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Hypoxanthine phosphoribosyltransferase deficiency: Association of reduced catalytic activity with reduced levels of immunologically detectable enzyme protein

(Lesch-Nyhan syndrome/immunoprecipitation/crossreactive material)

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ABSTRACT **In the present study hemolysates from fourteen patients with a genetically determined deficiency of hypoxanthine phosphoribosyltransferase (EC 2.4.2.8; IMP:pyrophosphate phosphoribosyltransferase) activity were examined immunologically for the presence of material that crossreacts with the normal enzyme. A quantitative assay for crossreacting material in enzyme-deficient hemolysates was based on the inhibition of the immunoprecipitation of the normal enzyme. As little as 3% of normal crossreacting material could be detected. One patient in this series was found to have a normal amount of crossreacting material, whereas the remainder had no detectable crossreacting protein. The lack of detectable crossreacting material in these patients raises the possibility that a defect in synthesis or degradation of enzyme protein may be present in many patients deficient in hypoxanthine phosphoribosyltransferase.**

The Lesch-Nyhan syndrome is an X-linked recessive disorder characterized clinically by spasticity, choreoathetosis, self-mutilation, and mental as well as growth retardation, and biochemically by hyperuricemia and hyperuricaciduria (1) secondary to a marked reduction in the activity of hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8; IMP:pyrophosphate phosphoribosyltransferase) (2). A second clinical syndrome is associated with a generally less striking reduction of HPRT activity (3). These patients exhibit hyperuricemia and hyperuricaciduria and eventually develop gout, though they uniformly lack the devastating neurologic and behavioral manifestations of the Lesch-Nyhan syndrome.

In a previous report from this laboratory (4) hemolysates from patients with HPRT deficiency were shown to contain material that was immunologically reactive with antiserum prepared in rabbits against a partially purified preparation of HPRT from normal hemolysate (crossreactive material), CRM^+). Recent preliminary experiments in our laboratory using a different antiserum preparation demonstrated that at least some samples of HPRT-deficient hemolysate did not compete with the normal hemolysate enzyme in an immunoprecipitation reaction. These findings led us to reexamine hemolysates from a number of patients with HPRT deficiency for the presence of CRM. Within this series of 14 patients, only one had a normal level of immunoreactive HPRT protein, whereas the remainder had no detectable CRM by this method.

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; CRM, crossreactive material (immunologically); PBS, phosphate-buffered saline.

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MATERIALS AND METHODS

[8-^{14}C]Hypoxanthine (48.6 mCi/mmol) and [8-^{14}C]adenine (6.8 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Tetrasodium PP-ribose-P was purchased from Sigma Chem. Co., St. Louis, Mo. All other chemicals were of the highest purity commercially available.

Preparation of Cell Lysates. Heparinized blood samples obtained from normal individuals, patients with the Lesch-Nyhan syndrome, and patients with "partial" HPRT deficiency were centrifuged at 8000 × *g* for 10 min, and the plasma and buffy coat were removed. The erythrocytes were washed twice with an equal volume of 0.15 M NaCl and lysed by freeze-thawing in a dry ice/acetone bath. The hemolysate was centrifuged at 10,000 × *g* for 30 min to remove the cell stroma and then dialyzed against 0.05 M potassium phosphate, pH 7.4, at 4° for 8 hr. Samples used in this study had been stored at −70° for 0–156 weeks.

Enzyme Assays. HPRT and adenine phosphoribosyltransferase (APRT) activity were assayed using radiochemical methods as described (5). Reaction mixtures containing normal hemolysate were incubated for 20 min using [^{14}C]hypoxanthine (5.0 mCi/mmol), whereas those containing only HPRT-deficient hemolysate were carried out for 2 hr using the same labeled compound with a higher specific activity (48.6 mCi/mmol).

Protein concentration was determined by the method of Lowry *et al.* (6), using bovine serum albumin as the standard.

Preparation of Antiserum. The antiserum was prepared as described by Arnold *et al.* (4) against the highly purified HPRT preparation from normal erythrocytes (5). This antiserum and control serum from nonimmunized rabbits had been stored in small aliquots at −70°. Rabbit sera were used either after dialysis against 1000 volumes of 0.15 M NaCl/0.05 M potassium phosphate, pH 7.4 (phosphate-buffered saline or PBS) or after partial purification by the following procedure. Both undialyzed antiserum and control serum were mixed with an equal volume of hemolysate (100 mg of protein per ml) from an HPRT-deficient patient found to have no detectable CRM in preliminary studies. After incubation at 37° for 30 min and at 4° for 24 hr, the precipitate was removed by centrifugation at 10,000 × *g* for 30 min. The crude immunoglobulin fraction remaining in the supernatant was precipitated with 50% saturated ammonium sulfate in 0.05 M potassium phosphate, pH 7.4, redissolved in PBS, dialyzed at 4° for 12 hr against 1000 volumes of PBS,

and diluted to a protein concentration of 20 mg/ml. This absorbed preparation was used in all immunologic experiments described below, unless indicated otherwise.

