Electrophoretic and Kinetic Studies of Mutant Erythrocyte Pyruvate Kinases

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Thin-layer polyacrylamide electrophoresis and kinetic studies were used to differentiate three different mutant erythrocyte pyruvate kinase (PK) in congenital nonspherocytic hemolytic anemia. In the first family, proband and father had PK which migrated faster than the normal and high Michaelis-Menten constants (Km), whereas mother had normal migration and kinetics, but low PK activity. In the second family, proband and mother had PK which migrated slower than the normal. Proband had markedly high Km and mother intermediate high Km. In the third family, proband and mother had faster migrating PK than the normal. Proband had high Km and mother had intermediate high Km. The parents in the second and third families were consanguineous. Inherited molecular abnormalities of erythrocyte PK were proven to exist by abnormal electrophoretic mobilities, as well as abnormal results of kinetic studies.

DEFICIENCY OF erythrocyte pyruvate kinase (PK) as a cause of hereditary nonspherocytic hemolytic anemia was first described by Valentine, Tanaka, and Miwa in 1961. In the 12 years since, more than 135 cases have been reported throughout the world. In addition to the classical type caused by quantitative PK deficiency, several types of functionally abnormal erythrocyte PK have been identified as the genetic variants associated with hereditary hemolytic anemia. However, electrophoresis, one of the standard methods for differentiating the structurally abnormal enzyme protein from the normal, has been difficult because of the instability of the enzyme and the lack of a suitable positive staining method for PK. Enzyme instability easily results in molecular conversion during purification, storage, or preparation for analysis, and abnormal enzymes seem to be more unstable than the normal. Artificial molecular alteration causes kinetic aberration. These facts would seem to require a more direct approach than kinetic studies to confirm the evidence of genetic polymorphism in this disease. Imamura et al. reported that the thin-layer polyacrylamide gel electrophoresis was possible for tissue PK isozyme.
and demonstrated clearly that, in tests of the liver PK of a patient with PK variant (case Y.K.), abnormal L-type PK (PK-L) migrated to the anode slower than the normal, whereas there was no demonstrable PK-L in the classical type of PK deficiency (case M.I.). There have been several previous reports of electrophoresis of kinetically abnormal erythrocyte PK, but we are unaware of any reports that clearly indicate inherited molecular abnormalities of erythrocyte PK with abnormal electrophoretic mobility. This paper describes the electrophoretic evidence and kinetic studies of the erythrocyte PK of three different inherited PK variants in three unrelated families. These inherited variants were tentatively designated PK "Kiyose," PK "Nagasaki," and PK "Sapporo," for the place of discovery.

MATERIALS AND METHODS

Subjects

Details of the patients' clinical history and laboratory data have been reported previously. Short summaries of the cases are as follows.

**Kindred Na.** (PK "Kiyose"). Proband At.Na. was an 8-mo-old Japanese boy when PK deficiency was detected. He was born at full term following an uncomplicated pregnancy and delivery, and received an exchange transfusion immediately after birth because of severe jaundice. Evaluation for etiology of jaundice was not done at that time. The patient was noted to have anemia and jaundice when 4 mo old. There was no history of anemia or jaundice in any relatives, and there was no evidence of consanguinity. When hospitalized, the patient was moderately anemic and subicteric (Hb 7.8 g/100 ml, Hct 24%, reticulocytes 11.9%, bilirubin 2 mg/100 ml). Red cell enzyme assay revealed that the patient had markedly low PK (0.97 U/10^10 RBC, normal, 3.2-5.9 U/10^10 RBC), and the mother had moderately low PK (2.9 U/10^10 RBC). Neither the father (5.8 U/10^10 RBC) nor the patient's brother (7.4 U/10^10 RBC) had PK deficiency, anemia, and jaundice, but both did have elevated phosphoenolpyruvate (PEP) in the red cells. The Michaelis constant (Km) for PEP of the patient (2.44 mM, normal, 0.23-0.73 mM), the father (1.61 mM) and the brother (1.07 mM) were elevated when measured by standard method with 50 mM triethanolamine-HCl buffer, pH 7.4, but the mother's Km (0.76 mM) was not significantly elevated. Because of progressive anemia and hyperbilirubinemia, splenectomy was performed at the age of 2 yr; the weight of the spleen was 510 g.

