Inherited Phosphofructokinase Deficiency in Dogs With Hyperventilation-Induced Hemolysis: Increased In Vitro and In Vivo Alkaline Fragility of Erythrocytes

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Although canine erythrocytes have many characteristics in common with other mammalian erythrocytes, including those of man, there are some peculiar differences. In a study performed by Waddell in 1956, canine erythrocytes were noted to be uniquely more fragile in alkaline media than erythrocytes from man.

Many hereditary erythrocyte enzyme defects have been described in man. The pathogenesis of hemolysis in erythroenzymopathies is not well understood, and the degree of hemolysis is quite variable from one defect to another, as well as within a single category of enzyme alteration. Some enzyme defects not only have adverse consequences to the red cells, but may compromise the functional integrity of other tissues as well. Inherited deficiency of phosphofructokinase (PFK) (EC 2.7.1.11), a key regulatory enzyme in the glycolytic Embden-Meyerhof pathway, is generally partial in the erythrocyte and complete in muscle cells and has been clinically characterized by hemolysis or myopathy on exertion or by asymptomatic states. PFK deficiency rarely occurs in man and has not been documented in other mammalian species.

The present study describes clinical features and hematologic data from two male English springer spaniel dogs with severely reduced PFK activity in the erythrocytes, and discusses the pathogenesis of hemolysis in canine PFK deficiency.

CASE REPORTS

Dog 1

A 5-year-old male English springer spaniel had a history of persistent bilirubinuria and mild anemia since the age of 5 months. Approximately every two months the owner of the dog noted an increase in the erythrocyte pH, which may partially explain the pathogenesis of hemolysis in canine PFK deficiency. Thus, these studies demonstrate a presumably inherited erythrocyte PFK deficiency in English springer spaniels, which causes an increased in vitro and in vivo alkaline fragility of erythrocytes in dogs with PFK deficiency and inducible hemolytic crises may become a valuable genetic animal model in which to study the pathophysiologic mechanism of hemolysis.
exercise-induced hyperventilation showing variation in intensity of brown color caused by bilirubin (Br) and hemoglobin (Hb).

Dog 2

Dog 2, a presumably unrelated male English springer spaniel, was referred to one of us (A.C.) when he was 5 years old with a lifelong history of intermittent severe pigmenturia. Splenomegaly had been noted for several years. Hematologic data collected over two years (Table 1) revealed a Coombs’ negative, partially compensated hemolytic anemia. No poikilocytosis was noted. Total serum globulin was increased at 4.0 to 5.6 g/dL; serum lactate dehydrogenase activity and serum potassium were also slightly increased. Dog 2 had splenic lymphoid follicular hyperplasia.

Studies of dog 1 (21 kg) were compared with results obtained from 15 healthy male dogs of various breeds, as determined by physical examinations and routine laboratory tests. Control dogs from 15 healthy male dogs of various breeds, as determined by physical examinations and routine laboratory tests. Control dogs used in in vivo experiments weighed between 18 and 26 kg and were kept in a research animal resource unit. All animals were fasted at least four hours before any experiment. Analytical-grade chemicals and special enzymes were purchased from Sigma Chemical Co. St Louis, and Boehringer Mannheim, Germany.

**Table 1. Hematologic Data of Dogs 1 and 2**

<table>
<thead>
<tr>
<th></th>
<th>Dog 1</th>
<th>Dog 2</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV (%)</td>
<td>29-35</td>
<td>15-37</td>
<td>37-55</td>
</tr>
<tr>
<td>RBC (x 10⁶/μL)</td>
<td>3.3-3.9</td>
<td>2.6-4.6</td>
<td>5.5-8.5</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>9.4-10.8</td>
<td>5.1-12.2</td>
<td>12-18</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>84-89</td>
<td>82-95</td>
<td>60-77</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>30-32</td>
<td>28-35</td>
<td>32-37</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>8-14</td>
<td>5-24</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>1-3</td>
<td>1-3</td>
<td>0</td>
</tr>
<tr>
<td>Nucleated RBC (per 100 WBC)</td>
<td>15-25</td>
<td>8-37</td>
<td>6-17</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.5-0.7</td>
<td>0.2-0.6</td>
<td>0.2-0.4</td>
</tr>
</tbody>
</table>

*Blood samples were collected almost daily during hospitalizations, n = 11 to 14.
†Data collected (A.C.) over two years, n = 9.
‡Normal range, University of Florida Veterinary Medical Teaching Hospital.

