy. There were sufficient blasts from the 44 hours bone marrow for measurement of MTX-PG in 42 ALL patients (31 B-lineage and 11 T-lineage).

FPGS activity in ALL and ANLL blasts. FPGS activity in ALL (n = 37) blasts at diagnosis (both B-lineage and T-lineage) was significantly higher than in ANLL blasts (n = 10; median, 689 ± 181 pmol/h/mg protein, P = .003; Table 1). Higher FPGS activity was seen in B-lineage blasts (CD10 or common ALL antigen [CALLA] positive) as compared with T-lineage blasts (median, 801 ± 261 pmol/h/mg protein;
FPGS activity was measured for the present study did not differ for leucovorin rescue. The subgroup of patients in whom to MTX when compared with pretreatment levels determined in leukemic lymphoblasts at diagnosis (0 hours) and after exposure to single-agent MTX progenitor cells (CD34+; median, 828). FPGS activity when compared with normal nonlymphoid lymphoid progenitor cells (CD10/CD19+) expressed higher metabolites such as MTX and to the new generation of TS Tumor sensitivity and resistance to "classical" folate antimitabolites such as MTX and to the new generation of TS

Table 1), although this did not reach statistical significance (P = .15) because of the heterogeneity of activity and small sample size.

FPGS activity in normal hematopoietic progenitors. Normal CD10+ cells, representative of lymphoid progenitors, were isolated from human volunteer donors using monoclonal antibodies (MoAbs) and immunomagnetic beads (see Materials and Methods). An 80% enrichment was confirmed using MoAb staining and flow cytometric analysis, and this population coexpressed the CD19 antigen (a B-lineage marker throughout all stages of differentiation). For comparison with early nonlymphoid progenitors, an anti-CD34 MoAb was used following the same methodology. This partially purified population also coexpressed the CD33 antigen (a myeloid marker) in 45% of isolated cells. As previously reported, in normal human bone marrow, only 4.9% ± 0.8% of mononuclear cells within the lymphoid-blast window are CD34+. Additionally, less than 0.6% of B-lineage progenitors (CD10/CD19+) will coexpress the CD34 antigen, thus indicating negligible cross-reactivity. Normal lymphoid progenitor cells (CD10/CD19+) expressed significantly higher FPGS activity when compared with normal nonlymphoid progenitor cells (CD34+; median, 828 ± 158 pmol/h/mg protein, P < .001; Table 1). These results are consistent with our finding of differences in expression of FPGS in leukemic cell populations of lymphoid versus nonlymphoid origin.

Pre- and post-MTX FPGS activity. Enzyme activity was determined in leukemic lymphoblasts at diagnosis (0 hours) and after exposure to single-agent MTX (44 hours), but before leucovorin rescue. The subgroup of patients in whom FPGS was measured for the present study did not differ significantly in presentation when compared with the overall study patient population (data not shown). A median 2.4-fold increase in FPGS activity was observed after exposure to MTX when compared with pretreatment levels (P < .02; Fig 1). FPGS activity increased in all patients studied, with no apparent difference between patients treated with HDMTX versus LDMTX.

FPGS activity in B-lineage versus T-lineage ALL. Although blast FPGS activity increased in all patients studied at 44 hours post-MTX (Fig 1), there was a significantly greater fractional increase in blasts from patients with B-lineage versus T-lineage ALL (median, 188% for B-lineage v 37% for T-lineage blasts, P = .003; Fig 2). Although there was not a significant difference in activity between B-lineage and T-lineage blasts obtained at diagnosis, there was significantly higher FPGS activity in B-lineage versus T-lineage ALL blasts obtained at 44 hours post-MTX exposure (median, 1,249 v 308 pmol/h/mg protein, P = .03).

