Human Hypoxanthine-Guanine Phosphoribosyltransferase

DEMONSTRATION OF STRUCTURAL VARIANTS IN LYMPHOBLASTOID CELLS DERIVED FROM PATIENTS WITH A DEFICIENCY OF THE ENZYME

JAMES M. WILSON, B. WADE BAUGHER, PAMELA M. MATTES, PETER E. DADDONA, and WILLIAM N. KELLEY, *Departments of Internal Medicine and Biological Chemistry, Human Purine Research Center, University of Michigan Medical School, Ann Arbor, Michigan 48109*

ABSTRACT We have explored the possibility of using cultured lymphoblasts from patients with a deficiency of hypoxanthine-guanine phosphoribosyltransferase (HPRT) as a source of cells for the isolation and characterization of mutant forms of the enzyme. HPRT from lymphoblasts derived from six male patients of five unrelated HPRT-deficient families was highly purified and characterized with regard to: (*a*) level of immunoreactive protein, (*b*) absolute specific activity, (*c*) isoelectric point, (*d*) migration during nondenaturing polyacrylamide gel electrophoresis, and (*e*) apparent subunit molecular weight. These experiments were performed on small quantities of lymphoblasts using several micromethods involving protein blot analysis of crude extracts as well as isolation and characterization of enzyme labeled in culture with radioactive amino acids. The lymphoblast enzymes from four of the patients exhibited structural and functional abnormalities that were similar to the recently described abnormalities found with the highly purified erythrocyte enzymes from these same patients. In addition, a previously undescribed HPRT variant was isolated and characterized from lymphoblasts derived from two male siblings. This unique variant has been called $HPRT_{Ann\ Arbor}$. We conclude that lymphoblastoid cell lines can be used as a source of cells for the detection, isolation, and characterization of structural variants of human HPRT.

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Dr. Mattes' present address is Department of Pediatrics, Johns Hopkins Hospital, Baltimore, Md.

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INTRODUCTION

Detailed studies on the structure and function of enzyme variants in man have been limited to the more abundant erythrocyte- or plasma-borne enzymes such as glucose-6-phosphate dehydrogenase (1), phosphoglycerate kinase (2), and carbonic anhydrase (3). Similar studies on mutant forms of the many other less abundant human intracellular enzymes have been unsuccessful because of the extreme difficulty in purifying the products of the mutant alleles in quantities necessary for detailed analyses.

An important factor limiting the isolation and characterization of mutant human enzymes is the absence of sufficient tissue that expresses the enzyme of interest. An easily accessible human tissue that has been shown to express many different intracellular enzymes is the cultured fibroblast. Indeed, at least 35 inborn errors of metabolism have been demonstrated in cultured fibroblasts (4). Fibroblasts have not proven useful, however, as a source for the isolation of normal and mutant gene products because of their finite life span and early senescence.

The establishment of permanent lymphoblastoid cell lines from human peripheral blood B lymphocytes by viral transformation provides a potentially useful new model system for the study of the genetic mechanisms responsible for inborn errors of metabolism in man. Lymphoblastoid cells appear to be ideal for genetic and biochemical studies because of their unlimited life span and relatively stable phenotype (5). Fujii et al. (6) have, in fact, recently isolated a mutant form of phosphoglycerate kinase from lymphoblasts derived from a patient with a deficiency of the enzyme. In this report, we have studied the relative expression and structure of hypoxanthine-guanine phosphoribosyl-

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transferase (HPRT)[1] in lymphoblasts derived from patients with a deficiency of the enzyme.

HPRT is an intracellular enzyme found in many tissues (7). It is normally expressed at very low levels as compared with the more abundant intracellular proteins. A complete deficiency of this X-linked enzyme is associated with the Lesch-Nyhan syndrome (8), while a partial deficiency of enzyme activity is found in some male patients suffering from a severe form of gout (9). Earlier attempts at characterizing the HPRT protein in patients with a deficiency of enzyme activity were mainly limited to the study of the residual enzyme activity in crude hemolysates (cf. 10). As a result, little was learned about the specific mechanisms responsible for a deficiency of HPRT in man.

We have recently succeeded in purifying HPRT from large quantities of erythrocytes from five unrelated male patients with a deficiency of enzyme activity (11). Detailed study of the structure and function of the highly purified enzymes from these patients indicates that at least four of the enzymes are unique structural variants. Three variants, $HPRT_{Toronto}$, $HPRT_{London}$, and $HPRT_{Munich}$, were from patients presenting with gout while $HPRT_{Kinston}$ was isolated from a patient with the Lesch-Nyhan syndrome.

Lymphoblastoid cells derived from patients with a deficiency of HPRT would indeed provide many theoretical advantages over erythrocytes as a source of tissue for the study of the genetic mechanism(s) responsible for the deficiency state. Lymphoblasts could be used as a endless source of tissue for the isolation and characterization of normal and mutant forms of the HPRT gene, messenger RNA (mRNA), and protein. In addition, since lymphoblasts are actively dividing and metabolizing cells, they should be amenable to the study of the dynamic processes involved in normal and abnormal expression of the HPRT gene (i.e., mRNA and protein synthesis, processing, and degradation).

We have established permanent lymphoblastoid cell lines from a variety of different HPRT-deficient patients and have developed the methodology necessary to study the structure and function of mutant forms of HPRT from small quantities of lymphoblastoid cells. HPRT was isolated and characterized using lymphoblasts from patients with the previously described erythrocyte enzyme variants: $HPRT_{Toronto}$, $HPRT_{London}$, $HPRT_{Kinston}$, and $HPRT_{Munich}$. Using lymphoblasts derived from two male siblings with a deficiency of enzyme activity, we have also identified a new structural variant of human HPRT ($HPRT_{Ann\ Arbor}$).

