

**Postnatal Hematologic Development in Phosphofructokinase-Deficient Dogs**

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Adult dogs with phosphofructokinase (PFK) deficiency have compensated hemolytic anemia, due to an absolute or functional deficiency of the muscle-type (M-type) subunit that normally accounts for a majority of total erythrocyte PFK activity in dogs. Potential effects of PFK deficiency on hematologic development were evaluated in dogs. Routine hematologic parameters were similar in normal and affected dogs when 1 day old, because all newborn dogs had erythrocyte PFK activities about three times that of normal adult dogs. Based on chromatographic separation of PFK isozymes and enzyme immunoprecipitation studies, the high PFK activity at birth was attributed to the predominance of the liver-type (L-type) subunit of PFK, which is negligible or absent in normal adult dog erythrocytes. Both total PFK activities and the amounts of L-type subunit present decreased dramatically during the first 6 to 8 weeks of life. The muscle-type subunit was negligible or absent at birth, but appeared and increased as the L-type decreased in normal dogs. These changes may result from the replacement of erythrocytes formed in the fetus with those formed after birth. A postnatal physiologic anemia developed to a similar degree in both affected and normal dogs because of decreases in both mean corpuscular volume and erythrocyte numbers. Reticulocyte counts were high in all dogs at birth and remained high in affected dogs, but decreased from 2 months of age onward in normal dogs. Erythrocyte 2,3-diphosphoglycerate (DPG) values were very low in all newborn pups and increased to values expected for adults in the respective groups by 2 to 4 weeks of age. A low 2,3-DPG concentration occurs in affected dogs because PFK deficiency inhibits glycolysis above the shunt that forms 2,3-DPG.

**PHOSPHOFRACTOKINASE (PFK) deficiency of erythrocytes and skeletal muscle has been reported in English springer spaniel dogs.** Based on erythrocyte assays of a limited number of related animals, it appears that the defect is transmitted as an autosomal recessive trait, as has been reported in humans. As in humans, PFK in dogs is under the genetic control of three structural loci that encode muscle (M)-, liver (L)-, and platelet (P)-type subunits. Various isozymes are formed by the random tetramerization of subunits that are variably expressed in different tissues. Normal dog muscle consists primarily (possibly exclusively) of the M₄ isozyme, and the M-type subunit accounts for 80% to 90% of the total subunit composition of PFK tetramers in normal dog erythrocytes. Since dogs with PFK deficiency have an absolute or functional deficiency of the M-type subunit, total PFK activities are markedly reduced in both erythrocytes and muscle from these animals.

Deficient dogs generally exhibit minimal evidence of myopathy because a small amount of activity is present that may be the result of a compensatory expression of the L-type subunit. In addition, dog skeletal muscle is less dependent on anaerobic glycolysis than human skeletal muscle is due to a lack of the classical fast-twitch glycolytic (type 1IB) fibers in dogs.

Like humans, dogs with PFK deficiency exhibit persistent compensated hemolytic anemia. In contrast to the disorder in humans, occasional episodes of intravascular hemolysis and hemoglobinuria occur in deficient dogs. Erythrocytes from normal dogs lyse at lower pH values than do human erythrocytes when incubated in a series of buffers of varying pH. Erythrocytes of PFK-deficient dogs are even more alkaline fragile in vitro and have occurred following hyperventilation-induced alkalemia in vivo in deficient dogs. The increased alkaline fragility occurs secondary to decreased 2,3-diphosphoglycerate (DPG) concentration within PFK-deficient erythrocytes. The concentration of 2,3-DPG is low because it is formed in a shunt off of the glycolytic pathway below the PFK step. This study was conducted to evaluate age-related hematologic changes that may occur secondary to PFK deficiency in dogs. Similar studies have not been conducted in humans with PFK deficiency because the disorder has not been diagnosed during the neonatal period. In addition to total PFK activity, the determination of PFK isozymes was of interest, because the PFK isozyme composition in normal human neonatal erythrocytes appears to be different from that of adult erythrocytes.

