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Determinants of Antifolate Cytotoxicity: Folylpolyglutamate Synthetase Activity during Cellular Proliferation and Development

JULIO BARREDO[1] and RICHARD G. MORAN

Department of Pediatrics and Biochemistry, University of Southern California, Los Angeles, California 90033 and Division of Hematology-Oncology, Children's Hospital of Los Angeles, Los Angeles, California 90027

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SUMMARY

Previous studies have documented the metabolism of a broad range of folate antimetabolites to polyglutamate derivatives by the enzyme folylpoly-γ-glutamate synthetase (FPGS). The activity of the more recently developed classes of antifolates directed against thymidylate synthase and *de novo* purine synthesis is sufficiently dependent on polyglutamation that these compounds should be specifically cytotoxic to any normal or malignant proliferating cell expressing this enzyme. We have studied the patterns of expression of FPGS in mammalian cells and tissues during rapid growth, growth arrest, differentiation, and embryonic development. During embryogenesis in the rat, FPGS levels in liver and brain were higher during the period of proliferative activity and then dropped to a level characteristic of the adult organs. However, the levels in liver were substantially higher than those in brain at any given time. This pattern was mimicked in mouse C3H 10T1/2 embryo fibroblast cells, in which FPGS activity decreased after cessation of growth but then remained at a lower steady state level during an extended period of postconfluent culture. Enzyme activity also dropped after the differentiation of human HL-60 promyelocytic leukemia cells. In a human homolog of these experimental systems, FPGS levels were below the limits of detection in circulating mature human hematopoietic cells of the granulocytic, lymphoblastic, and erythrocytic lineages. In striking contrast, substantial levels of FPGS were found in circulating lymphoblasts from eight patients with acute lymphoblastic leukemia. The levels of FPGS found in these transformed stem cells would help to explain the sensitivity of many acute lymphoblastic leukemias to folate antimetabolites. We concluded that expression of FPGS is regulated by at least two mechanisms, one of which is linked to proliferation and the other of which controls enzyme levels after differentiation and is tissue specific.

Folate antimetabolites have been involved in some of the major breakthroughs seen in the development of cancer chemotherapy during the last four decades. The dihydrofolate reductase inhibitor MTX has played a significant role in the increased survival of children with ALL (1–4) and in central nervous system chemoprophylaxis in children with this disease (5, 6). Combination of 5-formyltetrahydrofolate with 5-fluorouracil has been shown to prolong the inhibition of thymidylate synthase caused by this drug (7), and this combination has shown the promise of displacing 5-fluorouracil alone in the treatment of patients with metastatic colorectal carcinoma (8). More recently, folate analogs that are potent inhibitors of thymidylate synthase [e.g., CB3717 (10-propargyl-5,8-dideazafolic acid) and its second-generation derivative D-1694] (9, 10) and of *de novo* purine synthesis (e.g., DDATHF) (11) have been developed. Both of these new classes of folate antimetabolites were active antitumor agents in animal models of human disease (12, 13), as well as in early phases of clinical testing (14–17). The therapeutic activity of these several examples of metabolic inhibitors aimed at folate metabolism suggests an underlying metabolic difference between stem cells of normal tissues and those of folate antimetabolite-sensitive tumors.

A wide variety of "classical" antifolates, i.e., those with a fused heterocyclic ring linked to *p*-aminobenzoylglutamic acid, are substrates for the enzyme FPGS (18–21). A single enzyme appears to be responsible for the synthesis of di- to heptaglutamate derivatives of both folates and antifolates in mammalian cells (22). Several lines of evidence indicate that this polyglutamation is central to the action of classical antifolates, both as toxic compounds and as drugs that are therapeutically active for the treatment of human cancers. After exposure of proliferating mammalian tumor cells to either MTX, amino-

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[1] Present address: Medical University of South Carolina, The Children's Hospital, Division of Pediatric Hematology-Oncology, 171 Ashley Ave., Charleston, SC 29425.

ABBREVIATIONS: MTX, methotrexate; DDATHF, 5,10-dideaza-5,6,7,8-tetrahydrofolate; FPGS, folylpoly-γ-glutamate synthetase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RA, retinoic acid; DMF, dimethylformamide; ALL, acute lymphoblastic leukemia; FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate; NBT, nitroblue tetrazolium.

pterin, CB3717, D-1694, or DDATHF, essentially all intracellular drug is converted to polyglutamate forms (10, 23–29). These polyglutamate metabolites persist in sensitive tumor cells for substantial periods of time in the absence of extracellular drug (29–31). On the other hand, the polyglutamation of MTX has been reported to be very low in normal human bone marrow cells (32) and in intestinal epithelial cells (25). Although this earlier literature suggests that differences in FPGS activity, *per se*, between stem cells of drug-sensitive normal and neoplastic tissues are involved in the chemotherapeutic selectivity of MTX, this relationship now seems more complex. Thus, a more recent study has indicated that FPGS is, indeed, present in intestinal epithelial cells and that the activity of FPGS is substantially higher in the cells of the intestinal crypts than in the mature cells at the tips of the intestinal microvilli (33). Hence, the questions remain whether the characteristics of FPGS or the control of this enzyme differ between tumors and normal stem cell populations and whether any such differences are the root of the chemotherapeutic utility of folate antimetabolites.