[1] *Abbreviations used in this paper*: CRM, immunoreactive protein; HPRT, hypoxanthine-guanine phosphoribosyltransferase; PP-ribose-P, 5-phosphoribosyl-1-pyrophosphate.

METHODS

Radioisotopes and chemicals. ^{125}I-Protein A (89 μCi/μg) and [^{14}C]hypoxanthine (55 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.), and [^{35}S]methionine (1.1 Ci/μmol) was obtained from Amersham Corp. (Arlington Heights, Ill.). Gibco Laboratories (Grand Island, N. Y.) provided RPMI medium and horse serum, while Flow Laboratories (McLean, Va.) provided the fetal bovine serum. Ficoll type F-P was purchased from Sigma Chemical Co. (St. Louis, Mo.) and phytohemagglutinin M/P was obtained from Difco Laboratories (Detroit, Mich.). Eastman Kodak Co. (Rochester, N. Y.) provided the X-OMAT R x-ray film. Ampholites (pH 3.5–10.0) were obtained from LKB Instruments, Inc. (Rockville, Md.) and the nitrocellulose paper (GS type, 0.22 μM) was purchased from Millipore Corp. (Bedford, Mass.). Goat anti-rabbit antibody was supplied by Antibodies Inc., Davis, Calif. The Epstein-Barr virus was kindly provided by Dr. Jack Gruber of the National Cancer Institute, Bethesda, Md.

Patients. The clinical, genetic, and biochemical features of the HPRT-deficient patients, L.P. (12–15), I.V. (16–17), and E.S. (18–21), have been described.

Patient G.S. is a 31-yr-old white male who was well until 18 yr of age, when he first noted the onset of podagra. He subsequently developed arthritis in both of his feet and knees and suffered one episode of nephrolithiasis. In 1975 he was noted to have a partial deficiency of HPRT activity in his erythrocytes with a level of 45% of control. He was treated with allopurinol and colchicine. In 1977, after 3 mo without allopurinol, his serum urate was 7.6 mg/100 ml and his uric acid excretion was 16.1 mg/kg per 24 h. No other family members were available for study.

Patient K.C. is a 14-yr-old white male who first experienced intermittent episodes of reddish-brown urine with lower abdominal and back pain at the age of 5. Evaluation at that time revealed uric acid crystals in his urine and a serum urate of 16.5 mg/100 ml. An intravenous pyelogram revealed right ureteral obstruction. His erythrocyte HPRT activity was found to be decreased to 10% of the average control value. His brother T.C. (presently 16-yr-old) has experienced episodes of intermittent abdominal pain, nausea, and vomiting associated with dark urine since the age of 7. At the time of initial evaluation (1977) his serum urate was 16 mg/100 ml and his erythrocyte HPRT was also 10% of the average control value. An intravenous pyelogram demonstrated an atrophic right kidney with caliectasis. Neither boy has had joint symptomatology, mental retardation, or other neurologic or behavioral abnormalities. A similar deficiency of HPRT activity was found in their asymptomatic 5-yr-old brother (C.C.) and in a maternal uncle (R.H.). The latter subject had a history of symptomatic gout and nephrolithiasis. The properties of the purified erythrocyte enzyme from R. H. have been described (11).

Establishment and maintenance of lymphoblastoid cell lines. Fresh blood samples (20 ml) from the HPRT-deficient patients were anticoagulated with preservative-free heparin and diluted with an equal volume of buffer A (8.1 mM Na_2HPO_4, 0.62 mM KH_2PO_4, pH 7.4 containing 0.14 M NaCl, 2.7 mM KCl, and 1 g/liter glucose). The lymphocytes were isolated by centrifugation in a Ficoll type F-P gradient. The lymphocyte fraction was diluted with 7 ml of medium A (RPMI including 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin), centrifuged at 265 *g* for 5 min, and washed four times with medium A (10 ml). The cells were resuspended in medium A (5 ml) containing rehydrated phytohemagglutinin (0.1 ml) and placed in the

incubator for 72 h. After the phytohemagglutinin stimulation the cells were harvested by centrifugation, resuspended in medium A (5 ml) containing 100,000 U of B95-8 Epstein-Barr virus and placed in a 37°C water bath for 1 h. The culture was supplemented with 4 ml of medium A and transferred to the incubator. Transformants began to appear after 2–3 wk.

Two lymphoblastoid cell lines derived from normal males (MGL-8 and GM 130) and one mutagen-induced, HPRT-deficient lymphoblastoid cell line (GM 467) were obtained from the Human Mutant Cell Repository, Camden, N. J. The normal lymphoblastoid cell line, WI-L2, from another normal male was kindly provided by Dr. J. E. Seegmiller, La Jolla, Calif.

Each lymphoblast culture was maintained in medium B (RPMI containing 10% horse serum) at 37°C in the presence of 5% CO_2. All media was supplemented with fresh glutamine (2 mM final concentration) immediately before use.