**Kindred O.** (PK "Nagasaki"). Proband N.O. was a 6-yr-old Japanese girl when hemolytic anemia associated with defective PK was diagnosed. She had suffered from anemia and jaundice at the age of 4 mo. At the age of 6 yr, she was diagnosed as having congenital nonspherocytic hemolytic anemia, and the red cell enzymes were evaluated. Functionally abnormal PK was suggested by accumulation of PEP, 3-phosphoglycerate (3-PG), 2-phosphoglycerate (2-PG), and 2,3-diphosphoglycerate (2,3-DPG) in the red cells despite increased red cell PK activity (6.8 U/10^10 RBC). These accumulations were proved to be caused by increased Km (2.39 mM) of PK for PEP. The parents (PK activities: father 4.1 U/10^10 RBC, mother 4.2 U/10^10 RBC) are first cousins and had intermediate high Km values (father 1.06 mM, mother 1.25 mM). At the time of admission, the patient was a well-developed and well-nourished girl, but subicteric with hepatosplenomegaly. Laboratory studies showed Hb 7.7 g/100 ml, Hct 25%, reticulocytes 23.9%, bilirubin 3.4 mg/100 ml. Cr-t 1/2 was 15 days (normal, 25-30 days). The patient had no siblings and no family history of anemia or jaundice. This case was reported as a PK variant, PK "Nagasaki."23,24

**Kindred Ni.** (PK "Sapporo"). Proband K. Ni. was a 32-yr-old Japanese female. She had been well until the age of 21, when she suffered from jaundice which subsided with treatment for acute hepatitis, but reappeared at the age of 32. The patient was subicteric and slightly anemic without hepatosplenomegaly. The laboratory findings were as follows: Hb 8.5 g/100 ml, Hct 26%, reticulocytes 2.6%, and serum bilirubin 4.7 mg/100 ml. The patient's parents were first cousins. The father was dead and the mother, 62 yr old, had slight anemia of Hb 10.0 g/100 ml, Hct 31%, reticulocytes 1.5%, and serum bilirubin 1.6 mg/100 ml. The patient's brother, 35 yr old, had...
Hb 12.1 g/100 ml, Hct 35%, reticulocytes 1.8%, and serum bilirubin 2.0 mg/100 ml. The red cell enzymes were within normal limits at usual substrate levels for all family members (PK activities: proband 5.0 U/10^10 RBC, mother 3.5 U/10^10 RBC and brother 4.7 U/10^10 RBC), but there were accumulations of PEP, 3-PG, 2-PG, and 2,3-DPG in the red cells, and Kms for PEP were elevated (proband 1.67 mM, mother 1.07 mM, and brother 1.20 mM). This case was reported as a variant, PK “Sapporo,” which differed from PK “Nagasaki” in nucleotide specificity, optimal pH, and enzyme stability at 4°C.23,24

**Procedures**

Venous blood specimens from the patients, family members, and normal controls were anticoagulated with heparin and transported via airplane under refrigerated conditions for tests to proceed within 48 hr. PK was assayed by a method previously reported,25 in freshly prepared crude water hemolysate. The standard assay systems employed 1.33 mM PEP, 2.00 mM ADP, 0.15 mM NADH, 8.0 mM MgSO4, 6.0 mM EDTA, 75 mM KCl, and 6.0 U/ml LDH in 50 mM triethanolamine-HCl buffer, pH 7.4. The reaction was initiated by addition of the hemolysate. All assays were performed at 37°C at 340 nm in a Gilford Model 2400-S recording spectrophotometer. Michaelis constants (Km) presented in the clinical summary were obtained by the standard assay system with a series of different concentrations of PEP in 50 mM triethanolamine-HCl buffer, pH 7.4. Koster et al.26 reported that normal erythrocyte PK had greater allosteric effect and a sigmoidal kinetic curve in alkaline pH. In our standard method, the PK kinetic curve is hyperbolic. In order to produce a sigmoidal curve for allosteric effects, 100 mM Tris-HCl buffer, pH 8.0, was used instead of 50 mM triethanolamine-HCl buffer, pH 7.4. In the former system (pH 8.0), Km becomes higher and the maximum velocity lower, but normal erythrocyte PK has a sigmoidal curve. Substrates, enzymes, and coenzymes were purchased from Boehringer-Mannheim. Other chemical reagents were of analytic grade.