The new generations of antifolates aimed at thymidylate synthase and glycinamide ribonucleotide formyltransferase depend on polyglutamation for their cytotoxic activity to a greater degree than does MTX. As is the case with MTX, the retention of both D-1694 and DDATHF in cells is dependent upon polyglutamation, but, also, the activity of both D-1694 and DDATHF against their target enzymes is increased >100-fold by the addition of a polyglutamate side chain (10, 34). This is in contrast to the interaction of MTX with dihydrofolate reductase, which is essentially unchanged by polyglutamation (35). The effect of polyglutamation on DDATHF, D-1694, and the new thymidylate synthase inhibitor LY 231514[2] is sufficiently pronounced that we have concluded that these compounds are prodrugs. Hence, any cell that expresses sufficient FPGS would be recognized and potentially killed by these drugs.

The ongoing clinical trials of DDATHF and D-1694 (14, 36) mark the first time that such efficiently polyglutamated drugs have been administered to humans. The fact that these two compounds represent perhaps the most potent antimetabolites yet developed to clinical trial raises the question of the distribution of FPGS in mammalian tissues and the mechanism of control of expression of this enzyme. Using a new microassay for FPGS activity (37), we have studied the expression of this enzyme in model systems for mammalian cell proliferation and development. Our results indicate that the control of FPGS has at least two components, one of which is responsible for high enzyme levels in rapidly dividing cells, compared with nonproliferating cells of the same lineage, and one of which determines FPGS levels in a tissue-specific manner.

Experimental Procedures

Materials. Sephadex G-50 (30–80 μm), formaldehyde, ATP, FdUMP, sucrose, HEPES, magnesium chloride, EGTA, benzamidine, soybean trypsin inhibitor, type II ovomucoid trypsin inhibitor, monthioglycerol, and L-glutamic acid were purchased from Sigma Chemical Co. Inc. (St. Louis, MO). α-Toluenesulfonyl fluoride was from Eastman Kodak Co. (Rochester, NY). One-milliliter syringes were from Beckton-Dickinson (Rutherford, NJ). [3,4-^{3}H]Glutamic acid was purchased from New England Nuclear (Wilmington, DE) and was used without further purification. The scintillation cocktail used was Budget-Solve from Research Products International Corp. (Mount Prospect, IL). (6*S*)-Tetrahydrofolate was prepared from folic acid by an enzymatic procedure described previously (38). Thymidylate synthase was prepared either from *Lactobacillus casei* resistant to MTX (39) (from Dr. Bruce Dunlap, University of South Carolina) or from a strain of *Escherichia coli* bearing a plasmid (40) that allows expression of the *L. casei* thymidylate synthase (provided by Dr. Daniel Santi, University of California at San Francisco).

FPGS microassay. This assay relies on the formation of a covalently bound ternary complex of thymidylate synthase, 5,10-methylenetetrahydrofolate (or the polyglutamate forms of this compound), and FdUMP and has been described in detail (37). Briefly, this procedure involves two consecutive reactions. In the first reaction, which is carried out in a total volume of 10 μl for crude cytosols, any FPGS present in a sample mixture catalyzes the addition of L-[^{3}H]glutamic acid to tetrahydrofolate to form [^{3}H]tetrahydropteroyldiglutamate. In a second reaction, the [^{3}H]tetrahydropteroyldiglutamate is incorporated into a covalently bound ternary complex in the presence of thymidylate synthase, formaldehyde, and FdUMP. The reaction mixture is then passed through a Sephadex G-50 spin column to isolate macromolecular product from excess unreacted L-[^{3}H]glutamic acid. The eluate is collected into scintillation vials, 5 ml of scintillation cocktail are added, and the radioactivity is determined in a liquid scintillation counter. Protein was determined by either the Hartree (41) modification of the Lowry method or by a dye-binding procedure (Bio-Rad Laboratories, Richmond, CA).

Rat liver and brain FPGS cytosol preparations. Timed pregnant Sprague-Dawley rats were purchased from Harlan Sprague-Dawley Laboratories (Indianapolis, IN). Animals were anesthetized with CO_2 and sacrificed, and pups were quickly removed; embryonic liver and brain were excised and kept on ice. Two volumes of homogenizing buffer (20 mM HEPES, pH 8.5, 250 mM sucrose, 57 mM $MgCl_2$, 6.5 mM EGTA, 17 mM benzamidine, 0.7 mM ATP, 13.3 mg/liter soybean trypsin inhibitor, 13.3 mg/liter type II ovomucoid trypsin inhibitor, 4.3 ml/liter monothioglycerol, plus 10 μl of freshly prepared 0.1 M α-toluenesulfonyl fluoride per ml of solution) were added, and tissues were disrupted using a motor-driven homogenizer. Homogenate was centrifuged at 14,000 rpm for 5 min in a microcentrifuge to remove cellular debris. A 160,000 × *g* supernatant fraction was then prepared using a Beckman Airfuge driven at 30 psi for 18 min. The high-speed supernatant fraction was passed through a Sephadex G-50 spin column to remove any glutamic acid present in the sample that would interfere with the assay (37).