Preparation of lymphoblast extracts. Exponentially growing lymphoblast cultures were harvested and washed with buffer A. The cells were resuspended in a small volume of 10 mM Tris-HCl, pH 7.4, containing 154 mM NaCl (10^7 cells/250 μl) and lysed by six consecutive freeze–thaw cycles. Membrane-free extracts were obtained by centrifuging the cell lysates at 100,000 *g* for 30 min in a Beckman Airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

Enzyme assays. HPRT was assayed for enzyme activity by a previously described radiochemical method (22). Enzyme assays were performed in the presence of either normal or elevated concentrations of the substrates hypoxanthine and 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P) as described previously (11). 1 U of enzyme activity is defined as the formation of 1 μmol of inosine monophosphate/min. Protein was assayed according to the method of Lowry et al. (23) using bovine serum albumin as the standard.

Lymphoblast HPRT immunoreactive protein was measured using a previously described radioimmunoassay (11). The radioimmunoassay is based on the competition of nonradioactive HPRT with ^{125}I-labeled HPRT for binding to HPRT antibody. The least detectable dose of the assay was 0.01 ng based on a 50-μl sample and the slope of the standard curve was -1.03 ± 0.05 (mean±1 SD, $n = 5$). Identical slopes were obtained when dilutions of the lymphoblast extracts were used to compete with the ^{125}I-labeled HPRT for binding to the antibody, indicating that the lymphoblast enzymes and the HPRT standard bind to the antibody with the same affinity.

Preparation of insoluble anti-HPRT immunocomplex. HPRT was immunoprecipitated from lymphoblast extract with a preformed rabbit anti-HPRT, goat anti-rabbit immunocomplex. This immunocomplex was made by mixing 1.48 ml of goat anti-rabbit antibody (2.8 mg/ml), 0.36 ml of unfractionated, decomplemented, rabbit antiserum, and 2.63 ml of buffer B (25 mM Tris-HCl containing 154 mM NaCl and 0.02% sodium azide). The mixture was incubated at 37°C for 15 min and 4°C for 12 h. The precipitated complex was recovered by centrifugation at 400 *g* for 2 min and was washed four times with buffer B (5 ml). The washed immunocomplex was resuspended in 4.5 ml of buffer B and stored at 4°C for up to 6 mo without loss of binding capacity.

Labeling and purification of lymphoblast HPRT. Lymphoblasts from a normal male subject (MGL-8) and from patients G.S., L.P., and I.V. (10 ml; 10^6 cells/ml) were labeled with [^{35}S]methionine for 14 h in methionine-free RPMI medium containing horse serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), and [^{35}S]methionine (40 μCi/ml, 1.1 Ci/μmol). Membrane-free extracts of the labeled cells were obtained as described above. The extracts were supplemented with PP-ribose-P and $MgCl_2$ (final concentrations equal to 20 and 60 mM, respectively) and heated at 85°C for 15 min. The denatured proteins were removed by filtration over a 0.45-μM Millipore filter. HPRT recovered in the filtrate was further purified by immunoprecipitation with the anti-HPRT immunocomplex. The filtrate (~500 μl) was mixed with an equal volume of protein extract derived from the HPRT-deficient cell line GM 467 (10 mg/ml) and incubated at 37°C for 30 min. An aliquot of the anti-HPRT immunocomplex was added (20 μl) and the mixture was rotated overnight at 4°C. The immunoprecipitate was recovered by centrifugation at 400 *g* for 2 min and was washed once with 0.8 ml of buffer B containing bovine serum albumin (1 mg/ml) and four times with 0.8 ml of buffer C (25 mM Tris-HCl containing 7.5 mM $MgCl_2$, 500 mM NaCl, and 1% Triton).

HPRT in lymphoblasts from E.S. and T.C. was labeled and partially purified using a modified version of the procedure described above. The heat step at 85°C was omitted because the enzyme from E.S. and T.C. was heat labile (data not shown). The labeled protein extracts were directly immunoprecipitated using the procedure described above.

Polyacrylamide gel electrophoresis. Samples were prepared and electrophoresed in 12.5% polyacrylamide gels containing 0.33% *N,N'*-methylenebisacrylamide in the presence of SDS according to the method of Laemmli (24).

The lymphoblast extracts were also fractionated in polyacrylamide slab gels under nondenaturing conditions. The nondenaturing gels were prepared and run according to the method of Laemmli except that SDS was excluded from the buffers. The gels contained 6% acrylamide and 0.16% *N,N'*-methylenebisacrylamide.

Isoelectric focusing. Isoelectric focusing was performed under nondenaturing conditions in polyacrylamide slab gels (2 mm × 9.5 cm × 11 cm) containing 6.0% acrylamide, 0.16% *N,N'*-methylenebisacrylamide, 5% glycerol, and 5% ampholites (LKB, pH 3.5–10.0). Samples were prepared for focusing by the method of Johnson et al. (25). The gels were focused as described previously (11).

HPRT activity stain. HPRT was assayed for enzyme activity *in situ* after polyacrylamide gel electrophoresis, using a modification of the procedure of Zannis et al. (26). The only difference between the two methods was the composition of the reaction mixture. The reaction mixture used in our experiments contained 100 mM Tris-HCl, pH 7.4, plus 10 mM $MgCl_2$, 10 mM PP-ribose-P, and 36 μM [^{14}C]hypoxanthine (55 mCi/mmol).

Protein blot analysis. HPRT immunoreactive protein in lymphoblast extracts was detected after isoelectric focusing or nondenaturing polyacrylamide gel electrophoresis using the protein blot technique. Proteins were transferred from polyacrylamide slab gels to nitrocellulose paper using the procedure of Towbin et al. (27). After electrophoretic transfer, the blot (nitrocellulose paper with immobilized lymphoblast proteins) was incubated in 20 ml of buffer D (10 mM Tris-HCl, pH 7.4, containing 154 mM NaCl, 3% bovine serum albumin, and 0.02% sodium azide) overnight. The protein blot was then soaked for 4 h with shaking in 20 ml of buffer D containing a 1:500 dilution of HPRT antiserum followed by four 100-ml washes with buffer B. The protein blot was then incubated with 20 ml of buffer D containing ^{125}I-labeled protein A (20,000 cpm/ml) for an additional 4 h with shaking. The nitrocellulose paper was washed and air dried and the labeled immunocomplex was visualized by autoradiography at −70°C with X-OMAT R x-ray film for 24–72 h.