**MATERIALS AND METHODS**

This study was approved by the Institutional Animal Care and Use Committee of the University of Florida. Results from five normal English springer spaniel dogs were compared with those from 10 English springer spaniel dogs produced from matings of an affected male dog to two of his daughters having approximately half-normal erythrocyte PFK activity. Animals were housed in Animal Resources Facilities at the University of Florida. Pups were offered a moistened, commercially available dog food (Purina Puppy Chow, Ralston Purina Co, St Louis, MO) starting at 1 month of age and were weaned on this same food when 2 months old. A vitamin and mineral supplement (Pet Tinic, Beecham Laboratories, Bristol, TN) was administered at a dosage of 1 ml/kg orally from 8 to 14 weeks of age. Animals were given a routine series of vaccinations starting when 1 month old and were dewormed at 2 and 8 months of age.

Blood samples were collected in potassium ethylene diamine tetracacetate (EDTA) at selected time intervals from 1 day to 1 year of age. Complete blood cell counts (CBCs) were made with a
semi-automated cell counting system (ZBI-6 System, Coulter Electronics, Hialeah, FL). Reticulocytes were counted after staining with new methylene blue.\textsuperscript{11} Erythrocyte 2,3-DPG and adenosine-5'-triphosphate (ATP) concentrations were measured spectrophotometrically using trichloroacetic acid-precipitated extracts (Kits 35-UV and 366-UV, Sigma Chemical Co, St Louis, MO).

Erythrocyte PFK and pyruvate kinase (PK) activities were measured at 37°C in hemolysates prepared following the removal of leukocytes by filtration through microcrystalline cellulose and alpha-cellulose columns.\textsuperscript{12} Chromatographic separation of erythrocyte PFK isozymes was performed at 4°C using a DEAE-Sephadex A-25 column equilibrated with 0.1 mol/L Tris/phosphate buffer (pH 8.0), containing 0.2 mmol/L EDTA, 0.2 mmol/L adenosine-5-monophosphate, 25 mmol/L NaCl and 0.7 mmol/L dithiothreitol.\textsuperscript{13} Elution was done with a concave gradient using 0.5 mol/L NaCl in the same buffer. Chromatographic separations were conducted on hemolysates from two affected, two carrier and one normal pup at 1 day and 2, 4, 6, and 8 weeks of age. Selected blood samples were also analyzed by enzyme immunoprecipitation using a human anti-L monoclonal antibody that also reacts strongly with the dog L-type subunit of PFK.\textsuperscript{14}

Statistical comparisons were made between groups and over time using the repeated measures analysis of variance.\textsuperscript{15} When significant differences were found between groups, mean values for a specific age were compared by ANOVA and the Student’s t-test. Least squares regression analysis was used to compare different parameters to determine if a linear correlation existed between them. Unless otherwise indicated, differences discussed in the text were significantly different at $P < .05$.

**RESULTS**

**Pedigree analysis.** Although all pups had high erythrocyte PFK activities at birth, those produced from the matings of an affected male to two of his daughters with half-normal erythrocyte PFK activity resulted in five pups that were markedly deficient in erythrocyte PFK activity and five pups with half-normal activity when 3 months old. Male and female pups were present in both groups. Based on pedigree analysis, it is apparent that PFK deficiency in dogs is transmitted as an autosomal recessive trait, as it is in humans.\textsuperscript{5} For comparison purposes, animals were divided into three groups based on erythrocyte PFK activities: normals, heterozygous carriers, and homozygous affected. One or two mild episodes of intravascular hemolysis occurred between 6 months and 1 year of age in three of the five affected dogs, but no hemolytic episodes occurred near dates when dogs were bled.

**Routine hematology.** Most 1-day-old pups had packed cell volume (PCV) and hemoglobin (Hb) values within or near normal adult ranges of 37% to 55% and 12 to 18 g/dL, respectively (Figs 1 and 2). Mean values decreased to similar minimum values at 4 to 6 weeks and then increased through 6 months of age. Affected dogs had Hb values significantly below that of normal dogs from 6 months onward. Most of the decreases in PCV and Hb resulted from decreases in erythrocyte size rather than from decreases in erythrocyte numbers in blood (Fig 1). Erythrocyte mean cell volume (MCV) values were above normal adult dog values (60-75 FL) at birth and decreased to adult values between 3 and 6 weeks of age (Fig 1). After declining, MCV values of affected pups began to increase at 2 months of age and were significantly above normal and carrier animals from then on. Subsequent increases in PCV and Hb values in normal and carrier animals resulted primarily from increases in erythrocyte numbers.