**In Vitro Erythrocyte Studies**

Blood was collected in potassium EDTA or clot tubes and processed within three hours. The erythrocytes from dog 1 were tested on several days, each time simultaneously with erythrocytes from one or more control dogs. Erythrocytes were prepared by separating the plasma and white blood cells, washing three times, and resuspending with 0.15 mol/L NaCl solution in order to obtain a 50% suspension.

The acidified serum lysis and sucrose lysis tests were modified from established procedures used for the diagnosis of paroxysmal nocturnal hemoglobinuria in man. In the acidified serum lysis test, 0.05 mL of erythrocyte suspension was incubated with 0.5 mL of (1) 0.15 mol/L NaCl (pH 6.8), (2) autologous serum, (3) homologous serum, (4) acidified serum (pH 6.4), and (5) acidified and heat-inactivated serum. The pH of the serum was lowered by adding 0.2 N HCl and the serum was inactivated by incubation at 56°C for 30 minutes. The mixtures were incubated for one hour at 37°C. Four milliliters of 0.15 mol/L NaCl were then added to all tubes, and the cells remaining were removed by centrifugation. Complete lysis was achieved by adding 0.5 mL of 0.01 mol/L NaOH to 0.05 mL cells. The optical densities of the supernatant fluids were read against an H₂O blank at 540 nm with a Beckman spectrophotometer (model 25, Fullerton, Calif).

In the sucrose lysis test, 0.05 mL of erythrocyte suspension was added to 0.95 mL isotonic 0.28 mol/L sucrose-0.005 mol/L sodium barbital buffer with adjusted pH values at 6.8 as well as at recommended 7.3. Complete lysis was achieved by adding 0.95 mL of 0.01 mol/L NH₄OH to 0.05 mL cells. After the incubation at 22°C, hemolysis was measured as described.

The alkaline fragility test was modified from Lampietro et al. A series of isonic buffers, with pH values ranging from 6.8 to 8.3, was prepared. Incubation mixtures consisted of 0.02 mL erythrocyte suspension and 5 mL of isotonic buffer containing 0.15 mol/L NaCl, 0.005 mol/L dextrose, and 0.01 mol/L sodium phosphate. Owing to the limited buffering capacity of the phosphate, the actual pH of each tube was measured before and after the incubation period. No change in pH was observed during incubations. Incubations were performed at 22°C, 37°C, and 40°C in a water bath, and the incubation time varied from 20 to 80 minutes. After incubation, the tubes were centrifuged and the absorbances of supernatants were read at 540 nm. Cells added to 5 mL distilled water served as reference for 100% hemolysis. Erythrocytes from dog 2 and a control were simultaneously analyzed for alkaline fragility one day after collection of the blood samples.

Erythrocyte enzyme activities of dogs 1 and 2 were measured in microcrystalline cellulose-a-cellulose-filtered and washed erythrocytes according to the method of Beutler et al. Erythrocyte 2,3-diphosphoglycerate (2,3-DPG) concentration was determined in the neutralized supernatant of fresh whole blood mixed with perchloric acid by spectrophotometry (kit No. 35-UV, Sigma). Erythrocyte potassium and sodium concentrations were measured by a flame photometer, and erythrocyte chloride content was determined by a colorimetric using blood collected into heparinized tubes. A student’s t test was applied for statistical analysis.