TABLE I
Characterization of Lymphoblast HPRT

Cell lines	Specific activity		CRM level		Absolute specific activity
	mU/mg	*% average control**	*ng CRM/mg*	*% average control**	*mU/μg CRM*
Normal					
MGL-8	18.4±1.5 (6)‡	127	442±45 (8)	133	42±8§
WI-L2	13.6±2.2 (6)	94	290±23 (4)	87	47±11
GM-130	11.4±2.5 (12)	79	264±19 (3)	80	43±12
Average¶	14.5±3.6	100	332±96	100	44±3
Patients					
L.P.	4.8±0.3 (4)	33	172±20 (6)	52	28±5
G.S.	8.6±0.6 (6)	59	116±11 (7)	35	74±13
T.C.	1.5±0.2 (4)	10	37±3 (6)	11	41±8
K.C.	1.6±0.2 (4)	11	39±4 (6)	12	41±9
I.V.	0.37±0.09 (6)	3	263±17 (6)	79	1.4±0.4
E.S.	0.06, 0.04	0.3	240±29 (6)	72	0.21
E.S.‖	7.73±0.71 (4)	53	240±29 (6)	72	32±9

* Percentage of the average value for the three normal cell lines.
‡ Mean±1 SD, (*n*) = number of determinations.
§ Calculated absolute specific activity±the SD resulting from the propagation of the errors in the numerator (specific activity) and denominator (CRM level).
¶ Average value for the three normal cell lines.
‖ Results when assayed in the presence of elevated concentrations of substrates as described in Methods.

RESULTS

HPRT enzyme activity. The levels of enzyme activity and immunoreactive protein in lymphoblast lysates derived from three unrelated normal male subjects and from the six HPRT deficient patients are summarized in Table I. HPRT enzyme activity was measured in the presence of substrate concentrations that were saturating for the normal enzyme. The level of enzyme activity in lymphoblasts derived from the HPRT-deficient patients was, in each case, lower than the lowest normal value. The specific activity of HPRT from the normal controls and from patients L.P. and G.S. was unchanged when measured in the presence of concentrations of substrates which were higher than normally used (see Methods). Lymphoblasts from T.C., K.C., and I.V. exhibited slightly increased specific enzyme activities (~1.2–1.8-fold higher) when measured at these higher substrate concentrations. A more dramatic increase in the level of enzyme activity from 0.05 to 7.73 mU/mg, however, was measured in extracts from patient E.S. when assayed in the presence of higher concentrations of substrates.

HPRT immunoreactivity and absolute specific activity. HPRT immunoreactive protein was quantitated using a previously described radioimmunoassay (11) and the absolute specific activity of the enzyme was calculated from assays of enzyme activity and immunoreactive protein (Table I). The absolute specific activity of HPRT in the normal lymphoblastoid cell lines[2] (44±3 mU/μg immunoreactive protein, CRM) agrees very well with the previously reported absolute specific activity of HPRT in normal hemolysates, 38±5 mU/μg CRM (11), and the specific activity of the highly purified normal erythrocyte enzyme, 36±4 mU/μg protein (11).

The level of HPRT immunoreactive protein in the HPRT-deficient lymphoblasts ranged from 11% of the average control value (T.C. and K.C.) to near normal levels (I.V. and E.S.). The absolute specific activity of HPRT was markedly reduced in lymphoblasts from E.S. (0.3% of control) and I.V. (3% of control). However, the absolute specific activity of HPRT from E.S. was increased to 73% of normal when calculated from activity assays performed in the presence of higher concentrations of substrates.

Isoelectric focusing of lymphoblast HPRT. Lymphoblast extracts were focused in polyacrylamide gels under nondenaturing conditions and assayed for im-

[2] A normal lymphoblastoid cell line refers to lymphoblasts in long term culture that were derived from a patient who is clinically normal.

munoreactive protein using the protein blot technique. Fig. 1 shows autoradiographs of two representative experiments. Lymphoblast extract from the normal cell lines MGL-8 (Fig. 1A, lane N), GM 130 (data not shown), and WI-L2 (data not shown) demonstrated one immunoreactive isoelectric species with an isoelectric point equal to 6.0±0.1 (mean±1 SD, n = 3). Several observations indicate that this is HPRT. This band was absent when control antiserum was used in place of anti-HPRT serum or when lymphoblast extract from the cell line GM 467 (<0.1% of control immunoreactive protein) was analyzed (data not shown). In addition, an *in situ* activity stain of an identical gel demonstrated that HPRT enzyme activity and immunoreactive protein focus at the same pH (data not shown).