Immunologic Methods. HPRT immunoprecipitation was carried out by adding antiserum (0–50 μl) to increasing amounts of hemolysate protein (0.23–7.50 mg) in a 100 μl reaction mixture with a final concentration of 0.05 M potassium phosphate, pH 7.4, and 0.15 M NaCl. The immunochemical reaction mixtures, including controls with hemolysate protein but no antiserum, were incubated at 37° for 30 min and then at 4° for 18–24 hr. HPRT activity was assayed in the supernatant after centrifugation at 15,000 × *g* for 10 min.

The quantitative measurement of CRM in HPRT-deficient hemolysates was performed by an immunoprecipitation-inhibition assay. HPRT-deficient hemolysate protein (4.6 or 7.27 mg) was mixed with normal hemolysate protein (0.23 or 0.46 mg) before the addition of varying amounts of antiserum against HPRT. The immunochemical reaction was otherwise the same as described above. The level of CRM in the various HPRT-deficient hemolysates was determined by assessing the effect of each hemolysate on the antiserum-mediated precipitation of normal HPRT.

Immunodiffusion was performed by the method of Ouchterlony (7) in 1% agar containing 0.15 M NaCl. The center well was filled with 5–10 μl of antiserum, whereas the peripheral wells were filled with an equal amount of serially diluted normal hemolysate. The slides were stored at 4° and examined under darkfield illumination at 12-, 24-, and 48-hr intervals for the presence of precipitin lines.

RESULTS

In an earlier immunochemical study of HPRT in the Lesch-Nyhan syndrome, a group of HPRT-deficient patients, including D.C., was reported to have normal levels of CRM based on immunochemical methods including antibody removal and immunodiffusion (4). The antibody removal experiment was repeated using dialyzed antiserum against HPRT prepared against a highly purified preparation of normal erythrocyte HPRT (Fig. 1). Absorption with normal hemolysate caused a marked reduction in anti-HPRT activity. However, antiserum absorbed with hemolysate from the patient D.C. had essentially the same anti-HPRT activity as unabsorbed antiserum. In addition, immunodiffusion of normal hemolysate against antiserum absorbed with either normal or HPRT-deficient hemolysate did not yield a precipitin line as seen with unabsorbed antiserum (4). Thus, the absorption with D.C. hemolysate removed nonspecific antibodies from the antiserum while all of the anti-HPRT activity was retained. On this basis absorption with a different HPRT-deficient hemolysate (J. K.), available in larger quantities, was used as a step in the partial purification of antiserum (and nonimmune control serum) as described in *Materials and Methods*. This absorbed antiserum preparation also retained all of the anti-HPRT activity and thus was used in subsequent experiments (except when indicated otherwise).

Characterization of this antiserum preparation with respect to enzyme specificity is described in Figs. 2 and 3. As illustrated in Fig. 2A, the antiserum-mediated decrease of HPRT activity in the supernatant (85%) can be largely accounted for by the enzyme activity found in the immunoprecipitate (70%). Thus, HPRT did not appear to be inactivated when complexed with antibody. In addition, control

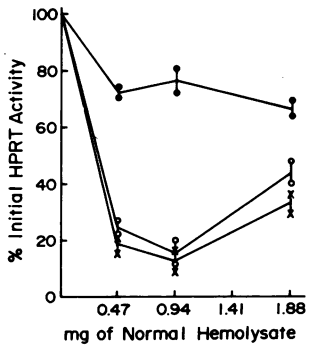

FIG. 1. Absorption of antiserum with Lesch-Nyhan hemolysate. Lesch-Nyhan hemolysate protein (4.69 mg) from patient D.C. was added to 25 μl of crude antiserum in a total volume of 100 μl. The sample was incubated at 37° for 30 min, at 4° for 24 hr, and centrifuged for 10 min at 15,000 × *g*. The supernatant (50 μl) was added to varying amounts of normal hemolysate protein in 10 μl total volume to test for inhibition of HPRT activity. Control samples included antiserum absorbed in the above manner with normal hemolysate protein and unabsorbed antiserum (25 μl in a total volume of 100 μl). The experiment was performed in duplicate as shown. The minimal product corresponding to 100% initial HPRT activity was 11,700 cpm. O, Absorption with Lesch-Nyhan hemolysate; ●, absorption with normal hemolysate; ×, unabsorbed antiserum.

