Hypoxanthine-Guanine Phosphoribosyltransferase Variant Associated with Accelerated Purine Synthesis

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ABSTRACT We have previously described a 14-yr-old boy with hyperuricemia, renal failure, and accelerated purine production resistant in vivo and in vitro to purine analogs. This patient demonstrated normal red cell hypoxanthine-guanine phosphoribosyltransferase (HPRT) heat stability, electrophoresis at high pH, and activity at standard substrate levels. In the present report an abnormal HPRT enzyme was demonstrated by enzyme kinetic study with phosphoribosylpyrophosphate (PRPP) as the variable substrate and inhibitory studies with sodium fluoride. Apparently normal HPRT activity in a patient with hyperuricemia and gout does not exclude a functionally significant HPRT mutation.

INTRODUCTION

The Lesch-Nyhan syndrome is characterized by severe neurologic dysfunction resembling cerebral palsy (1) and an acceleration of de novo purine synthesis resistant to inhibition by purine analogs (2, 3). In 1967 Seegmiller et al. (4) found this disorder was associated with almost complete absence of hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8; HPRT)[1] activity in red cells and fibroblasts from affected patients. The gene locus for HPRT is on the X-chromosome (5, 6), and to date only affected boys have been described. In recent years it has become apparent that some patients with mild neurologic dysfunction, gout, or renal uric acid calculi formation may have partial HPRT deficiency associated with accelerated purine synthesis and hyperuricemia (7, 8). We have recently described a boy (C. M.) with a mild neurological deficit, hyperuricemia, and accelerated purine production resistant in vivo, (9) and in vitro to purine analogs but with apparently normal HPRT activity in red cells and fibroblasts (10). Studies of red cell HPRT from this patient revealed normal heat stability at 80°C and normal electrophoretic mobility at pH 8.6. However, several properties of C. M.'s fibroblasts in culture resembled those of cells from patients with the Lesch-Nyhan syndrome. In the present study we find that the red cell HPRT from this patient has abnormal kinetic properties with phosphoribosylpyrophosphate (PRPP) as substrate. These findings extend the range of mutations described at the HPRT locus in man, and have implications for purine analog-resistant leukemia where HPRT appears normal (11).

METHODS

Forearm skin biopsy specimens were grown in Petri dishes and commercial F12 media (Gibco, Grand Island Biological Co., Grand Island, N. Y.) with supplemented 15% fetal calf serum. Cell strains were established from patients with the clinical symptoms described by Lesch and Nyhan (1) and almost complete HPRT deficiency (10). Cell strain 23 was derived from a patient (C. M.) with hyperuricemia and apparently normal HPRT activity, whose history has been described in detail (10). Cell strain 66 was obtained by Dr. William Kelley from a patient with the Lesch-Nyhan syndrome and a kinetic HPRT mutation (12). Cells were trypsinized and counted, and 0.2×10^5 cells were plated in plastic P30 Petri dishes to begin growth studies. Various media described below were added after 24 h. Cells were fed every other day, and duplicate cultures were counted after 7 days in a Coulter Counter (Coulter Electronics, Inc., Fine Particle Groups, Hialeah, Fla.).

Radiochemicals were obtained from New England Nuclear, Boston, Mass. Aza-L-serine was a gift of Dr. H. N. Wood of the National Cancer Institute, Bethesda, Md. Magnesium and disodium PRPP were purchased from P-L Biochemicals, Inc., Milwaukee, Wis., and Sigma Chemical

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[1] *Abbreviations used in this paper:* 8-AG, 8-azaguanine; HPRT, hypoxanthine-guanine phosphoribosyltransferase; *p*CMB, *p*-chloromercuribenzoate; PRPP, phosphoribosylpyrophosphate.