The isoelectric point of HPRT from G.S. was identical to normal (Fig. 1A, lane GS). In contrast, the isoelectric points of the enzymes from I.V. (Fig. 1A, lane IV) and E.S. (Fig. 1A, lane ES) were increased from the normal value of 6.0 to 6.4 and 6.2, respectively, while the isoelectric point of the enzyme from L.P. (Fig. 1A, lane LP) was decreased to 5.75. Preliminary study of the lymphoblast extract from T.C. demonstrated a faint band of immunoreactive protein that focused at pH 5.85 (Fig. 1A, lane TC). Repeated experiments indicated that the enzymes from the siblings T.C. (Fig. 1B, lane TC) and K.C. (Fig. 1B, lane KC) were indistinguishable from one another and were indeed more acidic than the normal enzyme (Fig. 1B, lane N). However, the enzymes from T.C. and K.C. were not as acidic as the enzyme from L.P. (Fig. 1B, lane LP).

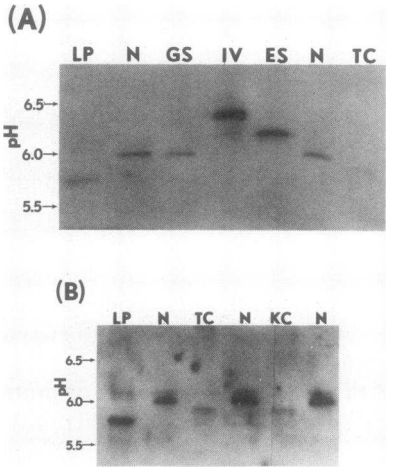

FIGURE 1 Protein blot analysis of lymphoblast HPRT after nondenaturing isoelectric focusing. Lymphoblast extracts were focused in polyacrylamide slab gels under nondenaturing conditions as described in Methods and HPRT immunoreactive protein was detected using the protein blot technique. This figure shows the area of the autoradiograph containing all detectable protein bands. The protein blots in panels A and B were exposed for 24 and 72 h, respectively. Panel A; lane LP—L.P. (40 μg protein, 6.9 ng CRM); lanes N-MGL-8 (20 μg protein, 8.9 ng CRM); lane GS—G.S. (53 μg protein, 6.1 ng CRM); lane IV—I.V. (73 μg protein, 19 ng CRM); lane ES—E.S. (50 μg protein, 12 ng CRM); and lane TC—T.C. (46 μg protein, 1.7 ng CRM). Panel B: lane LP— same as panel A; lanes N—same as panel A; lane TC—T.C. (69 μg protein, 2.5 ng CRM); and lane K.C. (80 μg protein, 3.1 ng CRM). These experiments were repeated twice with essentially identical results.

Nondenaturing polyacrylamide gel electrophoresis of lymphoblast HPRT. Membrane-free extracts from normal and HPRT-deficient lymphoblasts were fractionated under nondenaturing conditions in polyacrylamide slab gels and the HPRT enzymes were detected immunochemically using the protein blot technique. An autoradiograph of a representative protein blot is shown in Fig. 2. Lymphoblast extract from a normal cell line (MGL-8) demonstrated one immunoreactive protein band (Fig. 2, lane N). Careful inspection of the autoradiograph revealed that this rather broad band is composed of three closely spaced bands. This microheterogeneity is most likely due to noncovalent interactions since the lymphoblast enzyme has only one detectable isoelectric species (see Fig. 1). The identification of this band as HPRT was suggested by the following: (*a*) the band was absent when control rabbit serum was used in place of rabbit HPRT antiserum (data not shown); (*b*) a human HPRT-deficient

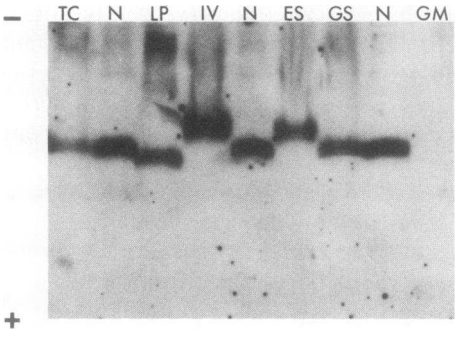

FIGURE 2 Protein blot of lymphoblast HPRT after nondenaturing polyacrylamide gel electrophoresis. Lymphoblast extracts were electrophoresed in 12 cm polyacrylamide gels under nondenaturing conditions and analyzed for immunoreactive protein as described in the Methods section. An autoradiograph of a representative protein blot experiment is shown in this figure. The film was exposed for 36 h. Lane TC—T.C. (584 μg protein, 22 ng CRM); lanes N-MGL-8 (159 μg protein, 70.3 ng CRM); lane LP—L.P. (384 μg protein, 66 ng CRM); lane IV—I.V. (585 μg protein, 154 ng CRM); lane ES—E.S. (480 μg protein, 115 ng CRM); lane GS—G.S. (507 μg protein, 59 ng CRM); and lane GM—GM 467 (800 μg protein; <0.03 ng CRM). Each lymphoblast extract was analyzed at least three times with essentially identical results.

lymphoblastoid cell line (GM 467) with undetectable HPRT immunoreactive protein (<0.1% of control) did not demonstrate this protein band (Fig. 2, lane GM); and (*c*) this band of immunoreactive protein comigrated with an area of HPRT enzyme activity (see Fig. 3).

The enzyme from T.C. (Fig. 2, lane TC) and G.S. (Fig. 2, lane GS) was indistinguishable from normal lymphoblast HPRT. In contrast, HPRT from L.P. (Fig. 2, lane LP) exhibited a more anodal migration while HPRT from I.V. (Fig. 2, lane IV) and from E.S. (Fig. 2, lane ES) exhibited a more cathodal migration. The migration of enzyme from I.V. was consistantly slightly more cathodal than was the migration of enzyme from E.S.