Mean cell Hb concentrations were lower in affected dogs than in the other groups (data not shown). Mean values ranged from 35 to 39 g/dL when 1 day old, decreased to 30 to 33 g/dL when 6 to 8 weeks old, and then increased to 33 to 38 g/dL at 1 year of age.

Reticulocyte counts were high in all groups when 1 day old (Fig 2) compared with values for normal adult dogs.
Fig 2. Age-related changes in reticulocyte counts, erythrocyte ATP concentrations, and Hb values from homozygous affected and heterozygous carrier dogs with PFK deficiency compared with normal control dogs. Values are mean ± SD.

Fig 3. Age-related changes in erythrocyte PK and PFK activities and 2,3-DPG concentrations from homozygous affected and heterozygous carrier dogs with PFK deficiency compared with normal control dogs. Values are mean ± SD.
adults for each respective group by 2 to 4 weeks of age (Fig 3). The mean value for normal pups reached a maximum at 6 weeks of age and then declined gradually throughout the remainder of the year. A less pronounced peak in 2,3-DPG concentration occurred in carriers, and affected dogs reached a plateau at 3 weeks of age. Affected dogs had lower concentrations than the other groups throughout the study, and all groups were significantly different from one another from 1 month of age onward.

Erythrocyte PFK activity in all groups at birth was approximately three times that of normal adult dogs (mean, 12 IU/g Hb). Activities generally decreased to expected adult values for each respective group by 2 to 3 months of age (Fig 3). Affected dogs were significantly different from the other groups from 2 weeks of age onward, and all groups were significantly different from one another from 2 months of age onward.

Erythrocyte PK activity was measured because the 2,3-DPG concentration within erythrocytes of a given species is controlled by the rate of the PK reaction relative to that of the PFK reaction. PK activities were high relative to adult values in all groups at birth (Fig 3). While PK activities in normal and carrier pups approached normal adult values during the study period, PK activity in affected dogs remained increased. There was a significant positive linear correlation ($r = 0.75, P < 0.0001$) between PK activities and the percentage of reticulocytes present in blood (data not shown).

**PFK isozyme analysis.** Results of chromatographic separations of PFK isozymes of erythrocytes are illustrated in Fig 4. At birth, most of the enzyme activity in each tested animal eluted in a peak at the position expected for $L_4$ species. Over the next 8 weeks, the amount of activity in this peak decreased and additional peaks in activity appeared. In the case of affected dogs, a major peak appeared which eluted in the $P_4$ region (Fig 4). In the case of heterozygous and normal dogs, multiple peaks appeared between the $M$ and $L$ isozyme regions.

From 85% to 91% of erythrocyte PFK activity from a homozygous and three heterozygous 1-day-old pups was precipitated using a monoclonal antibody that reacts with the $L$-type subunit of canine PFK. Only 0% to 4% of PFK activity was precipitated when this antibody was used in hemolysates prepared from three normal 2-month-old pups. These findings are in agreement with results from chromatographic separations and indicate that the high PFK activity in all groups at birth results from the presence of $L$-type subunits.

**DISCUSSION**

The physiologic anemia that developed after birth in all groups of dogs in this study has been recognized in other breeds of dogs and species of animals, and in human infants. The rapid postnatal decline in PCV and Hb values results from a combination of shortened fetal erythrocyte life-spans and expansion of total plasma volume, with inadequate compensatory erythropoiesis to maintain these values. The relative importance of these factors may vary depending on the species.

As has been reported in human infants, the physiologic anemia that developed in the present study resulted from decreases in average erythrocyte size (MCV), as well as erythrocyte number. In the case of carrier and affected dogs, the decrease in MCV was of primary importance. It appears that the macrocytes formed in the fetus were rapidly replaced by normocytes and/or microcytes.