For electrophoretic studies, heparinized blood was centrifuged at 3000 x g for 5 min. The white cells and platelet rich plasma were removed, and the red cells were alternately washed with isotonic saline and centrifuged three more times. After each washing, the buffy coat was removed with care so as not to disturb the top layer of red cells. Final packed cells were suspended in an equal volume of isotonic saline. One-tenth milliliter of 1%, white saponin (E. Merck, Darmstadt, Germany) in isotonic saline was added to 5 ml of the red cell suspension. This suspension was mixed well and allowed to stand at room temperature for 10 min to complete hemolysis. The hemolysate was centrifuged at 26,800 x g for 20 min in a refrigerated Hitachi Model 20 PR centrifuge to remove unlysed residual white cells and lysed erythrocyte stroma. The supernatant hemolysate was separated to avoid contamination of the sediment. A volume of toluene equal to 20%, of the hemolysate was added, and the solution was shaken vigorously for 10 min, then centrifuged at 4500 x g for 15 min. The toluene and stroma were removed. The hemolysate was aspirated into a capillary pipette and applied to the slot of the electrophoretic gel plate. Electrophoresis and staining of PK were performed according to the method of Imamura and Tanaka,20 slightly modified in that electrophoresis was carried out at 25 V/cm for 5 hr for the erythrocyte PK and at 40 V/cm for 2 hr for the liver extract.

Liver biopsy specimens were obtained at the time of splenectomy and a liver control specimen was obtained from the autopsy of a victim of Hodgkin’s disease. The specimens were immediately frozen in acetone-dry ice and kept in deep freeze until homogenization. Liver specimens were homogenized with a Universal Homogenizer (Nihonseiki Seisakusho Co., Tokyo, Japan) in about 5 volumes (w/v) of cold extracting solution at 0°C. Extracting solution consisted of 0.1 M KCl containing 20 mM Tris-HCl buffer (pH 7.4), 5 mM MgSO4, and 1 mM EDTA. The homogenate was centrifuged at 26,800 g for 30 min at 4°C in a refrigerated centrifuge. The supernatant was filtered through a membrane filter (Sartorius-Membranfilter, Göttingen, pore size 0.45 µ), and the filtrate was diluted according to the PK activity and used for PK electrophoresis.

**RESULTS**

**Electrophoresis**

Electrophoreses of erythrocyte PK are shown in Figs. 1 and 2. This system separates the erythrocyte PK into two bands, neither having the same mobility.
as liver PK-L, shown in Fig. 3. The cathodal band was designated as PK-R₁ and the anodal band as PK-R₂. The ratio of PK-R₁ to PK-R₂ seems to depend on the red cell aging; young red cells have more PK-R₁ and older cells have more PK-R₂, as Paglia et al. reported previously. The patients with hemolytic anemia (autoimmune hemolytic anemia, glucosephosphate isomerase deficiency, and hereditary spherocytosis) have increased PK-R₁, suggesting an
increased population of young red cells. PK-R1 and PK-R2 are fused to make only one broad band in cases of short-term electrophoresis or incomplete destromatizing, or when the enzyme reaction for staining lasts too long, as shown in Fig. 3. As previously reported by Imamura et al., the liver has two main bands in electrophoresis (Fig. 3). The cathodal band was designated M2-type PK (PK-M2), which is found in other tissues, the leukocytes, the spleen, the lymph node, the adipose tissue, and the tumor cells. The anodal band was named L-type PK (PK-L), which is only in the liver and has faster electrophoretic migration than the erythrocyte PK (PK-R1 and PK-R2). The muscle has another type of PK, M2-type PK (PK-M2), which is migrated between PK-M2 and PK-R2.