Cell culture. Two cell culture systems were used to assess changes in FPGS activity during cellular proliferation and differentiation. The mouse embryo fibroblast cell line C3H 10T1/2 was obtained at passage 6 from Dr. Shirley Taylor (University of Southern California) and was grown as a monolayer culture system at 37° in RPMI 1640 medium supplemented with 10% fetal bovine serum, in a 5% CO_2 atmosphere. Medium was changed three times per week in order to ensure cellular integrity. The viability of these cells after their growth had been arrested by contact inhibition was assessed by plating efficiency upon subculture. The HL-60 cell line, a human promyelocytic cell line, was obtained from the American Type Culture Collection (Rockville, MD) and was grown as a suspension culture at 37° in RPMI 1640 medium in the presence of 10% fetal bovine serum and a 5% CO_2 atmosphere. Suspension cultures of HL-60 cells were seeded at an initial density of 2.5×10^5 cells/ml; differentiation was induced by the addition of 1 μM RA at 24 hr and 60 mM DMF at 48 hr (42–45). This procedure is that classically used to direct HL-60 cells to the granulocytic pathway (45). Cell culture density was determined with a model Zf particle counter from Coulter Electronics Inc. (Hialeah, FL). The degree of cellular

[2] E. C. Taylor, D. Kuhnt, C. Shih, S. M. Rinzel, G. B. Grindey, J. Barredo, M. Jannatipour, and R. G. Moran. A dideazatetrahydrofolate analog lacking a chiral center at C-6, 2N-{4-[2-(amino-4(3*H*)-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5yl)ethyl] benzoyl}-L-glutamic acid, is an inhibitor of thymidylate synthase. Submitted for publication.

differentiation and viability were assessed using the reduction of NBT, by a modification of the method described elsewhere (43, 45). Cellular morphology was also analyzed after the cells were stained with Wright-Giemsa stain, and a differential count was performed. All slides were prepared with a Shandon Cytospin apparatus (Shandon Inc., Pittsburgh, PA) to avoid preferential selection of a given cell population due to changes in cellular adherence that accompany differentiation.

Hematopoietic cells. After informed consent was obtained, heparinized peripheral blood or bone marrow was collected from normal volunteer donors and from patients with ALL. Hematopoietic cells were centrifugally separated on a Ficoll-Hypaque gradient. Heparinized blood was diluted 1:2 with phosphate-buffered saline, and aliquots (35 ml) were layered over 15 ml of Ficoll-Hypaque and centrifuged at 1200 rpm for 35 min using a Beckman J-6B centrifuge. The mononuclear cell layer was then collected and washed by centrifugation first with RPMI 1640 medium and then with phosphate-buffered saline. Cells were then suspended in 2 volumes of homogenizing buffer, and high speed supernatants were prepared. For the erythrocyte-polymorphonuclear cell fraction, the erythrocytes were lysed on ice for 8 min in buffer containing 140 mM ammonium chloride, 10 mM potassium bicarbonate, and 13 μM EDTA, pH 7.4. Cells were then centrifuged at 1200 rpm for 10 min in a Beckman J-6B centrifuge. This procedure was repeated twice. A high speed supernatant fraction was then prepared from these cells. FPGS assays were performed on two separate sets of granulocytes, erythrocytes, and lymphoblasts prepared on different days, and the results reported for these assays (see Fig. 4) were supported by assays performed on total peripheral blood cell preparations. Peripheral blood or bone marrow was obtained from eight patients with ALL. All of the patients except one adult patient were studied at the time of initial diagnosis and were previously untreated. All of the leukemia samples studied were classified as common ALL antigen positive by flow cytometric analysis, which defined them as pre-B leukemias.

Results

FPGS expression during embryogenesis. The expression of FPGS activity was studied in developing rat embryos. Sprague-Dawley rats with timed pregnancies were sacrificed and liver and brain were dissected during late fetal, neonatal, and adult life. These tissues were chosen as representative of differentiated organs with very different stages of folate metabolism. Liver is known to be very rich in folate cofactors and folate-dependent enzymes, including FPGS (46). At the other extreme, undetectable (46) to low (47) levels of FPGS were found previously in adult mouse and rat brain, respectively. Of course, the potential for renewed cell growth is strikingly different for these two organs, with liver exhibiting the ability to regenerate via cellular proliferation as opposed to the nonre newable tissue that constitutes most of the brain.

The microassay used allowed even the low levels of activity found in adult rat brain to be measured without difficulty (Fig. 1). The expression of enzyme activity was higher in liver than in brain throughout development, implying a tissue-specific control of expression. Yet, in both tissues FPGS activity was higher during embryonic life and the neonatal period, although levels were never more than 3-fold higher than those in the adult organs. Thus, FPGS levels found 7 days before birth were 210 ± 50 pmol/hr × mg protein in liver and 65 ± 10 pmol/hr × mg protein in brain, compared with adult values of 90 ± 16 and 17 ± 10 pmol/hr × mg protein, respectively, in these tissues. A distinct peak of activity was observed in liver around the time of birth. In both rat liver and brain, the levels expressed in young adult animals were representative of the activity found throughout adulthood.