The HPRT enzymes were also detected by the *in situ* enzyme activity stain described in Methods. Fig. 3 shows an autoradiograph of a representative activity stain experiment. The radioactive bands in Fig. 3 representing areas of HPRT enzyme activity are, with one exception, coincident with the areas of immunoreactive protein shown in Fig. 2, thus, supporting the data derived from the protein blot experiments. The one notable exception is HPRT from E.S. The main band of enzyme activity (Fig. 3, lane ES) is slightly more cathodal than that of normal lymphoblast HPRT (Fig. 3, lane N) and is coincident with the single immunoreactive protein band detected by protein blot analysis (Fig. 2, lane ES). In addition, however, there is a very rapidly migrating band of enzyme activity that does not correspond to any analogous band of immunoreactive protein.

HPRT enzyme activity was virtually undetectable in this lymphoblast extract when measured in the presence of normal concentrations of substrates. This indicates that the rapidly migrating HPRT is most likely an altered molecular form of this patient's kinetically abnormal enzyme rather than a contaminating HPRT from bacteria or mycoplasma. This may, indeed, represent dissociation of the native tetramer (22) to a smaller molecular form (i.e., dimer or monomer) and suggests that the alteration in the primary structure of this variant enzyme may have affected its subunit–subunit interactions. The reason why this rapidly migrating form of HPRT was not detected in the protein blot experiments is unknown. This smaller molecular form may be less immunoreactive than the native tetramer. Alternatively, the enzyme may not transfer efficiently to the nitrocellulose paper from this area of the gel.

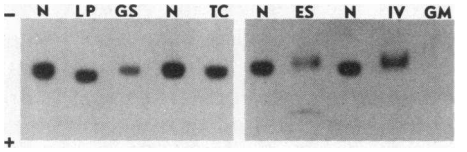

FIGURE 3 Activity stain of lymphoblast HPRT after nondenaturing polyacrylamide gel electrophoresis. Lymphoblast extracts were electrophoresed in nondenaturing polyacrylamide gels as described in the legend to Fig. 2. HPRT was detected by an *in situ* activity stain as described in Methods. This figure shows autoradiographs of two representative experiments. The films were exposed for 24 h. Lanes N-MGL-8 (7.7 μg protein, 3.4 ng CRM); lane LP—L.P. (19.2 μg protein, 3.3 ng CRM); lane GS—G.S. (24 μg protein, 2.8 ng CRM); lane TC—T.C. (81 μg protein, 3 ng CRM); lane ES—E.S. (479 μg protein, 115 ng CRM); lane IV—I.V. (578 μg protein, 152 ng CRM); lane GM—GM 467 (800 μg protein, <0.03 ng CRM). Essentially identical results were obtained when these experiments were repeated on two separate occasions.

Purification of normal and mutant lymphoblast HPRT. HPRT was labeled in culture with [^{35}S]-methionine and purified as described in Methods. Each lymphoblastoid cell line studied had taken up ~50% of the total radioactivity in the culture medium (3×10^8 cpm) after 14 h of labeling; one-third of this radioactivity (1×10^8 cpm) was usually found to be incorporated into soluble protein. Labeled HPRT in a normal lymphoblastoid cell line (MGL-8), and in lymphoblasts derived from patients L.P., G.S., and I.V. was isolated in near homogeneous form by a two-step procedure using a heat step and immunoprecipitation. The purified enzyme preparations contained the following total quantities of ^{35}S radioactivity: MGL-8, 45,074±9,431 cpm (mean±1 SD, four purifications); L.P., 17,275±275 cpm (mean±1 SD, three purifications); G.S., 9,546 cpm (one purification); and I.V., 35,600 (average of two purifications). SDS-polyacrylamide gel electrophoresis of these purified enzymes demonstrated one major band of radioactivity (Fig. 4, lanes N, IV, and LP, and Fig. 5 lane GS) which, in each case, comigrated with the highly purified erythrocyte enzymes from each patient (data not shown). Integration of densitometer scans of these autoradiographs indicated that the enzymes were radiochemically >95% pure (data not shown).

The heat step was omitted from the purification of HPRT in lymphoblasts of patients T.C. and E.S. because these variant enzymes were more labile to heat inactivation than was the normal enzyme (data not shown). The immunoprecipitates from these cell extracts demonstrated numerous ^{35}S-labeled protein bands after SDS-polyacrylamide gel electrophoresis (Fig. 4, lanes ES, ES′, and TC). The specific HPRT protein was identified as the single protein band that competed with excess unlabeled highly purified erythrocyte enzyme for binding to the HPRT antibody during the immunoprecipitation step (Fig. 4, lane ES′ for E.S.; and data not shown for T.C.). The labeled lymphoblast enzymes from E.S. and T.C. also comigrated with the

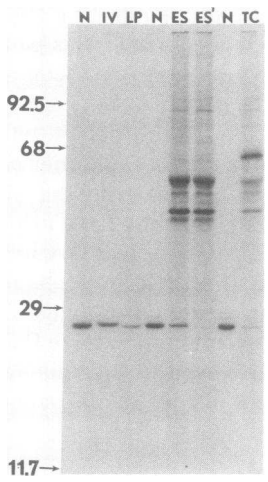

FIGURE 4 SDS-polyacrylamide gel electrophoresis of labeled lymphoblast HPRT. Lymphoblast HPRT labeled in culture with [^{35}S]methionine was denatured and subjected to SDS-polyacrylamide gel electrophoresis in an 18-cm slab gel as described in Methods. The gel was dried and autoradiographed for 48 h. Lanes N—purified normal enzyme from MGL-8 (2,200 cpm); lane IV—purified enzyme from I.V. (1,780 cpm); lane LP—purified enzyme from L.P. (540 cpm); lane ES—partially purified enzyme from E.S. (10,160 cpm); lane ES'—labeled extract from E.S. that was immunoprecipitated in the presence of 100-fold excess of unlabeled purified erythrocyte HPRT (14,960 cpm); and lane TC—partially purified enzyme from T.C. (9,060 cpm). This experiment was repeated at least three times with each mutant using different purified preparations, with essentially identical results.