As is evident in Fig. 1, the mother in kindred Na. showed normal electrophoretic mobility with decreased intensity of the two bands, whereas the proband and father in kindred Na. showed faster migration to the anode than the normal. The classical type of severe homozygous PK deficiency, case M.I., had no demonstrable PK-R (Fig. 1). Moreover, the liver of the case M.I. had no demonstrable PK-L when tested by electrophoresis, and in that respect was similar to case M.T. (Fig. 3). Case M.I. had received massive blood transfusion for a splenectomy, and it was not possible to do electrophoresis with his own erythrocyte PK at the time of splenectomy. Mobilities of the proband and mother in kindred O. were slower than the normal control (Figs. 1 and 2). The proband and mother in kindred Ni. also showed abnormal mobility, both faster than the normal. Mixed samples of normal control and proband in kindred Ni. developed three bands, the middle band being a combination of PK-R1 from the control and PK-R1 from proband (Fig. 2), which clearly indicated that the PKs of the normal control and proband in kindred Ni.
had completely different electric charge. Leukocytes and platelets have PK-M₂ but no PK-R. Contamination of the hemolysate by leukocytes and platelets caused an additional band to appear at the site of the M₂ band. Saponin affects neither PK activity nor PK electrophoretic mobility. In this study, saponin hemolysis was preferred to prevent leukocyte contamination. In Fig. 3, electrophoresis of the liver extract is shown. The proband in kindred Na. had PK-M₂ and PK-L with normal mobility, but case M.T., a classical type of PK deficiency, had only one band of PK-M₂ and no PK-L.

**PK Kinetic Studies**

The data were converted from absolute values to the rate of change curves in Figs. 4-6, which describe the celerity with which the PK activity approached the maximum (1.0) as the substrate levels increased with or without stimulation of fructose-1,6-diphosphate (FDP). These kinetic studies were done in 100 mM Tris-HCl buffer, pH 8.0. In Fig. 4, the mother in kindred Na. had a kinetic curve within normal range with normal response to FDP stimulation. However, the proband and father in kindred Na. had high Km and PK activity which approached the maximum more slowly. The proband and father in kindred Na. had saturation curves with sigmoid shape, but the presence of FDP converted this sigmoid curve to a hyperbolic one and lowered the Km for PEP, although Kms in the presence of FDP of the proband and father were still higher than the normal Km in the presence of FDP, suggesting that the variant of this family had an allosteric effect. The similarly impaired curves of the proband and father and the normal curve of the mother in kindred Na. suggest that the proband inherited a paternal gene for kinetically aberrant PK and a maternal gene which caused quantitative PK deficiency. Absence of anemia in the father and mother may be due to the fact that the father is heterozygous for variant PK and the mother is heterozygous for deficient PK.

In kindred O. (Fig. 5), the proband has high Km, no sigmoidicity in the
curve, and incomplete FDP activation, suggesting that the proband in kindred O. has high Km variant with less allosteric property. In addition, the mother's curve lies between the normal and proband, suggesting that the mother is heterozygous for the variant PK.

In kindred Ni., the proband had an affected kinetic curve, and the mother seemed to have a normal curve, but in the presence of FDP, the mother had an abnormal curve with high Km. In addition, Km for PEP measured in triethanolamine-HCl buffer, pH 7.4, was higher than the normal in the absence of FDP, as presented in a clinical summary. This phenomenon might be caused by different optimal pH in kindred Ni. These are sigmoid curves in the absence of FDP but become hyperbolic in the presence of FDP.