The pattern of cellular proliferation in normal liver and brain is summarized from earlier literature in Fig. 1B. The mitotic index in brain tissue steadily decreases during the latter third of embryonic life to very low levels during the neonatal period and thereafter (48–52). Concomitantly, a downward trend in the expression of FPGS in brain was seen during late fetal life (Fig. 1A). The liver follows a more complex pattern of proliferation in the three distinct cell types present within this organ. The hematopoietic and hepatocyte cell compartments exhibit a steady decrease in their proliferative activity before birth (53–55), whereas the Kupffer cells show an increase in proliferation in the immediate perinatal period (53, 55). However, the Kupffer cells make up a small proportion (perhaps 1–2%) of the bulk of the liver and are very unlikely to contribute to the total levels of FPGS found in this organ (Fig. 1). The initial decrease in FPGS levels (prenatal days −7 to −5) reflects the changes in the hematopoietic and hepatocyte compartments, but the peak seen in the immediate perinatal period (days −5 to +1) remains unexplained at this time. However, it could be said that the higher expression of FPGS during early development occurred during periods of cellular proliferation in both liver and brain but that the level of FPGS after differentiation was substantially different in the two organs.

FPGS levels in growth-arrested cells. Interpretation of the mechanism(s) involved in control of FPGS in developing and proliferating versus mature and differentiated liver and brain (Fig. 1) is complicated by the multiple cell types found in liver during embryogenesis and the diversity of the cell types emerging in the brain. The influence of growth rate on the expression of FPGS can be more easily dissociated from the effects of differentiation using cloned cells in culture. Previous studies on H35 hepatoma cells (56) indicated a decrease in FPGS levels at high culture density, in contrast to earlier studies on Chinese hamster ovary cells (57), which indicated that FPGS levels were not affected by growth state. This important question was reevaluated in mouse embryo C3H 10T1/2 cells, a primitive but nontransformed fibroblast cell line that grows in monolayer culture and strictly obeys postconfluent inhibition for extensive periods without loss of viability (58). Cultures of C3H 10T1/2 cells were grown to confluence and then were maintained for 30 days after contact inhibition had been achieved. Medium was changed three times per week to ensure culture viability. FPGS activity was measured in unfractionated cytosols (after removal of small molecules with centrifugally eluted Sephadex columns), both during exponential cell growth and during postconfluent culture. During the period of rapid growth, the levels of enzyme activity were high and remained stable (Fig. 2). As confluence was reached, FPGS activity decreased and remained constant at a lower level in cultures maintained for as long as 30 days. Thus, in this *in vitro* model system in which nonproliferating cells retained the ability to reinitiate growth upon subculture, FPGS activity was stably expressed, but at a lower level than that characteristic of proliferating 10T1/2 cells.

FPGS expression during cellular differentiation *in vitro*. The expression of FPGS was studied in the HL-60 human promyelocytic leukemia cell line after induction of differentiation towards the polymorphonuclear lineage with RA and DMF. Culture medium was changed every 72 hr to ensure cellular viability, and FPGS was measured in unfractionated but desalted cytosol preparations. The growth of treated cul-