serum (Fig. 2B) did not produce significant inhibition of HPRT activity except when added in large volumes; the failure to produce more than 15% inhibition and the absence of concomitant precipitation of HPRT activity indicates that this may be a nonspecific effect. The effect of control and antiserum against HPRT on the inhibition of adenine phosphoribosyltransferase (APRT), a functionally similar hemolysate protein, is compared in Fig. 3. Although antiserum against HPRT inhibited APRT activity, the same degree of inhibition was observed with an equal amount of control serum. Moreover, no APRT activity appeared in the precipitate.

Fig. 4 (solid lines) demonstrates that under conditions of antigen excess there was a direct relationship between the amount of normal hemolysate protein present in the immunoreaction mixture and the amount of antiserum required to produce immunoprecipitation. Using this relationship, the quantitative determination of CRM was based on the inhibition of immunoprecipitation of the normal enzyme by HPRT-deficient hemolysate. Mutant hemolysate was mixed with normal hemolysate and the degree of competition determined by comparison of the resulting immunoprecipitation curve with the series of curves obtained with different amounts of normal hemolysate protein alone. The addition of 4.60 mg of hemolysate protein from the patient E.S. (Lesch-Nyhan syndrome) to 0.46 mg of normal hemolysate protein produced an immunoprecipitation curve similar to that obtained with 5.0 mg of normal hemolysate protein alone (Fig. 4A). Thus, 4.60 mg of E.S. hemolysate protein appeared to have a level of CRM equivalent to 4.53 mg of normal hemolysate protein, or approximately 98% of normal. Fig. 4B, however, demonstrates virtually no CRM in the hemolysate of M.S., another patient with the Lesch-Nyhan syndrome. The mixture of 7.27 mg of hemolysate protein from M.S. and 0.23 mg of normal hemolysate protein produced an immunoprecipitation curve indistinguishable from that obtained with 0.23 mg of normal hemolysate protein alone. If the level of CRM in the hemolysate of the latter patient had been as little as 3% of normal, the immu-

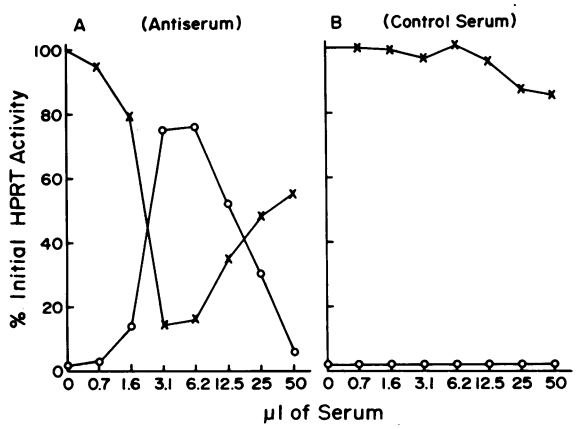

FIG. 2. Precipitation of HPRT activity with antiserum against HPRT. A constant amount of normal hemolysate protein, 0.23 mg, was incubated with varying amounts of antiserum against HPRT (A) and control serum (B) as described under *Materials and Methods*. After centrifugation, the supernatant and precipitate were separated and the latter was washed once with 200 μl of PBS and resuspended in 50 μl of PBS. The percent of initial HPRT activity remaining in the supernatant and that appearing in the precipitate was determined. The minimal product corresponding to 100% initial HPRT activity was 7580 cpm. ×, Supernatant; O, precipitate.