**Hyperventilation Studies in Dog 1**

In order to assess the effect of "stress," hyperventilation was induced for 30 minutes by various means on different days. First, the dogs were exercised alongside a bicycle at a pace of approximately eight to ten miles per hour. Second, the animals were placed in a water bath with a temperature of 39°C (as deep as the middle of the scalp). Finally, doxapram hydrochloride (Dopram-V Injectable, AH Robins, Richmond, Va) was administered intravenously, start-
ing with an initial bolus of 20 mg, followed by 10 mg every five minutes. Rectal temperature was monitored before and after experiments. Urine samples were collected by free catch or urinary catheterization before, immediately after, and one hour after each experiment, and urinalysis was performed (N-Multistix, Ames Co., Miles Laboratories, Elkhart, Ind). A urine pH of 7.0 was a prerequisite for starting an experiment. Venous blood (3 ml) was collected in heparinized syringes before, during (depending on the type of experiment), and after each experiment. Venous blood pH was immediately measured (Blood pH Gas Analyzer 813, Instrumentation Laboratory, Lexington, Mass). Packed cell volumes (PCV) were determined using a microhematocrit centrifuge. Plasma hemoglobin was measured spectrophotometrically using Drabkin’s solution to convert hemoglobin to cyanmethemoglobin. This method allowed accurate measurements to 3 mg/dL hemoglobin and more in the plasma. During the exercise experiment, serum creatine kinase and potassium were measured at 0 (prior), 0.5, 1.5, 8, 14, and 20 hours.

RESULTS

In Vitro Alkaline Fragility of Erythrocytes From Dog 1

The findings on erythrocytes from dog 1 in saline, variably treated serum, and sucrose is shown in Table 2. In the acidified serum lysis test, erythrocytes from control animals showed little or no hemolysis during incubation with any type of serum. In contrast, the presence of both the patient’s (autologous) and control dogs’ (homologous) serum caused similarly enhanced lysis of dog 1 erythrocytes. Acidification with or without heat-inactivation of serum prevented the accelerated lysis of the patient’s erythrocytes. In the presence of isotonic sucrose, erythrocytes from dog 1 were fragile compared with erythrocytes from controls (Table 2). However, the sucrose lysis of the patient’s cells was significantly reduced at a pH 6.8 compared with a physiologic pH of 7.3 (P < .05). Furthermore, the sucrose lysis of both controls’ and patient’s erythrocytes was time dependent over 80 minutes but was not influenced by the presence or absence of serum (data not shown). No mechanical force, such as a shaking water bath, was used to induce this in vitro lysis.

To further assess the influence of pH on canine erythrocytes, an alkaline fragility test was performed. The large difference in the extent of hemolysis associated with a small difference in pH is apparent (Fig 2). When percent hemolysis was plotted linearly against increasing pH values, symmetrical, sigmoid curves were obtained from the patient and controls. Erythrocytes from dog 1 exhibited an increased fragility in alkaline media as indicated by a significant shift of the sigmoid curve to the left. Thus, erythrocytes from dog 1 started to lyse markedly at a pH of 7.5, whereas erythrocytes from controls lysed only at a pH of 7.7 or more, when incubated for one hour at 37 °C, as shown previously. In addition, the alkaline fragility of canine erythrocytes was time and temperature dependent (data not shown).

Table 2. Saline, Serum, and Sucrose Lysis of Dog 1’s and Canine Controls’ Erythrocytes In Vitro

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH Value of Medium</th>
<th>% Hemolysis</th>
<th>Dog 1</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6.8</td>
<td>5.3 ± 0.6 (3)</td>
<td>5.9 ± 0.6 (3)</td>
<td></td>
</tr>
<tr>
<td>Autologous serum</td>
<td>7.7</td>
<td>24.2 ± 6.5 (3)*</td>
<td>1.0 ± 0.8 (6)</td>
<td></td>
</tr>
<tr>
<td>Homologous serum</td>
<td>7.7</td>
<td>30.0 ± 11.8 (5)*</td>
<td>1.2 ± 0.7 (6)</td>
<td></td>
</tr>
<tr>
<td>Acidified serum</td>
<td>6.5</td>
<td>0.4 ± 0.8 (6)</td>
<td>0 (6)</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>7.3</td>
<td>65.9 ± 4.4 (7)*</td>
<td>2.7 ± 0.9 (7)</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>6.8</td>
<td>34.4 ± 6.5 (4)*</td>
<td>1.9 ± 1.0 (4)</td>
<td></td>
</tr>
</tbody>
</table>

Canine erythrocytes were incubated for one hour in various media in vitro and percentage of lysis was measured. The incubation temperature was 37 °C, except for the sucrose lysis test, which was performed at 22 °C. The data are expressed as mean ± SD, with the number in parentheses representing the number of determinations done in the patient and control animals.