The high ATP concentrations in blood of neonatal pups is partially related to the presence of reticulocytes. Canine reticulocytes have higher ATP concentrations than mature erythrocytes, and ATP concentrations correlated to some extent with the percentage of reticulocytes present in this study. Erythrocyte ATP content in affected dogs is not low when measured in whole blood because of the presence of reticulocytes, which can generate ATP oxidatively within mitochondria. In contrast to dogs, erythrocyte ATP concentration in newborn humans is equal to that of adults. It increases slightly at 1 month of age and remains relatively constant during the first year of life.

Fig 4. Chromatographic separations of erythrocyte PFK isozymes from 1-day-old and 8-week-old pups. (A) Homozygous deficient. (B) Normal control. (C) Heterozygous deficient. Circled letters M, P, and L demonstrate the expected elution positions of $M_4$, $P_4$, and $L_4$ tetramers of canine PFK.
Erythrocyte PFK activities in all groups were approximately three times normal adult values when 1 day old. Based on our studies of PFK isozymes, this high activity at birth is attributable to the predominance of the L-type subunit, which is negligible or absent in normal adult dog erythrocytes. Both total PFK activities and the amounts of L-type subunit present decreased dramatically in all groups during the first 6 to 8 weeks of life. The M-type subunit was negligible or absent in all groups at birth, but appeared and increased as the L-type decreased in carrier and normal dogs. It was not possible to determine whether the P-type subunit of PFK changed over time. The decreases in enzyme activity appeared to correlate with decreases in MCV; consequently, it is suggested that these decreases result from the replacement of erthrocytes formed in the fetus with those formed after birth. Potential age-related changes in PFK activity have not been studied previously in dogs, but similar postnatal decreases in activity have been reported in cattle, sheep, and goats.

In contrast to dogs and ruminants, erythrocyte PFK in newborn humans is lower than that in adults. The M-type and L-type subunits each account for approximately half of the PFK activity in adult human erythrocytes. Travis and Garvin studied PFK activities in density-separated erythrocytes from cord blood and adults and speculated that the relative PFK deficiency resulted from the normal synthesis of an unstable fetal isozyme of PFK. Rather than indicating the presence of a unique fetal enzyme, it appears that PFK in human fetal erythrocytes is deficient in the M-type subunit and that the L-type subunit accounts for the majority of activity present.

The rapid increase in 2,3-DPG after birth in dogs is considered to be beneficial in the delivery of oxygen to tissues by decreasing the Hb oxygen affinity. Since affected dogs had significantly lower 2,3-DPG than the other groups from 1 day of age onward, it is surprising that they did not have a compensatory reticulocyte response higher than the other groups until 6 weeks old. Meugger and Black studied the postnatal control of 2,3-DPG levels in erythrocytes of normal Labrador retriever pups. A decrease in PFK activity was measured concomitant with an increase in phosphoenolpyruvate concentration that correlated directly with 2,3-DPG concentration. Their interpretation was that 2,3-DPG concentration in postnatal canine erythrocytes was controlled by the activity of PFK, which functions as a sink reaction.

In contrast to the above study, PK activity changed minimally in our study during the first month of life when the most dramatic increases in 2,3-DPG concentration occurred. This finding suggests that a factor(s) other than a decline in total PK activity may be important in causing the postnatal increase in 2,3-DPG. However, we cannot rule out the possibility that a decline in PK activity in mature erythrocytes was masked by the presence of reticulocytes with high PK activity.

Besides inhibiting the PK reaction, factors that stimulate glycolysis have the potential of increasing erythrocyte 2,3-DPG. The PFK reaction appears to be the most important step in controlling the glycolytic rate of erythrocytes under physiologic conditions. The finding that total PFK activity decreases substantially during the period that 2,3-DPG increases would suggest that the PFK reaction might not be important; however, it is possible that there is a change in enzyme kinetics associated with the decrease in the L-type subunit and increase in the M-type subunit in normal and heterozygous animals. Indirect support for PFK reaction involvement in controlling the postnatal increase in 2,3-DPG is provided by the observation that the erythrocyte 2,3-DPG content of affected dogs at 2 weeks of age reached the value expected for affected adults and was significantly lower than that of control or carrier dogs, yet the total PFK activity was equal to that found in normal adult dogs.

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