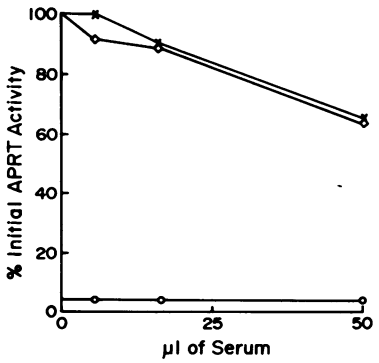

FIG. 3. Specificity of antiserum against HPRT. In an experiment similar to the one described in Fig. 2, 1.1 mg of normal hemolysate protein was incubated with 5.5–50 μl of antiserum against HPRT and control serum. The rabbit sera used here were not partially purified but dialyzed. APRT activity was assayed in the supernatant and precipitate of samples incubated with immune serum and only in the supernatant of those samples incubated with control serum. The minimal product corresponding to 100% initial APRT activity was 1710 cpm. ◊, Antiserum against HPRT, supernatant; O, antiserum against HPRT, precipitate; ×, control serum, supernatant.

noprecipitation curve of the hemolysate mixture would have been expected to have approximated the curve obtained with 0.46 mg of normal hemolysate protein. As illustrated in Fig. 4B, the immunoprecipitation curves for 0.23 and 0.46 mg of normal hemolysate protein were clearly distinguishable. Thus, the level of CRM in the hemolysate of M.S. was estimated to be less than 3% of normal.

The hemolysates of 10 unrelated patients with the Lesch-Nyhan syndrome and four patients with "partial" HPRT deficiency from two families were assayed for CRM according to the procedure described. These data are summarized in Table 1. Only the hemolysate from the patient E.S. had a normal level of CRM. Hemolysates of all the other patients tested had less than 3% of normal CRM. The mixing of the hemolysate of E.S. with HPRT-deficient hemolysate containing less than 3% of normal CRM had no significant effect on the CRM value of either sample when assayed separately. Variation in the time of storage at −70° did not affect the level of CRM in hemolysate (Table 2).

DISCUSSION

In the present study we have examined hemolysates of patients with reduced activity of HPRT for the presence of material that immunologically crossreacts with normal HPRT protein (CRM). Of 10 patients with the Lesch-Nyhan syndrome and four patients with the clinical syndrome associated with a "partial" deficiency of HPRT, hemolysate from only one patient had a normal level of CRM. The hemolysates from the remaining 13 patients had less than 3% of normal CRM.

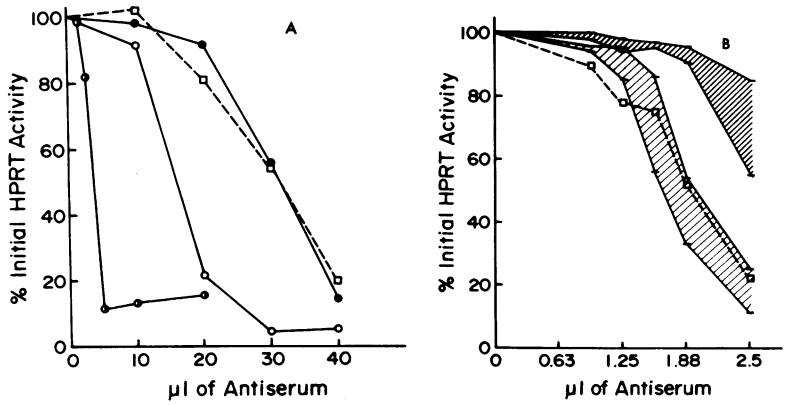

FIG. 4. Determination of CRM in HPRT-deficient hemolysates. Immunoprecipitation of HPRT activity by antiserum against HPRT was performed at several different concentrations of normal hemolysate protein, ranging from 0.23 to 7.50 mg. An immunoprecipitation curve was also obtained for a mixture of 0.47 mg of normal hemolysate protein and 4.60 mg of hemolysate protein from HPRT-deficient patient E.S. (A) and a mixture of 0.23 mg of normal hemolysate protein and 7.27 mg of hemolysate protein from HPRT-deficient patient M.S. (B). These experimental immunoprecipitation curves were compared with normal, and the amount of CRM in the experimental hemolysates was calculated as described in the *text*. The minimal product corresponding to 100% initial HPRT activity was 5139 cpm. (A) ●, 5.0 mg of normal hemolysate protein; O, 3.75 mg of normal hemolysate protein; ◑, 0.47 mg of normal hemolysate protein; □, 0.47 mg of normal hemolysate protein + 4.60 mg of HPRT-deficient hemolysate protein (patient E.S.). (B) ▨, 0.47 mg of normal hemolysate protein (mean ± 1 SD); ▨, 0.23 mg of normal hemolysate protein (mean ± 1 SD); □, 0.23 mg of normal hemolysate protein + 7.27 mg of HPRT-deficient hemolysate protein (patient M.S.).