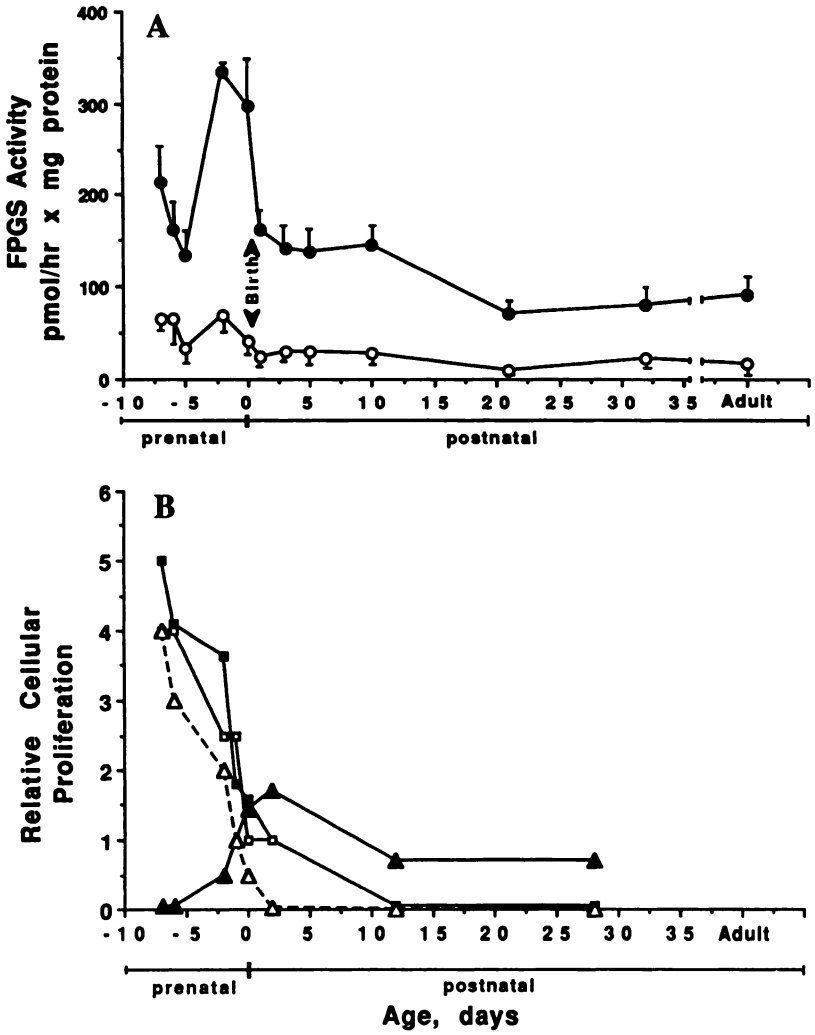

Fig. 1. Modulation of FPGS activity in rat liver and brain during normal development. A, Sprague-Dawley rats with timed pregnancies were anesthesized with CO_2 and sacrificed at intervals, following the guidelines described in Experimental Procedures. The levels of FPGS activity in unfractionated cytosols from liver (●) and brain (○) were determined at intervals during late fetal (days −7 to −1), neonatal (days 1 to 10), adolescent (day 21), early-adult (day 32), and late-adult (day 56) life. Each *point* represents the mean of eight independent determinations; *error bars*, standard deviations. B, Literature summary of proliferative activity during the development of the rat brain (■) (48–52) and cellular compartments of the rat liver, i.e., hepatocytes (□), hematopoietic cells (△), and Kupffer cells (▲) (53–55). The mitotic index decreases in brain and in hepatocytes and hematopoietic cells within the developing liver during late embryonic life but does not in the Kupffer cells, which represent a small fraction of the total hepatic cell mass. For details, see text.

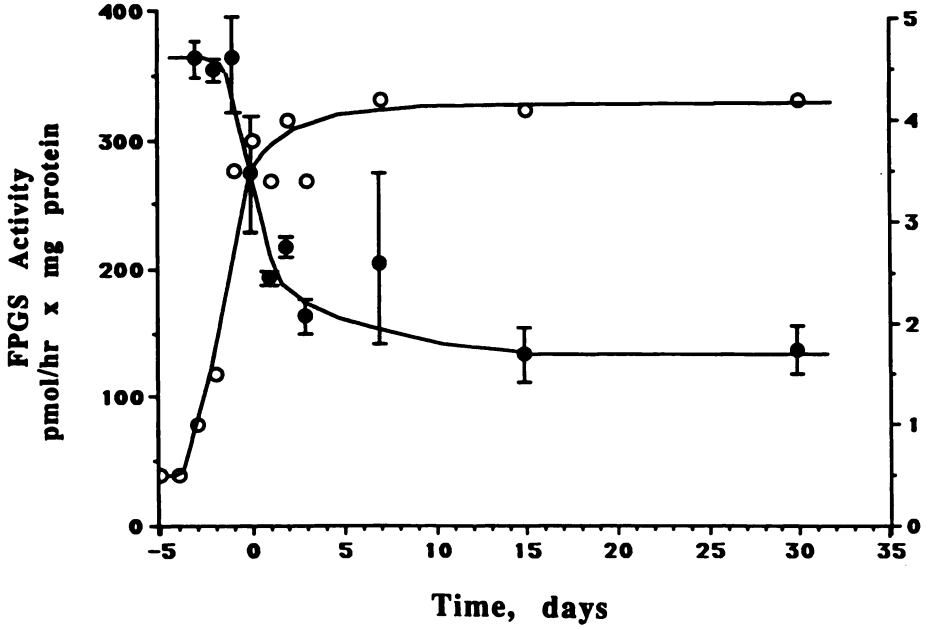

Fig. 2. FPGS activity expressed by exponentially dividing and post-confluent C3H 10T1/2 cells. Exponentially growing C3H 10T1/2 cells were plated at 750,000 cells/150-cm^2 flask and were fed three times per week with RPMI 1640 medium supplemented with 10% fetal calf serum. Viability was determined by plating efficiency upon subculture and did not change during the course of these experiments. *Left vertical axis*, FPGS activity expressed in pmol/hr × mg of protein (●); *right vertical axis*, culture density (○). Each *point* represents the mean of three experiments, with triplicate flasks at each time point in each experiment; *error bars*, standard deviations.