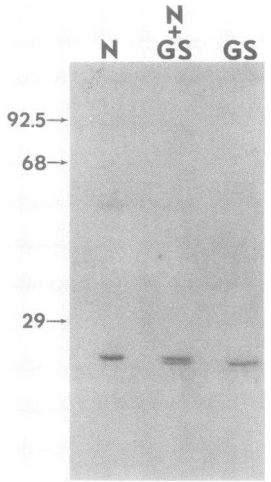

FIGURE 5 SDS-polyacrylamide gel electrophoresis of labeled lymphoblast HPRT. The experimental conditions were identical to those described in the legend to Fig. 4 except that a 12-cm slab gel was used and the electrophoresis was continued for 30 min after the bromphenol blue front reached the bottom of the gel. Lane N—purified normal enzyme from MGL-8 (636 cpm); lane N + GS—a mixture of purified enzymes from MGL-8 (424 cpm) and G.S. (300 cpm); and lane GS—purified enzyme from G.S. (400 cpm). An identical decrease in the apparent subunit molecular weight of the enzyme from G.S. was observed in four separate experiments.

purified erythrocyte enzymes from these patients (data not shown).

SDS-polyacrylamide gel electrophoresis of lymphoblast HPRT. Differences in the apparent subunit molecular weight of the variant lymphoblast enzymes were identified by comparing the relative migration of the purified ^{35}S-labeled enzymes during SDS-polyacrylamide gel electrophoresis. Normal lymphoblast HPRT exhibited an apparent subunit molecular weight equal to 26,000 (Fig. 4, lane N). The apparent molecular weight of HPRT from L.P. (Fig. 4 lane LP), E.S. (Fig. 4, lane ES), and T.C. (Fig. 4 lane TC) was indistinguishable from that of the normal lymphoblast enzyme (Fig. 4. lane N). In contrast, HPRT from I.V. and G.S. exhibited small but reproducible differences in their migration during SDS-polyacrylamide gel electrophoresis. HPRT from I.V. exhibited an apparent molecular weight that was increased by ~400 (Fig. 4, lane IV). While the altered mobility of this enzyme variant was reproducible, it was not great enough to be clearly resolved from the normal enzyme when electrophoresed in the same lane (data not shown). The apparent molecular weight of the enzyme from G.S. was decreased by ~1,000 as compared to normal (Fig. 5). This variant enzyme was indeed clearly resolved from the normal lymphoblast enzyme when electrophoresed in the same lane (Fig. 5).

DISCUSSION

The potential use of lymphoblastoid cells in the study of the genetic mechanisms and biochemical consequences of inborn errors of metabolism in man has only recently been realized (5). At least 17 different inborn errors of metabolism have been demonstrated in cultured human lymphoblasts (28). Recently, Fujii et al. (6) were the first to report the use of lymphoblasts as a source of cells for the isolation of a mutant gene product. These investigators purified and characterized a structurally altered form of phosphoglycerate kinase from lymphoblasts derived from a patient with a deficiency of the enzyme. This putative mutant enzyme, however, has not been studied from a nontransformed tissue, such as erythrocytes, from this patient. Thus, comparison of the enzyme from lymphoblasts with that from another source has not been possible. In this report, we have explored the possibility of using cultured lymphoblasts from HPRT-deficient patients as a source of cells for the isolation and characterization of variant forms of the enzyme.

Using selected immunochemical and radiochemical

techniques we studied the structure and function of HPRT in lymphoblasts derived from six patients in five unrelated families with a deficiency of this enzyme. The lymphoblast enzymes were compared to each other in terms of their concentration in cell lysates (level of immunoreactive protein), catalytic function (absolute specific activity), isoelectric point, migration during nondenaturing polyacrylamide gel electrophoresis, and apparent subunit molecular weight. These experiments clearly indicated that the lymphoblast enzyme from each HPRT-deficient family studied was indeed a unique structural variant. A summary of these results is provided in Table II.

We have recently reported the purification and characterization of HPRT from erythrocytes of these same patients (11). A detailed study of the function and subunit structure of the highly purified erythrocyte enzymes indicated that four of the enzymes were unique structural variants. These variants were named $HPRT_{Toronto}$ (patient L.P.), $HPRT_{London}$ (patient G.S.), $HPRT_{Munich}$ (patient I.V.), and $HPRT_{Kinston}$ (patient E.S.). As summarized in Table II, the major functional and structural abnormalities detected in these four erythrocyte enzyme variants were similar to those observed for the corresponding lymphoblast enzymes.

An earlier study of the erythrocyte enzyme from the C. family was limited in scope because of the difficulty in obtaining sufficient quantities of the purified enzyme (11). A limited study of the subunit structure of this purified erythrocyte enzyme failed to detect any structural abnormalities. In the present study, using micromethods of enzyme structural analysis, we clearly demonstrated that HPRT in lymphoblasts derived from T.C. and K.C. has an apparently normal subunit molecular weight and an isoelectric point that is lower than the normal value. Furthermore, this enzyme variant was shown to be electrophoretically distinct from the other known structural variants of HPRT. We therefore suggest that this unique enzyme variant be called $HPRT_{Ann\ Arbor}$.