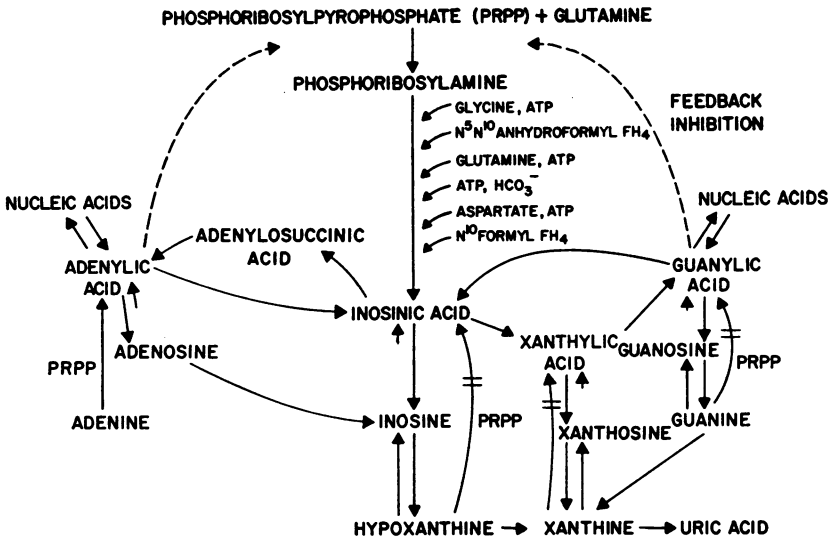

FIGURE 1 Pathways of purine metabolism. The enzyme defects in the Lesch-Nyhan syndrome are indicated by the bars (‡).

Co., St. Louis, Mo., respectively. All other chemicals were of the highest quality available.

HPRT activity was assayed at 37°C as previously described (13, 14). Twice-washed red cells were frozen and thawed and dialyzed for 3 h against 0.005 M Tris pH 7.4 at 4°C. The final reaction mixture consisted of 50 mM Tris, 5 mM $MgSO_4$, 1 mM PRPP, 0.6 mM hypoxanthine and enzyme at pH 7.7 in a final volume of 100 μl. The reaction was stopped with 20 μl 4 M formic acid, and elution on descending paper chromatography with 5% NaH_2PO_4 was used to separate nucleotide product from labeled base (14).

Red cell hemolysates were dialyzed for 3 h in 0.01 M sodium phosphate buffer, pH 6.05, and Pevikon block electrophoresis (15) was performed. The tray buffer consisted of 0.393 M sodium phosphate pH 6.2 (16), and the gel buffer was diluted 1:40 giving a pH of 6.05. Electrophoresis was run at 1 mA/cm for 4 h. 1-cm slices were cut out, the enzyme was eluted with 1 cm^3 of 0.5 M Tris, pH 7.4, containing 0.01 M $MgCl_2$, and the HPRT assay was carried out as described above. Hemoglobin was determined by converting it to cyanmethemoglobin and reading the optical density at 540 nm in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

pH 7.4 dialyzed red cell hemolysates were added to prepared reaction mixtures to begin inhibition studies according to the general outline of Adye and Gots (17). Kinetic studies were performed when HPRT enzyme activity was linear with time and proportional to enzyme concentration. Erythrocyte enzyme activity was measured with 2×10^{-4} M hypoxanthine for PRPP kinetic studies. Hypoxanthine and guanine kinetic studies were carried out at 1×10^{-3} M PRPP for control HPRT and 3×10^{-3} M magnesium-PRPP for C. M. HPRT unless otherwise noted. Protein was estimated with bovine serum albumin as a standard (18).

RESULTS

Tissue culture studies. C. M. fibroblasts and control fibroblasts differ from cells obtained from patients with the Lesch-Nyhan syndrome in their ability to grow in aminopterin and commercial F12 media containing thymidine and hypoxanthine (10). Aminopterin inhibits the two tetrahydrofolate-requiring steps in de novo purine synthesis (Fig. 1) and forces cells to obtain hypoxanthine from the media for growth. In Fig. 2 it may be seen that C. M. fibroblasts develop sensitivity to 8-azaguanine (8-AG) in the presence of aminopterin. C. M. cells in aminopterin demonstrate a threefold increase in purine transport (19). A similar increase in 8-AG transport with conversion to its toxic ribonucleotide would explain growth inhibition of C. M. cells in aminopterin and 8-AG. It can be seen in Fig. 2 that cell strain 66, a kinetic HPRT mutant strain associated with the Lesch-Nyhan syndrome (12), is resistant to 8-AG as has been described for cell strains from patients with classical Lesch-Nyhan syndrome (10).

Aza-L-serine, a second inhibitor of de novo purine synthesis, has a unique partial inhibitory effect on C. M. cell growth when compared to control fibroblasts (Fig. 3). Lesch-Nyhan fibroblasts do not grow in the presence of this inhibitor. It could have been predicted that C. M. fibroblasts would demonstrate full growth in aza-L-serine as they do in aminopterin. We have not determined whether aza-L-serine affects other metabolic pathways in C. M. fibroblasts or if aza-L-serine inhibition of de novo purine synthesis is greater than that of aminopterin.