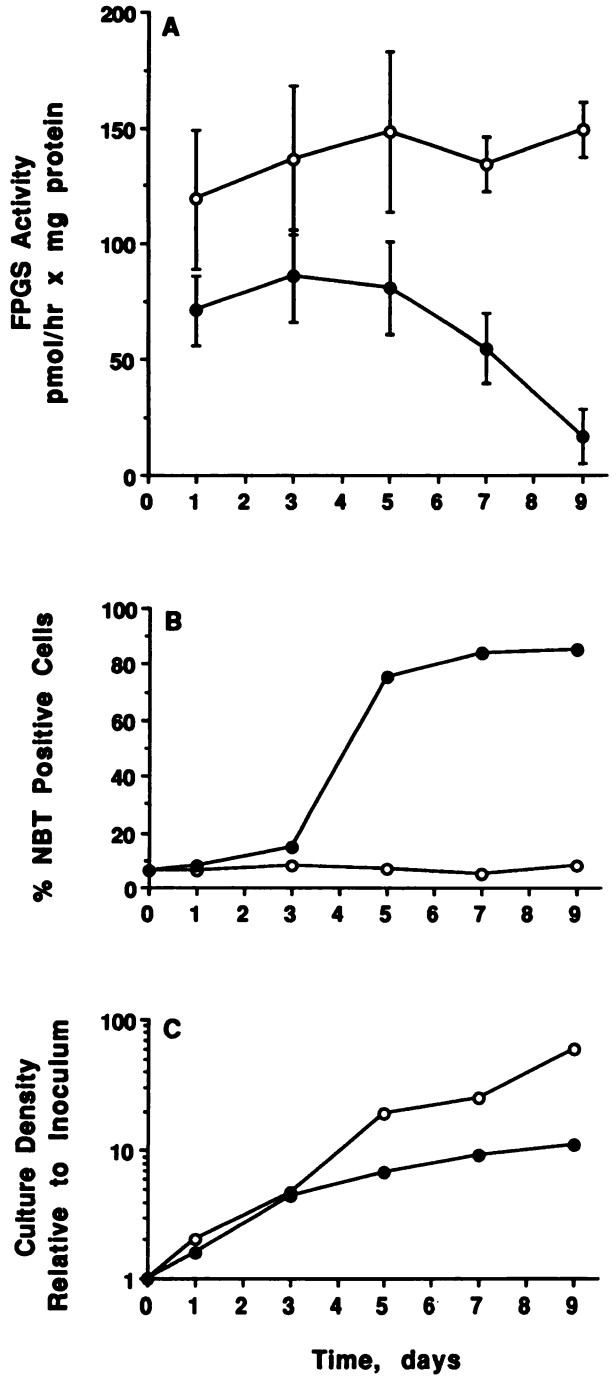

Fig. 3. Levels of FPGS activity in HL-60 cells after induction of differentiation. HL-60 cells were inoculated at a density of 2.5×10^5 cells/ml and differentiation was induced by the addition of 1 μM RA at 24 hr and 60 mM DMF at 48 hr, as described in Experimental Procedures. Fresh medium was added to all cultures, with readdition of RA/DMF to differentiating cells, on days 3 and 5. At intervals, FPGS activity expressed in pmol/hr × mg of protein (A), percentage of cells capable of reducing NBT (B), and culture density relative to the initial inoculum (C) were determined. At these same time points, Wright-Giemsa stains were prepared for morphological analysis under light microscopy (data not shown). ●, Cells exposed to RA/DMF; ○, controls. Each *point* represents the mean of data drawn from three experiments performed on separate days. Enzyme levels found after 7 and 9 days of treatment were significantly different from those in untreated control cultures ($p < 0.02$).

tures arrested after 5 days, when the majority of the cells were already expressing the mature phenotype (>80% of the cells reduced NBT) (Fig. 3, B and C). After 7 or 9 days of exposure to RA/DMF a substantial decrease in FPGS was consistently observed, whereas enzyme levels were maintained in untreated cells. On day 9, when most cells expressed the mature phenotype and growth had arrested, the level of FPGS activity decreased 6–9-fold compared with enzyme activity in cells treated with RA/DMF and 15-fold relative to untreated controls (Fig. 3A). It should be noted that even at the last time point cells were viable, as assessed by their morphology (data not shown) and their ability to undergo an oxidative burst with reduction of NBT. These experiments could be interpreted to indicate that the expression of FPGS was altered by the induction of differentiation of HL-60 cells but that either mRNA specific for FPGS or the enzyme itself had a stability that delayed the decline of FPGS levels after differentiation.

FPGS in hematopoietic cells and tumors. The down-regulation of FPGS during the development of liver and brain tissue prompted us to study the levels of this enzyme in mature cells of the hematopoietic lineages. FPGS activity was found to be below the limits of detection (<5 pmol/hr × mg of protein) in mature erythrocytes, polymorphonuclear cells (granulocytes), and mononuclear cells isolated from peripheral blood from normal volunteers (Fig. 4). These results are consistent with our data in HL-60 cells, suggesting that differentiated cells of the myeloid lineage express very low levels of FPGS activity. In contrast, when lymphoblasts from patients with ALL were analyzed, very high levels of FPGS activity were found, ranging from 440 to 1800 pmol/hr × mg of protein (Fig. 4). A total of eight patients with ALL were studied, six children (ages 2 months to 18 years) and two adults (ages 26 and 41 years). The distribution of the levels of enzyme activities seemed bimodal, but this could easily be due to chance, given the number of patients studied. Treating the data as a single distribution, there was an mean ± standard deviation of 1018 ± 554 pmol/hr × mg of protein. No differences in the levels of FPGS activity in Ficoll-Hypaque-isolated lymphoblasts were found that correlated with age or sex in this initial limited survey. There also was no correlation noted between white blood cell count and the levels of FPGS found in the leukemic blasts. High levels of FPGS activity were found in eight of eight ALL samples and were, on average, >200 times higher than the maximum estimate for FPGS expression in the normal, differentiated, nonproliferating lymphocytes studied.