MTX-PG accumulation in B-lineage versus T-lineage ALL. The percentage of intracellular long chain MTX-PG (Glu3-MTX) was significantly higher (P = .02) in B-lineage ALL (92%) compared with T-lineage ALL (65%; Fig 3). This finding is consistent with higher FPGS activity in B-lineage versus T-lineage ALL blasts, and may reflect the greater fractional increase in post-MTX FPGS activity in B-lineage compared with T-lineage ALL.

DISCUSSION

Tumor sensitivity and resistance to "classical" folate antimitabolites such as MTX and to the new generation of TS

![Fig 1. Changes in blast FPGS activity after treatment with MTX. Activity in B-lineage (n = 14) and T-lineage (n = 6) lymphoblasts was measured at diagnosis immediately before MTX (Dx) and 44 hours after the start of MTX single-agent therapy (44 h). Open symbols represent LDMTX and closed symbols represent HDMTX patients; lines connect paired samples for each patient; horizontal lines and numbers are median values. A median 2.4-fold increase in post-MTX (44 hours) ALL blast (B- and T-lineage) FPGS activity was observed compared with diagnosis (Dx, P < .02).](image1)

![Fig 2. Percentage change in FPGS activity from pretreatment (diagnosis) to 44 hours after MTX therapy in B-lineage (n = 14) versus T-lineage (n = 6) blasts from children with ALL. Data were derived from paired samples. Horizontal lines (and numbers) represent median percentage of change; boxes span 25th to 75th percentile for patients with B-lineages (n = 14) and T-lineage (n = 6) ALL.](image2)
inhibitors (ie, tomudex, LY231415) and GARFT inhibitors (ie, lometrexol) is highly dependent on polyglutamation by FPGS. To our knowledge, this is the first study to characterize the in vivo differential changes in blast FPGS activity that occur after MTX therapy in children with B-lineage and T-lineage ALL.

Our findings indicate that lymphoblasts from most children with ALL at diagnosis express high in vivo FPGS activity, providing a biochemical basis for their intrinsic sensitivity to antifolates, ie, MTX. Moreover, Whitehead et al21 showed a survival advantage for children with ALL whose blasts at diagnosis accumulated higher levels of MTX and MTX-PG in vitro. Our study provides one mechanism by which higher MTX-PG accumulation would occur in this patient population, ie, higher ALL blast FPGS activity. In contrast, MTX has proven to have no significant therapeutic activity in ANLL.22 Whitehead et al21 suggested increased MTX-PG catabolism, whereas Lin et al22 reported normal MTX transport and suggested decreased FPGS activity leading to decreased long chain MTX-PG accumulation in ANLL blasts as a mechanism for resistance in vitro. Our data indicate that ANLL blasts have low FPGS activity, suggesting decreased FPGS as a mechanism for their lower accumulation of long chain MTX-PG and a biochemical basis for their resistance to antifolates. Thus, the significant differences in FPGS activity in ALL versus ANLL blasts could provide a biochemical basis for their differential intrinsic sensitivity to antifolates.

The expression of FPGS activity in normal and malignant tissues appears to play a critical role in the sensitivity and selectivity to antifolates. Barredo and Moran previously reported undetectable levels of FPGS activity in circulating mature hematopoietic cells, whereas enzyme activity was found to be high in blasts from adult and pediatric ALL patients. In a related study, Koizumi et al27 reported very low in vitro accumulation of MTX-PG in an unpurified population of normal human bone marrow cells. To address these differences between normal and transformed cells, we isolated partially purified populations of normal bone marrow progenitor cells representative of lymphoid (CD10/CD19+) and nonlymphoid (CD34+) lineages. Normal lymphoid progenitor cells (CD10/CD19+) have high FPGS activity, similar to enzyme activity in childhood ALL blasts, which are CD10 (CALLA or common ALL) antigen positive in 90% of cases.23 In contrast, normal nonlymphoid progenitors (CD34+) express lower FPGS activity; likewise, in the same range as those in ANLL blasts. It is unclear whether these differences in FPGS activity found in normal and leukemic cells of lymphoid versus nonlymphoid origin reflect changes in gene expression, or result from posttranscriptional or posttranslational modification(s). Nonetheless, these differences suggest a cell lineage-specific control for FPGS within the human lymphohematopoietic system, probably independent of the events leading to leukemogenesis. The proliferation, differentiation, and survival of hematopoietic progenitor cells is sustained by a variety of hematopoietic growth factors. Even though little is known about the precise cascade of events initiated by the binding of these glycoproteins to their cell surface receptors, it is tempting to speculate that they may also play a role in the lineage-specific control of FPGS. These data support the hypothesis that the control of expression of FPGS is regulated by at least two mechanisms, one of which is linked to proliferation and another that controls enzyme levels during differentiation and is tissue (or cell lineage) specific.