Table 1. HPRT activity and crossreactive material in hemolysates of HPRT-deficient patients

Patient	CRM (% control)	Weeks of storage −70°	Specific activity HPRT (nmol/mg of protein per hr)
Normal*			98 ± 14
Lesch-Nyhan syndrome			
D.G.	<3	33	0.003
M.S.	<3	>52	0.004
D.C.	<3	3	0.017
J.F.	<3	>52	0.006
H.D.	<3	0	0.007
V.P.	<3	>52	0.005
M.B.	<3	>52	0.001
J.K.	<3	>52	0.001
W.E.	<3	0	0.001
E.S.	98	0	0.076
Partial HPRT deficiency			
Mark D.	<3	16	0.107
Mike D.	<3	0	0.353
B.D.	<3	0	0.333
R.G.	<3	>52	0.002

* Normal is the mean ± SD of 119 subjects.

The undetectable levels of CRM in the hemolysates of most of the patients studied could be the result of either a reduction in the concentration of HPRT protein or the presence of structurally defective enzyme molecules with altered antigenic sites. Consistent with either possibility is earlier evidence suggesting that many of the spontaneous mutations leading to HPRT deficiency in man occur at a locus that affects the structure of the enzyme (8, 9). In regard to the first possibility, Cappechi *et al.* (10) have shown that the reduced level of CRM in mutagenized HPRT$^-$ mouse cells can be attributed to an enhanced rate of degradation of the mutant protein. In many patients with HPRT deficiency the enzyme appears more labile than normal to thermal inactivation *in vitro* (3, 8, 9, 11) and to inactivation in aging mature erythrocytes *in vivo* (4). Although there is no information on the rate of degradation of HPRT protein in human cells, a phenomenon similar to that described by Cappechi *et al.* seems possible in at least some patients with HPRT deficiency. In addition, a decrease in the rate of synthesis of HPRT could also explain the observed decrease in CRM. Possibly a mutation in the putative regulatory gene for HPRT (12, 13) could lead to a decrease in transcription or translation of the HPRT messenger. The fact that there is substantial genetic heterogeneity among the mutations leading to HPRT deficiency suggests that examples of various mechanisms may be found.

Table 2. Effects of storage on crossreactive material in hemolysates of HPRT-deficient patients

Patient	Weeks of storage −70°	Specific activity HPRT (nmol/mg of protein per hr)	CRM (% control)
D.C.	3	0.017	<3
	48	0.004	<3
	>52	0.004	<3
E.S.	8	0.076	98
	>52	0.076	98
H.D.	0	0.006	<3
	36	0.007	<3
Mike D.	0	0.353	<3
	16	0.143	<3
B.D.	0	0.333	<3
	16	0.090	<3

Interestingly, the results of this study are similar to those reported by Beaudet *et al.* (14) for HPRT$^-$ mutant strains of cultured Chinese hamster cells. These investigators examined the level of CRM in several 8-azaguanine-resistant clones after chemical mutagenization and found that most of the clones had less than 4% CRM. This may indicate that the spontaneous mutations occurring in man at the HPRT locus are similar to those induced at the same locus with mutagens in non-human cultured cells.

The one patient, E.S., found in the present study to have a normal level of CRM is the K_m mutant described previously (15). At a saturating concentration of PP-ribose-P and purine base, HPRT in erythrocytes from this patient can be calculated to have 94% of normal HPRT activity. Thus, this patient represents one example in which the mutant form of the enzyme, though catalytically defective *in vivo*, may exhibit both normal antigenicity and maximal catalytic activity using appropriate conditions *in vitro*.

Prior to the present study, all patients with HPRT deficiency studied immunologically had been found to be CRM$^+$ (4, 16, 17). While we have not studied samples from other laboratories, three samples previously reported by us to be CRM$^+$ have no detectable CRM by our current procedure. There may be several explanations for this difference. The antiserum used in the present study differed from that used by us previously in that it was prepared against a more highly purified preparation of HPRT, and it had greater anti-HPRT activity. In addition, the immunochemical reactions described in this report were carried out under more stringent conditions with respect to the maintenance of a constant ionic strength. Finally, as described here, there was substantial contamination of the unabsorbed antiserum with immunoprecipitating material unrelated to HPRT antibodies which was not appreciated at the time of the earlier study. This finding negates the previous data from this laboratory using immunodiffusion or immunoelectrophoresis techniques upon which the earlier "semi-quantitative" assay for CRM was based.

The results of this study provide a basis for further investigations into the control of synthesis and degradation of normal and mutant HPRT in human tissue. The assay for immunoreactive protein that has been developed also has potential for use in the purification of an HPRT$^-$ CRM$^+$ human enzyme for biochemical characterization.

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