* Indicates significant statistical difference of lysis between patient’s and controls’ erythrocytes by Student’s t test, P < .01.

In Vitro Alkaline Fragility of Erythrocytes From Dog 2

Erythrocytes from dog 2 were also markedly more fragile in alkaline media, as shown in Fig 3. In fact, at a physiologic pH of 7.4, there was already an extreme hemolysis, further suggesting an enhanced fragility of the patient’s erythrocytes after storage at 4 °C for one day.

Biochemical Studies of the Erythrocytes

Table 3 summarizes the biochemical studies of dog 1’s and dog 2’s erythrocytes. The erythrocyte PFK activity of both patients was severely reduced to...
approximately 10%, compared with normal canine erythrocyte activity. The activities of all other erythrocyte enzymes, such as pyruvate kinase and glucose-6-phosphate dehydrogenase, appeared to be normal or increased in dogs 1 and 2, presumably because of the marked reticulocytosis. A diminished PFK activity led to a decreased erythrocyte concentration of 2,3-DPG in dog 1. In addition, dog 1’s erythrocyte potassium and chloride contents were markedly higher than those of control dogs. The chloride concentration ratio (erythrocyte chloride to plasma chloride) was 1.03 in dog 1 of control dogs. The chloride concentration ratio (erythrocyte chloride to plasma chloride) was 1.03 in dog 1 compared with 0.85 ± 0.06 (mean ± SD) in controls.

**Hyperventilation Studies in Dog 1**

Exercise, placement of dog 1 in a 39 °C water bath, or intravenous infusion of a potent respiratory stimulant (doxapram hydrochloride) for 30 minutes immediately induced panting and hyperventilation. The duration of hyperventilation involved approximately 75% to 90% of the exposure time, and the extent of hyperventilation appeared to be similar in the patient and in the control dogs. Within a few minutes after terminating each experiment, the animals stopped panting, thus indicating a transient effect of the experimental stress. No muscle fatigue and only a mild and transient increase in creatine kinase to less than 300 IU/L was observed during the exercise experiment.

Hyperventilation, induced by all three means, caused a mild but significant increase in venous blood pH (Table 4). The increase in blood pH was transient and occurred to a similar extent in control animals and in dog 1.

Intravascular hemolysis was assessed by (a) hemoglobinemia, (b) a decrease in packed cell volume, (c) hemoglobinuria, and (d) bilirubinuria. Control dogs had plasma hemoglobin concentrations of less than 10 mg/dL before, during, and after the in vivo experiments, and no significant changes in the packed cell volume were observed (Fig 4). In addition, neither hemoglobinuria nor bilirubinuria were detected during or after the experiment.

In contrast, the patient’s initial plasma hemoglobin levels were slightly elevated, comparable to the steady-state plasma concentrations of 11 to 21 mg/dL (range) observed during the entire time of hospitalization (Fig 4). Hyperventilation, induced by the various experiments, caused increases in plasma hemoglobin concentration of twofold to threefold over the patient’s baseline values. Concomitantly, a 4% to 5% decrease in PCV was noted, eg, a PCV of 34% decreased to 29% and 30%. In addition, serum potassium levels transiently rose to 8.1 mEq/L in dog 1 during the exercise experiment. The patient’s urine was persistently strongly positive for bilirubin, but the different intensities of brown color suggested an increase in bilirubin concentration after experimental challenges (Fig 1).