When comparing blast cell FPGS activity at diagnosis (0 hours) and after in vivo exposure to MTX (44 hours) in children with ALL, we found a 2.4-fold increase in activity (P < .02). The mechanism by which antifolates such as MTX increase FPGS activity remains to be defined, but could involve translational and/or transcriptional modification(s) resulting in accumulation of the FPGS protein, as was recently observed for thymidylate synthase in vitro. Nimec and Galivan reported a 1.5- to 2-fold increase in the rate of MTX polyglutamation in folate-depleted H35 hepatoma cells in vitro, which may be due to increased FPGS activity or less competition by intracellular folates resulting in a consequent increase of FPGS sites available for MTX binding. Alternatively, drug-induced or nutritional folate deficiency might upregulate the expression of FPGS as a compensatory mechanism to insure cellular integrity. In addition, MTX-induced alterations in cell cycle regulation and/or quantitative changes in the proliferative cell fraction (percentage of S-phase cells) in the bone marrow could result in the changes in FPGS activity detected at 44 hours (post-MTX).

Children with B-lineage ALL have a better prognosis than both children with T-lineage ALL and adults with B- or T-lineage ALL. Goker et al25 reported higher in vitro accumulation of total MTX and MTX-PG in pediatric B-lineage blasts than in blasts from T-lineage ALL patients and adults with B-lineage ALL. They suggested decreased polyglutamate formation or increased breakdown as responsible for the reported low levels of MTX-PG in T-lineage and adult B-lineage ALL. Our results indicate that blasts from children with B-lineage ALL tend to have higher constitutive FPGS changes in ALL

Fig 3. Percentage of intracellular MTX metabolized to long chain polyglutamates (MTX-PGn) in vivo, in patients with B-lineage (n = 31) and T-lineage (n = 11) ALL. Horizontal lines (and numbers) represent median values; boxes span 25th to 75th percentile. Blasts were obtained 44 hours after starting therapy with MTX and before leucovorin rescue (see Materials and Methods for details).
activity, but, more importantly, have a significantly higher increase in their FPGS activity after in vivo exposure to MTX when compared with blasts from children with T-lineage ALL \((P = .003)\). Furthermore, B-lineage ALL blasts accumulated a higher percentage of long chain MTX-PG compared with T-lineage ALL blasts \((P = 0.02)\), consistent with higher FPGS activity in B-lineage versus T-lineage ALL. Long chain PG are preferentially retained inside the cell; thus, drug efflux would be expected to have negligible effects in the analysis of our data. However, it remains to be determined whether multiple mechanisms (eg, transport, gamma-glutamyl hydrolase, intracellular folate content, and FPGS) contribute to the lineage differences in MTX-PG accumulation. These long chain MTX-PG are considered to be the active metabolites and responsible for enhancing the cytotoxicity of the parent compound.\(^6\)\(^\text{26}\

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Differences in constitutive and post-methotrexate folylpolyglutamate synthetase activity in B-lineage and T-lineage leukemia

JC Barredo, TW Synold, J Laver, MV Relling, CH Pui, DG Priest and WE Evans