In Fig. 7, PK activities were inhibited more as the ATP concentration increased. The three probands in kindred Na., O., and Ni. suffered more inhibition than the normal control. This figure also shows that variant PKs with ele-
Fig. 7. ATP inhibition test. N.C., normal control. At. Na., proband in kindred Na.; N.O., proband in kindred O.; K.Na., proband in kindred Na. Assay system: 1.33 mM PEP, 2.00 mM ADP, 0.15 mM NADH, 8.00 mM MgSO₄, 6.00 mM EDTA, 75 mM KCl, 6.0 U/ml LDH, and varied concentrations of ATP in 100 mM Tris-HCl buffer, pH 8.0, at 37°C.

vated Km for PEP were more affected by competitive inhibition of ATP for PEP.

DISCUSSION

In our electrophoretic system, PK is stained positively and hemoglobin negatively, whereas in Blume’s method, hemoglobin appears positively. In Figs. 1.2, and 3, hemoglobin was stained negatively and migrated faster than PK. Blume et al. and Paglia et al. previously reported two bands of PK in the red cells. Our system also gave two bands that were dependent on the red cell aging. Reticulocytosis increases PK-R₁, but this should not be regarded as an abnormal pattern. As shown in Figs. 1 and 2, all probands had increased PK-R₁, which suggests an increased young red cell population due to hemolysis and active erythropoiesis in these patients. PK-R₁ may be converted to PK-R₂ in red cell aging. It does not seem to be plausible that PK-R₁ and PK-R₂ are isozymes that are produced by different structural genes, because it has been observed that both PK-R₁ and PK-R₂ are converted to PK-L electrophoretically under special conditions (long standing at 4°C, repeated freezing and thawing, and addition of liver extract). As is evident in Figs. 1 and 2, all probands and family members have both PK-R₁ and PK-R₂. However, the proband and father in kindred Na. and the proband and mother in kindred Ni. have faster migrating PK than the normal, while those in kindred O. have slower migrating PK than the normal. Of kindred Na., the proband and father have PK-R₁ and PK-R₂ that migrated faster than the normal, while the mother has normal migration. The father and mother had no anemia or jaundice. These facts suggest that the
father is heterozygous with a variant PK gene and a normal PK gene, that the mother is also heterozygous with a deficient PK gene and a normal PK gene, and that the proband has a double heterozygote with a variant PK gene from the father and a deficient PK gene from the mother. This theory is supported by the kinetic studies, which indicated a normal kinetic for the mother and abnormal kinetic curves (Fig. 4) for the proband and father. Paglia et al.\textsuperscript{5,16} and Brandt et al.,\textsuperscript{12} detected four families with double heterozygote, mainly on the basis of kinetic evidences, but none of these investigators reported any abnormal electrophoretic evidence. In this case, the presence of a double heterozygote detected by kinetic studies was confirmed by electrophoretic evidence. In addition, the brother in kindred Na. is also considered to be heterozygous, with a variant PK gene and a normal PK gene, because of rather high PK activity and high Km, as presented in the clinical summary.

In contrast to kindred Na., the parents in kindred O. are first cousins, and previous kinetic studies\textsuperscript{23} revealed similar results for both parents. As shown in Fig. 5, the kinetic curve for the mother was intermediate to the normal control and proband. It is concluded that the proband in kindred O. is pure homozygous with two identical variant genes (PK "Nagasaki").

In kindred Ni., the parents are first cousins. It is impossible to obtain complete information because of the father's death, but the intermediately aberrant kinetic curve of the mother and consanguinity of the parents strongly suggests that the proband in kindred Ni. has also a pure homozygote with two identical variant PK genes (PK "Sapporo"). PK "Sapporo" had already been distinguished from PK "Nagasaki" by differences in optimal pH, nucleotide specificity, and enzyme stability in two previous reports.\textsuperscript{23,24} Now, these variants can be distinguished by differences of electrophoretic migration rates.