**DISCUSSION**

The present investigation describes a presumably inherited erythrocyte PFK deficiency in two male English springer spaniel dogs that is characterized (a) clinically by a chronic compensated hemolytic anemia with hyperventilation-induced hemolytic crises and (b) experimentally by an increased in vitro and in vivo alkaline fragility of erythrocytes. PFK is a multimeric protein with a tetrameric acting as the smallest functional unit that has extensively been studied in man and rabbit. In man, muscle PFK contains only muscle-type subunits, whereas erythrocyte PFK consists of muscle-type and liver-type subunits in approximately equal amounts, forming five isozymes. Muscle PFK from 1 vertebrate species, including man and dog, exhibited a strong cross-reactivity with a rabbit antihuman muscle-type antibody, indicating the highly conserved nature of the vertebrate muscle PFK. In addition, one monoclonal antibody against human liver-type subunits (V44-08) also precipitated 90% of liver PFK activity of dogs, whereas others did not cross-react. These indirect
30
MINUTES

PHOSPHOFRUCTOKINASE DEFICIENCY IN DOGS

Table 4. Effect of Hyperventilation on Venous Blood pH in Dog 1 and Controls

<table>
<thead>
<tr>
<th></th>
<th>Venous Blood pH at</th>
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<tbody>
<tr>
<td></td>
<td>0 Minutes</td>
<td>15 Minutes</td>
<td>30 Minutes</td>
<td>90 Minutes</td>
</tr>
<tr>
<td>Exercise</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 1</td>
<td>7.380</td>
<td>ND</td>
<td>7.428</td>
<td>7.408</td>
</tr>
<tr>
<td>Controls</td>
<td>7.371 ± .022</td>
<td>ND</td>
<td>7.435 ± .023*</td>
<td>7.393 ± .008</td>
</tr>
<tr>
<td>Water bath</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 1</td>
<td>7.378</td>
<td>7.419</td>
<td>7.438</td>
<td>7.402</td>
</tr>
<tr>
<td>Controls</td>
<td>7.364 ± .050</td>
<td>7.438 ± .009*</td>
<td>7.443 ± .019*</td>
<td>7.386 ± .010</td>
</tr>
<tr>
<td>Doxapram IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 1</td>
<td>7.378</td>
<td>7.445</td>
<td>7.438</td>
<td>7.401</td>
</tr>
<tr>
<td>Controls</td>
<td>7.384 ± .013</td>
<td>7.431 ± .018*</td>
<td>7.428 ± .015*</td>
<td>7.393 ± .009</td>
</tr>
</tbody>
</table>

Venous blood samples were collected before (0 minutes), during (15 minutes, 30 minutes), and one hour after each experiment (90 minutes). Controls: mean ± SD, N = 3 to 5; *P < .01 compared with presample (0 minutes) and postsample (90 minutes); ND, not done.

Less than 30 human patients with PFK deficiency have been reported. Most patients show a complete lack of muscle PFK activity and partial reduction (approximately 50%) in erythrocyte PFK activity (glycogen storage disease type VII, Tarui syndrome) owing to a deficiency or a catalytically inactive mutant of muscle-type subunit of PFK. In addition, labile variants of muscle-type subunits, as well as catalytically inactive and labile mutants of liver-type subunits, have also been shown to cause markedly decreased erythrocyte PFK activity. The erythrocyte enzyme activity in the two dogs described here was particularly low (approximately 10% of normal); however, the molecular basis of this PFK deficiency has not yet been elucidated. Because both affected dogs were of the English springer spaniel breed and had a life-long history of pigmenturia, an inherited defect, rather than an acquired disorder of erythrocyte PFK, is considered likely.

The heterogeneity of the molecular lesions in inherited PFK deficiency in man may explain the variety of clinical manifestations, including signs of hemolysis or myopathy of varying severity as well as asymptomatic states. The two dogs with erythrocyte PFK deficiency had almost identical hematologic findings and suffered from a chronic, partially compensated hemolytic anemia similar to the clinical features described in man. The marked reticulocytosis despite mild anemia may be explained by the metabolic block in the erythrocyte glycolysis at the level of PFK, resulting in decreased concentrations of 2,3-DPG, as shown in this study and in human studies. Reduced 2,3-DPG levels are associated with increased hemoglobin-oxygen affinity and compensatory accelerated erythrocyte production.