Red cell HPRT. Previous starch gel studies have demonstrated that migration of C. M. HPRT is similar to control enzyme at pH 8.6 (10). Studies at a second pH value were required to definitively establish this point. C. M. red cell HPRT migration was identical to

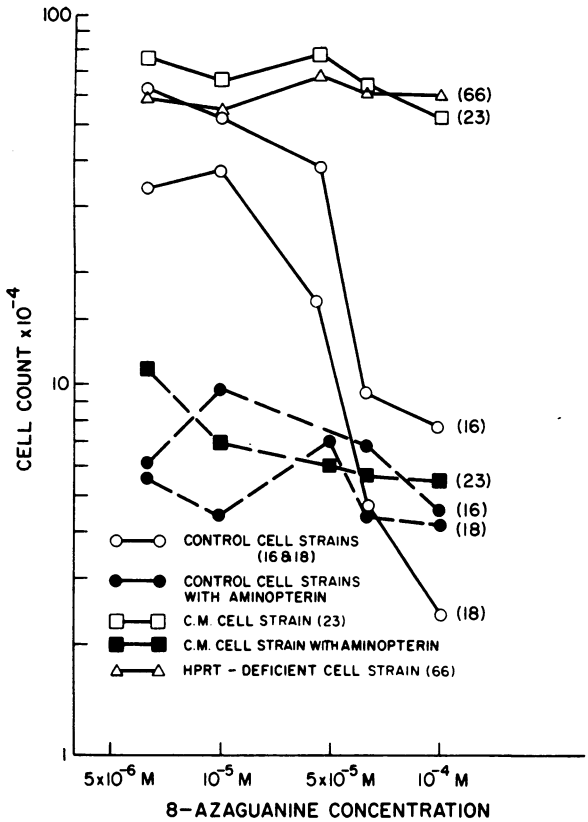

FIGURE 2 Cell growth in aminopterin and 8-AG. 0.2×10^5 cells were plated in Falcon plastic P-30 dishes with F12 and 15% fetal calf serum. Control media with 8-AG, or control media with 8-AG and 5.5×10^{-5} M aminopterin were added after 24 h of culture. Cells were fed daily and counted in duplicate in a Coulter counter after 7 days of growth.

control HPRT in two of three experiments at pH 6.2, suggesting similar net charge composition at this pH. A slight difference in migration was obtained in the third experiment (Fig. 4), which is probably not significant. Elution of HPRT from Pevikon block was greater than that for starch block. Pevikon block electrophoresis did not identify isoenzymes of HPRT as suggested by studies with acrylamide gel electrophoresis (20), DEAE cellulose chromatography (21), and isoelectric focusing (22). Other mutant HPRT enzymes may have altered electrophoretic mobility (7, 23).

Krenitsky, Papaioannou, and Elion (14) have demonstrated that partially purified red cell HPRT has a wide pH range of maximal catalytic activity. C. M. HPRT from red cell hemolysates is similar to control enzyme preparations in this regard.[2]

Adye and Gots (17) have described an interesting HPRT mutant of *Salmonella typhimurium* with normal maximal velocity but altered kinetic properties and marked sensitivity to the sulfhydryl reagent *p*-chloromercuribenzoate (*p*CMB) compared to other enzyme inhibitors. Similar studies were carried out with enzyme from red cell hemolysates. Table I demonstrates that HPRT from C. M. hemolysates is sensitive to sodium fluoride. More recently, DeMars and Held (24) have extended these observations to cell strains with induced HPRT mutations, and sodium fluoride sensitivity is again found.

HPRT kinetics. It can be seen in Fig. 5 that high PRPP levels inhibits control HPRT activity. This was not described in the kinetic studies of Henderson, Brox, Kelley, Rosenbloom, and Seegmiller (25) and Krenitsky et al. (14) but has been noted by Arnold and Kelley (22). Fig. 5 shows that C. M. red cell HPRT has an altered kinetic response to variable PRPP levels. The apparent PRPP K_m for control HPRT is $1.1–1.4 \times 10^{-4}$ M when determined by Lineweaver-Burke plots, whereas the apparent PRPP K_m for C. M. HPRT is $1.0–1.4 \times 10^{-3}$ M, an order of magnitude higher. The apparent hypoxanthine K_m was $7.7 \pm 2.3 \times 10^{-6}$ M and $1.0 \pm 0.2 \times 10^{-5}$ M for control and C. M. enzyme, respectively ($P > 0.05$ compared to control). Fig. 6 demonstrates that a similar apparent K_m for hypoxanthine may be obtained with different levels of magnesium PRPP with C. M. HPRT. The apparent guanine K_m is 6.0 and 6.8×10^{-6} M for control and C. M. HPRT, respectively. Thus, C. M.'s gene mutation affects the enzyme affinity for PRPP and not for purine bases.