To approach the question of whether the high FPGS levels in malignant lymphoblasts reflected a simple linkage of FPGS expression to growth rate, the activity of FPGS was studied in several human cell lines representing rapidly proliferating tumor cell populations. A T cell leukemia (CCRF-CEM), a myeloid leukemia (HL-60), a pediatric osteosarcoma (TE-85), a pediatric rhabdomyosarcoma (RD), and a fibrosarcoma (HT-1080) cell line were chosen as a sampling of the spectrum of hematological and solid malignancies. In all five human tumor cell lines studied, FPGS levels (ranging from 63 to 156 pmol/hr × mg of protein) (Fig. 4) were significantly lower than those seen in malignant lymphoblasts *in vivo*.

Discussion

It appears that levels of expression of FPGS respond to at least two control mechanisms, one tissue specific (Figs. 1 and

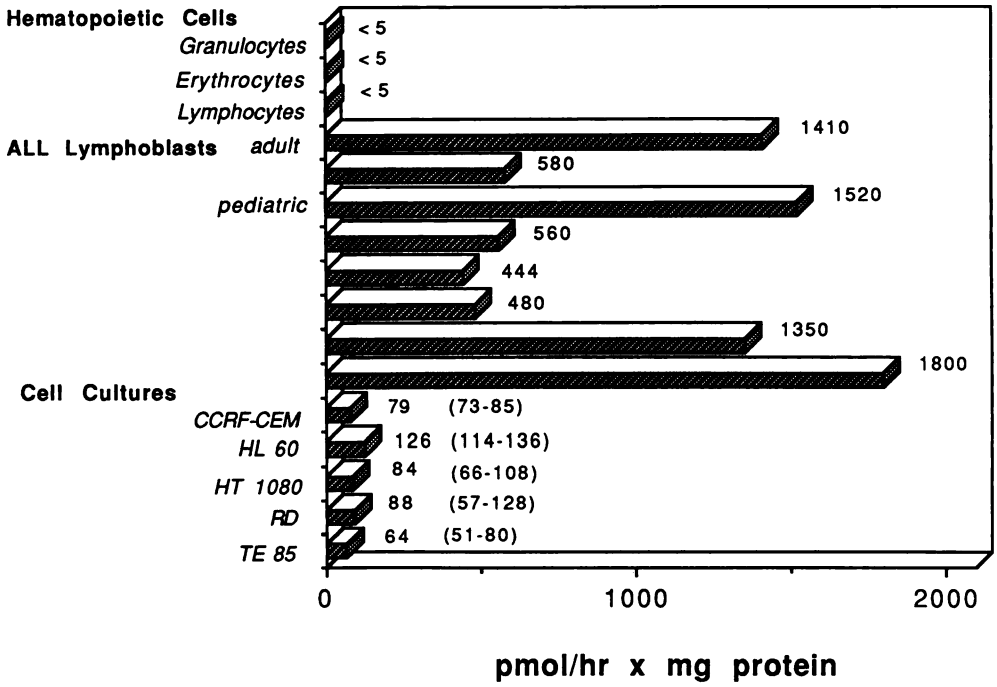

Fig. 4. Levels of FPGS in mature circulating hematopoietic cells, ALL lymphoblasts, and related human cell cultures. The enzyme activity was measured on at least 2 separate days using fresh, desalted, high speed supernatant fractions for mature cells and cell cultures. The values shown for ALL lymphoblasts represent data for freshly prepared cytosol fractions of each isolate; however, a repeat measurement of each ALL sample using frozen cytosolic protein gave results that agreed within 15% for all specimens.

4) and the other proliferation dependent (Fig. 2). As dividing mammalian cells respond to stimuli towards differentiation, the expression of FPGS decreases, with changes ranging between a 50% reduction and decrease to a level that appears to be zero for several cell lineages. In these studies, we have observed systems that illustrate the extremes of this behavior both *in vivo* and *in vitro*. Thus, the stable maintainence of FPGS at fractional levels in nondividing C3H 10T1/2 cells is reminescent of the relative expression of FPGS in fetal and adult liver (Figs. 1 and 2). On the other hand, the changes in expression of FPGS in HL-60 cells after differentiation in culture are comparable to the differences in enzyme levels in lymphocytes and lymphoblasts (Figs. 3 and 4). The modulation of FPGS activity seen as C3H 10T1/2 cells reached confluence (Fig. 2) represents evidence that the expression of FPGS is influenced by the state of growth *per se*, in support of previous direct (56) and indirect (59) evidence for this linkage. This was in contrast to the sharp decrease (ranging from 6- to 15-fold) in the levels of FPGS activity expressed by HL-60 cells when 80% of this population was expressing the mature phenotype and division had ceased (Fig. 3). This residual FPGS activity seen in the population predominantly composed of terminally differentiated HL-60 cells (day 9) probably represented either enzyme activity in the 20% of cells that were not yet expressing the differentiated phenotype or slow enzyme turnover after the synthesis of FPGS had ceased. It is interesting to note that the drop in FPGS seen in RA- and DMF-treated HL-60 cells lagged behind the differentiation of these cultures; we interpret this as an indication of the turnover of FPGS or its mRNA. Hence, these results indicate that events related both to cellular proliferation and to the cell lineage exert influence on the regulatory elements that control the expression of mammalian FPGS.