From electrophoretic and kinetic studies, there is no doubt that kindred O. and kindred Ni. have different variant genes. The heterozygous father in kindred Na. and the heterozygous mother in kindred Ni. have similar PK migration and kinetic behavior. In other biochemical parameters, i.e., pH curve, nucleotide specificity, and enzyme stability tests, there was no distinct evidence that revealed differences between the father in kindred Na. and the mother in kindred Ni. But in clinical features, the heterozygous mother and brother in kindred Ni. had slight hemolytic anemia, whereas the heterozygous father and brother in kindred Na. had neither anemia nor jaundice. These clinical features should be regarded as in vivo reflections of abnormal functions of the variant enzymes, because previously reported cases with qualitative and quantitative defects of the erythrocyte PK had different clinical features, according to the natures of the defective PKs.\textsuperscript{22} Sachs et al.\textsuperscript{7} reported a family with mutant PK in which heterozygous family members had hemolytic anemia. Due to the different clinical features, it is impossible to consider the genetic defects in PK variants of kindred Na. and kindred Ni. identical, despite the similar biochemical parameters. At the present time, it is not feasible to determine the amino acid sequence of the PK molecule. Tentatively, the genetic defects in kindred Na. and kindred Ni. should be considered different, with separate designation (PK "Kiyose" and PK "Sapporo"), until more advanced procedures can be devised.
The evidence of inhibition by ATP (Fig. 7) indicated that probands with high Km were more inhibited than the normal, suggesting that ATP inhibition affects the active site of the enzyme for PEP and that probands have abnormal molecules.

It has been reported20,22 that the normal liver contains two PK isozymes, PK-L and PK-M2, as shown in Fig. 3. However, the classical homozygous PK deficiency, case M.T., had only the PK-M2 band and no PK-L. Bigley and Koler3 reported that PK-L was absent in the PK-deficient liver by chromatographic studies. The autopsy case used here as a control had died of Hodgkin’s disease, and the PK-L seemed to be more decreased than in other autopsy cases. Imamura et al.20 found that noncancerous liver in the cancer-bearing rat contained less PK-L and more PK-M2 than the normal. This supports the finding that the autopsy case of Hodgkin’s disease had less PK-L and more PK-M2 in the liver, which was free from tumor infiltration. There are also two faint minor bands between the L and M2 bands in the normal liver, which are main bands in the brain and the intestine.20 In this autopsy case, the decrease of PK-L might cause the minor bands and M2 bands to become relatively more prominent than usual. These minor bands could be due to erythrocyte contamination, or may be charge isomers of PK-L, because no minor bands were observed in the liver of case M.T. A previously reported variant PK “Tokyo-I” (case Y.K.)21,22 had an abnormal PK-L band migrating more slowly than the normal, whereas a classical type of PK deficiency (case M.I., same case in Fig. 1) had no PK-L in the liver. PK-L in the liver of the proband in kindred Na. is probably a product of a variant gene, despite the evidence of normal electrophoretic migration. Koler and his co-workers31 reported that erythrocyte PK and PK-L of the liver are the same, but in our electrophoretic studies of the mixture of normal hemolysate and normal liver extracts, PK-R and PK-L moved independently, and respective bands were additive.22 PK-R1 and PK-R2 can convert to PK-L under special conditions.28 In addition, because of the discrepancy between the normally migrating PK-L and the faster moving PK-R of the proband in kindred Na. and in consideration of the absence of PK-L in the classical type of PK deficiency (case M.T. in Fig. 3) and slower moving PK-L and PK-R in a variant, PK “Tokyo-I,”21,22 it is conceivable that PK-L and PK-R are not completely identical, but have somewhat similar composition. It is still uncertain why heterozygotes and homozygotes of the PK variants have similar electrophoretic migration, whereas heterozygotes and homozygotes of smaller enzyme variants, such as glucosephosphate isomerase variant,32,33 have different migrations. This may probably arise because PK has a large molecule with four subunits (mol wt 2 x 105),34,35 and the net electric charge of the heterozygotes may be determined by one or more abnormal subunits.

ACKNOWLEDGMENT

We wish to thank Dr. Kaneo Yamada, Department of Pediatrics, Keio University, Dr. Yoshiro Tsuji, Department of Pediatrics, Nagasaki University, and Dr. Mitsuo Miyahara, Department of Medicine, Sapporo Medical School for kindly permitting us to study patients under their care. We also gratefully acknowledge the expert technical assistance of Miss Hitomi Ogawa.
REFERENCES


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