Difficulty was encountered in interpreting data like that shown in Fig. 5. The first problem was that apparent PRPP K_m values for C. M. HPRT, obtained from double reciprocal plots, were always higher than expected by inspection of plots such as the insert, Fig. 5. The second problem was that the apparent K_m for PRPP with C. M. HPRT was the same as the PRPP substrate level routinely used in the enzyme assay. One possible explanation was that total activity of C. M. HPRT was not being determined when magnesium ion concentration was 5 mM. Magnesium ion concentrations were then raised, with PRPP levels constant at 1 mM. C. M. HPRT activity rose by 57% when magnesium ion levels were doubled to 10 mM. At 10-mM magnesium PRPP levels, C. M. HPRT activity rose from 75 to 138 μmol hypoxanthine catalyzed/mg protein/h, significantly higher than the control activity of 80±18 μmol/mg protein/h. Increased levels of HPRT activity in C. M. red cell hemolysates may represent enzyme induction and are currently under investigation. These studies suggest that kinetic analysis of HPRT activity is more important than considerations of absolute levels of enzyme activity.

[2] Benke, P. J., and N. Merrick. Unpublished results.

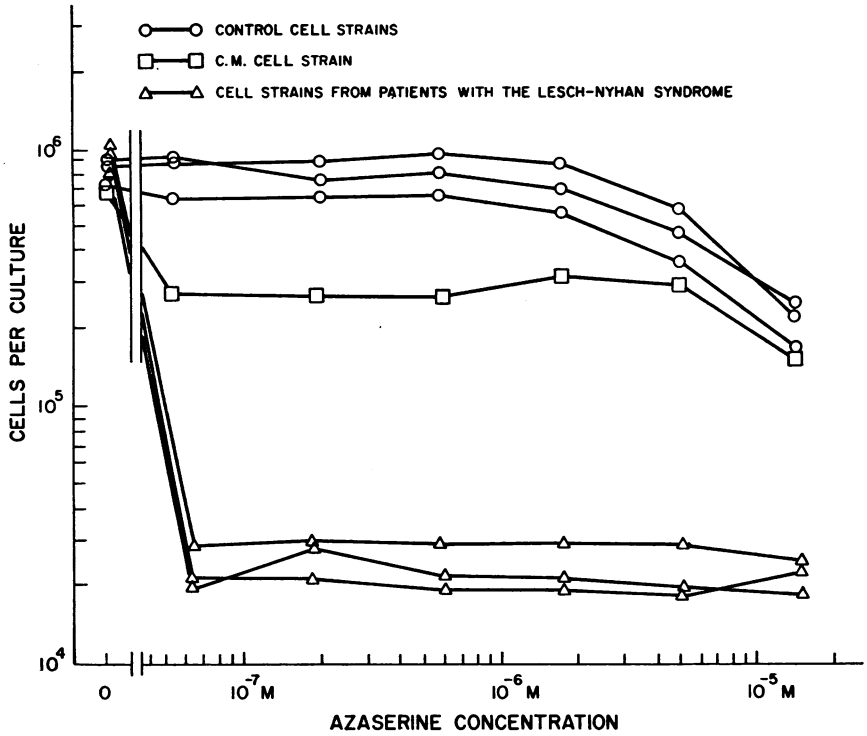

FIGURE 3 Cell growth in aza-L-serine. Cells were plated as described in Fig. 2 and aza-L-serine was added after 24 h of culture. Fresh media were added daily, and cells were counted as in Fig. 2.