It is noteworthy that FPGS activity in adult brain is significantly lower than that seen in adult liver (Fig. 4) but that both are substantially higher than levels found in mature erythrocytes. Yet, essentially all of the intracellular folates in these three tissues are present as polyglutamate cofactors. All available evidence indicates that metabolism to folylpolyglutamates containing ≥3 mol of glutamate/mol of pteridine is essential for the intracellular retention of the naturally occurring folates and of classical folate antimetabolites (18, 25, 29). It seems likely that the predominance of folates as polyglutamate derivatives in tissues that have very low levels of FPGS activity reflects the intracellular entrapment of folylpolyglutamates and their slow rate of turnover. Nondividing cells would retain cellular folates as polyglutamates even in the presence of a level of FPGS activity barely sufficient to equal the rate of cleavage of glutamates from the γ-chain. This rate of loss has never been quantitated *in vivo* but might indeed be quite low. For a dividing cell population, FPGS levels would have to be at least high enough to balance the rate of turnover and the rate of loss of polyglutamates by dilution during cell division.

The regulation of FPGS activity has been studied previously by Galivan and co-workers (56, 60–62). In their studies on hepatocytes and hepatoma cells in culture, the rate of MTX polyglutamation was inversely proportional to the amount of folates present in the medium. Interestingly, FPGS activity *per se* was found to drop quickly after exposure of H35 cells to reduced folates or MTX (56). Insulin and dexamethasone increased the accumulation of MTX and folinic acid polyglutamates, whereas the opposite effect was seen using dibutyryl-cAMP and theophylline (to mimic the effects of glucagon). For both insulin and dexamethasone, the changes observed appeared to be dependent on protein synthesis, because cycloheximide and actinomycin D blocked the effects. The molecular mechanisms by which FPGS activity is controlled are unknown. We would conclude from a synthesis of our results with earlier literature that both enzyme levels and the activity of cellular FPGS are modulated.

It has generally been concluded that mammalian FPGS is encoded by a single genetic locus (22). However, a very intriguing recent study by Rumberger *et al.* (63) reported fundamen-

tally different kinetic behavior of FPGS extracted from normal murine intestinal epithelium and from two antifolate-sensitive mouse tumors. It remains to be seen whether these differences reflect FPGS species that differ in sequence or by post-transcriptional or -translational modification(s). However, it would be interesting if the enhanced levels of FPGS found in fetal tissues (Fig. 4) were due to a fetal form of enzyme that was detected in tumors by Rumberger *et al.* (63).

Accumulated evidence indicates that control of expression of FPGS in normal and malignant tissues plays a crucial role with respect to the cytotoxicity and therapeutic selectivity of folate antimetabolites (23, 25–28, 63). Three classes of clinically relevant folate analogs are currently available, i.e., inhibitors of dihydrofolate reductase (e.g., MTX), inhibitors of thymidylate synthase (CB3717 and D-1694), and inhibitors of *de novo* purine biosynthesis (DDATHF). For all three classes of drugs, longer chain polyglutamates are responsible for intracellular retention in tissues that express sufficient levels of FPGS (10, 25–29) and, in addition, polyglutamation of antifolates that target thymidylate synthase and glycinamide ribonucleotide formyltransferase increases enzyme inhibition by ≥100-fold, compared with the monoglutamate compounds (10, 34). Although it is known that FPGS plays a role in the cytotoxicity of these antifolates to tumors and normal tissues, it is not clear at this time whether the levels of enzyme activity in malignant tissues would be predictive of sensitivity to chemotherapy with these agents. Nevertheless, there are some indications that this might be the case. Whitehead *et al.* (64) have reported a significant survival advantage for children with ALL, treated with MTX as part of multimodal chemotherapeutic regimens, when stratified on the basis of the initial ability of their lymphoblasts to accumulate MTX and MTX polyglutamates. At first glance, the very high levels of FPGS activity reported here in lymphoblasts from patients with ALL (Fig. 2) would help to explain the sensitivity of this disease, as well as other tumors, to therapy with folate antimetabolites. On the other hand, leukemic cells from patients with both high and low white blood cell counts had high levels of FPGS. The nondetectable levels of FPGS in nondividing circulating lymphocytes (Fig. 2) contrast sharply with our findings in lymphoblasts, raising the possibility that expression of high levels of FPGS is intrinsic to the patterns of gene expression in some transformed cells. If this is proven to be the case, the levels of expression of FPGS in different tumor types or the properties of the enzymes being expressed could have significant implications in the clinical use of currently available folate antimetabolites and the development of novel, more selective, therapeutic applications of antifolates.

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Send reprint requests to: Richard G. Moran, University of Southern California Cancer Center, Cancer Research Laboratories, 1303 N. Mission Road, Los Angeles, CA 90033.