The PFK-deficient erythrocytes from both dogs were much more fragile in alkaline media than canine erythrocytes from controls, as shown by several in vitro lysis tests. Erythrocytes from the canine species in general are known to be uniquely alkaline fragile, whereas erythrocytes from man and all studied animal species do not undergo hemolysis within two hours, even in media of pH values as high as 8.2. Parker suggested that the enhanced alkaline fragility of canine erythrocytes might be related to facilitated calcium entry under conditions of increased pH.

The pathogenesis of hemolysis in canine PFK deficiency was at least partially caused by increased erythrocyte pH. As mentioned, PFK-deficient erythrocytes have low levels of 2,3-DPG. Reduced concentrations of intracellular impermeant organic phosphate anions lead to increased erythrocyte chloride contents, as shown in dog 1. The ratio of cell chloride to plasma chloride content is equal to the hydroxyl concentration ratio and thereby determines the transmembrane pH gradient. Dog 1 had an increased chloride...
ratio, indicating that PFK-deficient erythrocytes contain higher hydroxyl concentrations and therefore have a more alkaline intracellular pH value than normal at any external pH, thereby favoring the alkaline lysis of canine erythrocytes. In the sucrose lysis test, in which the medium is depleted of chloride, the erythrocytes are more alkaline compared with the medium, and therefore, PFK-deficient erythrocytes show an increased fragility even at neutral or acidic pH values of the medium.

The observed in vitro alkaline fragility at physiologic pH values of the PFK-deficient erythrocytes contributed to the shortened red cell survival in vivo, as shown by various experiments mimicking physiologic situations. Hyperventilation induced by exercise, a warm water bath (39 °C), or infusion of a potent respiratory stimulant, doxapram, occurred readily in all dogs owing to their tendency to pant and regulate their body temperature by this means and resulted in a mild respiratory alkalosis. The mild increases in blood pH were sufficient to cause intravascular hemolytic crises in dog 1, as evidenced by hemoglobinemia and hemoglobinuria, a decrease in PCV, and increased bilirubinuria, whereas no hemolysis was observed in the control dogs. The experimentally induced hemolytic crises correlated well to the previously recognized “stress-precipitated” episodes in dog 1. Thus, the enhanced alkaline sensitivity of PFK-deficient erythrocytes appears to be important in relation to the acute hyperventilation-induced hemolysis and may also play a role in the observed chronic hemolytic anemia.

Interestingly, mature canine erythrocytes contain low potassium and high sodium concentrations owing to a lack of a membrane Na-K-ATPase, whereas erythropoietic stem cells have high potassium and low sodium levels. Reticulocytes with high potassium concentrations may have caused the elevated erythrocyte potassium content in dog 1. Consequently, lysis of erythrocytes with high potassium concentration may have contributed to the increased serum potassium levels, as seen in both dogs.

Muscle-type PFK deficiency can cause myopathy, characterized by elevated creatine kinase and myoglobinuria on exertion. Although there is a lack of muscle PFK activity, the clinical signs are generally mild, probably because muscle cells can generate energy by other pathways and have active protein synthesis. Dog 1 had mildly elevated resting serum creatine kinase activity and minimal transient increases of this enzyme during exertion. These findings may suggest muscle involvement, but neither myoglobinuria nor muscle fatigue was observed.

NOTE ADDED IN PROOF

While this manuscript was in press, our subsequent studies with Dr S. Vora at Scripps Clinic and Research Foundation have determined that dog 1 also has muscle PFK deficiency. Detailed findings will be described elsewhere.

ACKNOWLEDGMENT

We acknowledge Dr J.C. Parker, University of North Carolina, many clinicians, and the staff of the VMTH, University of Florida, and the owners of the PFK-deficient dogs for their kind cooperation and support.

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