DISCUSSION

The presence of multiple gene mutations at the HPRT locus in man is suggested by the varying catalytic and kinetic properties of red cell and fibroblast HPRT preparations from different probands with hyperuricemia. Kelley and Meade (26) have found at least three different mutant forms of HPRT in the small amount of enzyme obtained from fibroblasts cultured from patients with the Lesch-Nyhan syndrome, and at least nine additional mutant forms of HPRT were suggested by studies

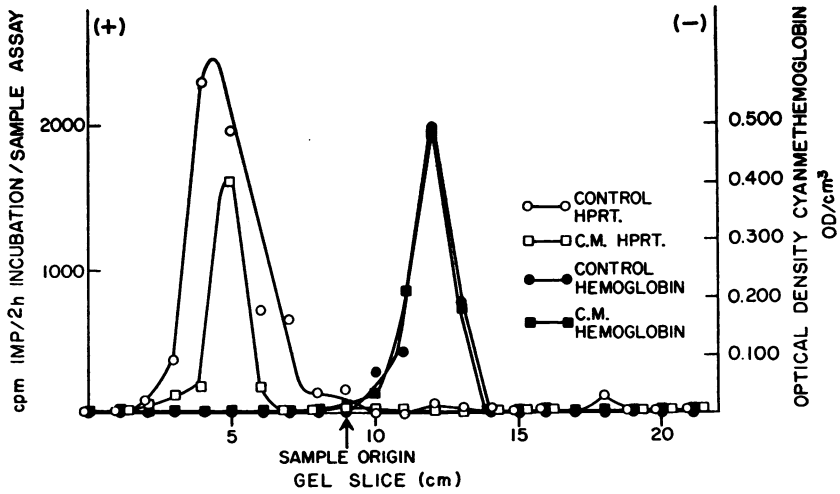

FIGURE 4 Pevikon block electrophoresis of HPRT from dialyzed red cell hemolysates of C. M. and a control. Migration of HPRT was toward the cathode. Activity was demonstrated by eluting the enzyme from 1-cm slices of gel. IMP, inosinic acid.

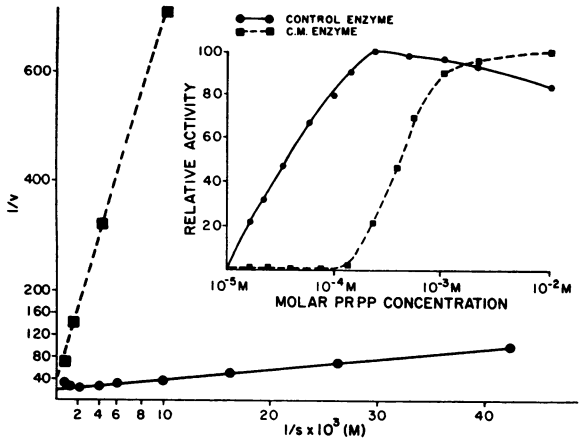

FIGURE 5 Lineweaver-Burke plots of dialyzed control and C. M. red cell hemolysate HPRT with 2.0×10^{-4} M hypoxanthine, 5×10^{-3} M $MgSO_4$ in Tris buffer, pH 7.7 and PRPP as the variable substrate.

on red cell enzyme obtained from patients with partial enzyme deficiency and gout (7). The original experiments in C. M. in vivo, demonstrating accelerated purine synthesis resistant to azathioprine, were interpreted to suggest that C. M. had a defect in the feedback regulation of purine synthesis (9). This proved incorrect, for subsequent experiments in vitro demonstrated that C. M. fibroblasts were more sensitive to feedback effects of adenine nucleotides than control cell strains, in a manner similar to cell strains grown from patients with the Lesch-Nyhan syndrome (10). We now find that C. M. has a distinct kinetic HPRT mutation, similar to but not as severe as that found in a patient with the Lesch-Nyhan syndrome (12). The enzymatic defect alters the enzyme affinity for PRPP (Fig. 5), and not the apparent Michaelis constants of hypoxanthine or guanine. Similar kinetic defects in other enzymes have been demonstrated. Some pseudocholinesterase enzymes have apparently normal maximal velocities but altered kinetic

TABLE I
Inhibition of HPRT by sodium fluoride (NaF), sodium pyrophosphate, and pCMB

	Concentration	Percent Inhibition		
		Control	Control	C. M.
	mM			
NaF	20	14.4	6.9	68.3
	5	0	5.3	23.0
Sodium	30	0.5	17.5	29.5
pyrophaphale	10	0	0	25.3
*p*CMB	0.10	67.9	93.0	98.0
	0.01	37.9	84.9	96.4

The enzyme reaction was initiated by the addition of erythrocyte hemolysate to the incubation mixture.

and inhibitory properties (27, 28). Matsuda, Arashima, Nambu Takekoshi, and Anakura (29) have described a mutant ornithine transcarbamylase with a decrease in affinity of the enzyme for carbamyl phosphate but normal affinity for ornithine, the second substrate. Kinetic and other analyses are required to demonstrate these various forms of enzyme mutations.