The only notable difference between the lymphoblast and erythrocyte enzymes was that none of the lymphoblast enzymes demonstrated the electrophoretic heterogeneity previously reported for the erythrocyte enzymes from these patients. Normal erythrocyte HPRT has been shown to exhibit complex

TABLE II
Comparison of Lymphoblast and Erythrocyte HPRT*

Subject	Enzyme	Source	Absolute specific activity	Migration during native polyacrylamide gel electrophoresis (relative to normal)	Apparent subunit molecular weight‡ ($\times 10^{-3}$)	Isoelectric points§
			mU/μg CRM			
Normal	Normal	Lymphoblast	44±3	—	26.0	6.00
		Hemolysate	38±5	N.D.¶	26.0	6.6, 6.2, 6.0
L.P.	$HPRT_{Toronto}$	Lymphoblast	28±5	Anodal	26.0	5.75
		Hemolysate	42±8	N.D.	26.0	6.2, 6.0, 5.8
G.S.	$HPRT_{London}$	Lymphoblast	74±13	No Change	25.0	6.00
		Hemolysate	51±7	N.D.	25.0	6.6, 6.2, 6.0
I.V.	$HPRT_{Munich}$	Lymphoblast	1.4±0.4	Cathodal	26.5	6.40
		Hemolysate	1.3±0.1	N.D.	26.5	6.9, 6.6, 6.2
E.S.	$HPRT_{Kinston}$	Lymphoblast	0.21	Cathodal	26.0	6.20
		Hemolysate	N.D.‖	N.D.	26.0	6.9, 6.6, 6.2
C. Family**	$HPRT_{Ann\ Arbor}$	Lymphoblast	41±9	No Change	26.0	5.85
		Hemolysate	41±11	N.D.	26.0	N.D.‖

* Erythrocyte data summarized from reference 11.

‡ Estimated from SDS-polyacrylamide gel electrophoresis experiments.

§ The isoelectric points of the lymphoblast enzymes were determined under nondenaturing conditions while the isoelectric points of the three major isozymes of the erythrocyte enzyme subunits were determined under denaturing conditions.

¶ Not determined for the erythrocyte enzymes because of the complexity of the electrophoretic patterns.

‖ Not determined because of technical reasons described in reference 11.

** Lymphoblast data were from cells derived from T.C. and K.C. and the erythrocyte data were from hemolysate of patient R.H. who is the maternal uncle of T.C. and K.C.

isoelectric profiles when focused under nondenaturing conditions in either preparative columns (12–15, 17, and 29) or polyacrylamide gels (26). The demonstration of at least three major isoelectric forms of the normal HPRT subunit (26) indicated that the complexity of the native enzyme is caused in part by heterogeneity in the enzyme subunit itself. Although the precise mechanism of this subunit heterogeneity is unknown, several findings suggest that it is derived from posttranslational events. Erythrocytes from a single hemizygous male demonstrate three isoelectric forms of the HPRT subunit in spite of having only one HPRT allele (11 and 26). In addition, there is only one detectable isoelectric form of HPRT in cultured human fibroblasts (26) and lympholasts (30).

The isoelectric properties of HPRT in normal and HPRT-deficient lymphoblasts were further evaluated, in the present study, by protein blot analysis. Crude lymphoblast extracts were focused under nondenaturing conditions and HPRT was located by an *in situ* immunoassay. These experiments demonstrated a single isoelectric form of HPRT in each lymphoblastoid cell line studied. Our previous studies of the isoelectric properties of the erythrocyte enzymes from these patients were carried out on the highly purified enzymes under denaturing conditions because the isoelectric profiles of the native erythrocyte enzymes were too complex to interpret (unpublished observations). These experiments indicated that the normal and variant erythrocyte enzymes individually exhibit three isoelectric forms of the enzyme subunit (11). The relative changes observed in the isoelectric points of the erythrocyte subunit isozymes of $HPRT_{Toronto}$, $HPRT_{London}$, $HPRT_{Munich}$, and $HPRT_{Kinston}$ are qualitatively similar to the changes measured in the isoelectric point of the corresponding native lymphoblast enzyme (Table II). This suggests that neither the erythrocyte specific posttranslational modifications nor the lymphocyte transformation and in vitro propogation has obscured the genetically derived alteration in the primary structure of the variant enzymes.

In this report, we have focused on the structural and functional properties of HPRT in lymphoblasts derived from patients with a deficiency of HPRT activity in erythrocytes. We conclude from these studies that cultured lymphoblasts are an excellent source for the detection, isolation, and characterization of structural variants of human HPRT.

The investigation of the structural properties of lymphoblast HPRT was greatly simplified through the use of the recently described protein blot technique. Using this technique, we were able to study the electrophoretic and isoelectric properties of normal and catalytically nonfunctional forms of HPRT from small quantities of unfractionated lymphoblast extracts. The *in situ* immunochemical detection of HPRT was extremely specific and sensitive. For example, following nondenaturing isoelectric focusing, ~2–5 ng of HPRT was specifically detected from lymphoblast extracts in which HPRT comprised ~0.004% of the total protein. Similar analyses could theoretically be performed on any protein antigen for which a highly specific and sensitive antiserum is available. Using the method described in this paper, we have routinely achieved similar sensitivity and specificity in protein blot experiments of other relatively nonabundant human enzymes including adenine phosphoribosyltransferase (31), adenosine deaminase (32), and the binding protein for adenosine deaminase (unpublished observation).

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