That HPRT is important in the economy of purine metabolism in man is suggested by the loss of large amounts of uric acid and the accelerated purine synthesis from simple precursors in patients with the Lesch-Nyhan syndrome. Adenine nucleotides are degraded to inosine and hypoxanthine (30), and HPRT reutilizes the adenine purine ring and conserves the energy of four ATP molecules (Fig. 1). It has not yet been established that the accelerated purine synthesis in these patients is an attempt to compensate for adenine nucleotide loss. The control HPRT enzyme operates at kinetic disadvantage since in vivo levels of PRPP are about $1\text{–}10 \times 10^{-6}$ M (31). It can be seen in Fig. 5 that this disadvantage is accentuated in C. M. hemolysate preparations. C. M.-like HPRT defects may be missed because of artifically high PRPP levels in the in vitro assay. It would be interesting to perform similar studies in patients with partial HPRT activity and normal amounts of uric acid production (32) to correlate the HPRT mutation and the magnitude of purine synthesis.

8-AG is used in cell culture in a classical selection system for determining mutations at the HPRT locus. Recently, HPRT mutations have been induced in lymphocytoid cell lines with ethyl methane sulfonate and nitro-

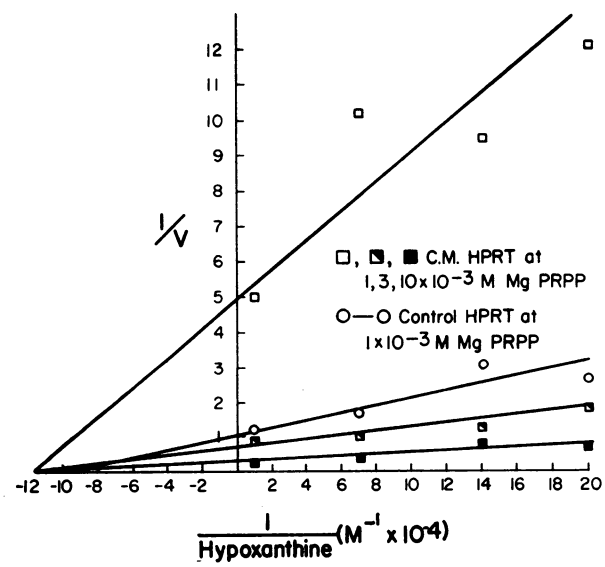

FIGURE 6 Lineweaver-Burke plots of dialyzed control and C. M. red cell hemolysate HPRT with hypoxanthine as the variable substrate and various levels of magnesium PRPP.

soguanidine, and almost all mutants had considerably less than 1% of the HPRT activity of wild-type cells (33). Initial results were similar in X-irradiated human fibroblasts (34), but more complete study reveals that 8-AG-resistant low HPRT activity mutants resembling Lesch-Nyhan cells are the minority of mutant cell lines isolated (24); at least 90% of them can utilize hypoxanthine for growth and have residual HPRT activities that range from low but significant levels to that found in one mutant cell strain, where HPRT activity could not be distinguished from control. It has been known for several years that purine analog resistance in human leukemia is associated with an apparently normal HPRT activity (11). The spontaneous incidence of HPRT mutations in cultured human cells is about 5×10^{-6} (24). This makes selection for mutants in leukemic cell populations similar to C. M. cells probable, if in vitro conditions are an indicator of the in vivo mutation rate. Kinetic HPRT mutants similar to the one described here would explain the purine analogue resistance and apparently normal HPRT activity of cultured tumor cell lines (35), hamster cells (36), and human fibroblasts (24).

Partial HPRT deficiency in man is currently thought to be a rare cause of accelerated purine synthesis and gout. In a recent series of 425 cases of hyperuricemia with gouty arthritis or uric acid stone formation, Yü et al. (32) found only seven subjects, including five in one family, with partial HPRT deficiency. Sperling et al. (37) have identified only one partial HPRT mutation out of 52 patients with primary gout and uric acid overproduction. However, two other brothers with gout in this series of patients had accelerated PRPP synthesis associated with accelerated uric acid production (38). The mutation in this family may be X-linked, since the mother of these patients also has accelerated uric acid production (38). A mutant HPRT would explain these findings. Kinetic or inhibition studies, or tests of purine analog resistance may identify more HPRT mutations in patients with gout or uric acid stone formation.

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