

Hereditary Elliptocytosis With Protein Band 4.1 Deficiency in the Dog

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A dog with persistent elliptocytosis was studied. The dog had membrane protein band 4.1 deficiency, microcytosis, shortened erythrocyte lifespan, increased osmotic sensitivity, and a mild glutathione deficiency. Erythrocyte deformability and membrane stability were adversely effected.

The dog's parents had decreased band 4.1, decreased stability, and some elliptocytosis. This disorder in dogs closely resembles human patients with band 4.1 deficiency and should provide a valuable animal model to study the erythrocyte membrane cytoskeleton.

THE ERYTHROCYTE contains a cytoskeletal network under the membrane that modulates several membrane properties. Erythrocyte shape, membrane flexibility, structural integrity, and deformability seem to be the consequence of this cytoskeleton.¹ It is formed from the following membrane proteins: spectrin (bands 1 and 2), actin (band 5), ankyrin (band 2.1), and protein band 4.1.^{2,3} The two polypeptides of spectrin associate side-to-side as heterodimers. The spectrin dimers, which are long worm-like molecules, join head-to-head with other spectrin dimers to form a tetramer.^{4,5} One end of the spectrin tetramer is bound through ankyrin to an integral membrane protein, band 3.⁶ That links the membrane skeleton to the lipid bilayer. The other end of the spectrin is bound to actin.⁷ Protein band 4.1 modulates the spectrin-actin interaction.^{8,9}

Several abnormalities of the cytoskeleton occur in man. Because the erythrocyte has a limited repertoire of possible shapes, a particular shape may result from several primary defects. Hereditary elliptocytosis can result from protein band 4.1 deficiency^{10,11} or abnormal spectrin structure.¹² Some cases of hereditary spherocytosis result from an inadequate amount of spectrin;¹³ others from an abnormal spectrin that does not bind protein band 4.1.¹⁴ Some patients with hemolytic poikilocytic anemia have erythrocytes with an abnormal binding site for ankyrin;¹⁵ others with hereditary pyropoikilocytosis have an altered spectrin dimer-dimer association.¹⁶

Relatively few inherited abnormal erythrocyte shapes occur in animals. A spectrin deficiency in the common house mouse causes a recessively inherited hemolytic anemia.¹⁷ Four mutants with varying degrees of spectrin deficiency have anemias that correlates with the amount of residual spectrin.¹⁸ In this report, we describe a dog with elliptocytosis due to abnormality in protein band 4.1. This animal should provide another animal model for disorders involving the erythrocyte membrane cytoskeleton.

MATERIALS AND METHODS

Erythrocyte numbers, PCV, and hemoglobin were measured with a Coulter Counter, Model S, Sr (Coulter Electronics, Hialeah, Fla.).

Reticulocytes were counted after they had been stained with new methylene blue.¹⁹ Red cell sodium and potassium determinations were made on an IL 343 flame photometer (Instrumentation Laboratory, Watertown, Mass.). ⁵¹Cr-erythrocyte lifespan,²⁰ osmotic fragility,²¹ and erythrocyte enzymes²² were determined with standard methods. Serum iron and total iron-binding capacity were measured coulometrically;²³ erythrocyte water, gravimetrically; and reduced glutathione, as a 5,5'-thiobis-(2-nitrobenzoic acid) derivative.²⁴

Membranes were prepared from washed erythrocytes by hypotonic lysis with 5 mM sodium phosphate buffer, pH 8.0.²⁵ Proteolysis was minimized by removing leukocytes with cellulose²⁶ and by adding 1 mM phenylmethylsulfonyl fluoride to the phosphate buffer. In a few experiments, 1 mM diisopropylfluorophosphate was substituted for phenylmethylsulfonyl fluoride. Membrane proteins were separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). Studies were done with gels of 8% polyacrylamide.²⁷ After protein staining with Coomassie blue, the cylindrical gels were scanned with a Gilford Model 2520 gel scanner attached to a Gilford Model 2400 spectrophotometer. Individual peaks of chart paper were photocopied, cut separately, and weighed.

Erythrocyte deformability²⁸ and membrane fragmentation²⁹ were measured with an ektacytometer. The deformability index is a measure of the average cellular ellipticity when the erythrocytes or ghosts are subjected to a shear stress.²⁸ Fragmentation time is defined as the time required for the deformability index to decay to half of its maximum value when resealed ghosts are subjected to a high constant shear stress.²⁹

Blood samples were prepared for scanning electron microscopy by the method described by Bessis and Weed.³⁰ Erythrocytes were separated from white cells by passing them through cellulose²⁶ and from plasma by centrifugation. They were washed 3 times with isotonic saline. An aliquot (100 μ l) of washed erythrocytes was mixed with 0.5 ml of phosphate-buffered (0.01 M, pH 7.4) saline (PBS) that had been prewarmed at various temperatures (2 θ intervals from 37°C to 51°C). After incubating 10 min, 250 μ l of the solution was added to 1 ml of 1% glutaraldehyde-PBS and processed for scanning electron microscopy. The remaining solution was

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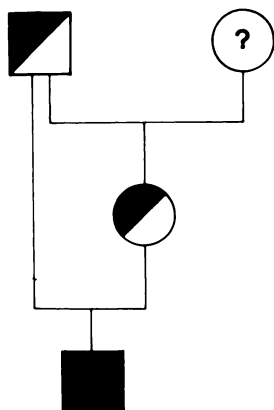


Fig. 1. Pedigree of the dog with canine hereditary elliptocytosis.

centrifuged, the supernatant removed, and plasma added. Blood smears from the plasma-heated erythrocyte suspension were examined by light microscopy.¹¹

CASE REPORT

The dog is a crossbred male and the offspring from a father-daughter mating (Fig. 1). He was seen initially because of emaciation secondary to malnutrition and chronic bacterial dermatitis. The first hemogram revealed an anemia (PCV, 32%) and elliptocytosis. He responded dramatically to adequate nutrition and antibiotic therapy, but elliptocytosis persisted. Only limited studies could be made on the parents.

RESULTS

The proband has a well compensated hemolytic disease. The PCV, erythrocyte number, and hemoglobin became normal, but the reticulocyte number remained abnormally high (Table 1). The ⁵¹Cr-labeled erythrocyte lifespan (16.4 days) is shorter than that of normal dogs (24.6 ± 0.8).³² Serum iron, total iron-binding capacity, and erythrocyte Na⁺, K⁺, and water were normal. Erythrocyte enzyme activity reflected the shortened mean cell age. Hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase, phosphofructokinase, and phosphoglycerate kinase were increased, but adenylate kinase, enolase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydroge-

nase, aldolase, diphosphoglycero-mutase, monophosphoglyceromutase, glutathione reductase, glutathione peroxidase, glucose phosphate isomerase, and 6-phosphogluconate dehydrogenase were not different from normal canine erythrocytes. Erythrocyte glutathione (GSH), which usually is increased with shortened erythrocyte life span, was unexpectedly lower than normal (Table 1).

When blood smears from the proband were examined by light microscopy or scanning electron microscope, numerous elliptocytes were seen (Fig. 2). Fragmented erythrocytes, budding cells, and spherocytes were also seen. The mean cell volume (MCV) was smaller than normal (Table 1). An increased sensitivity to hypotonic lysis (Fig. 3) reflected an altered surface-to-volume ratio.

The proband's elliptocytes had normal thermostability. Fragmentation began at 51°C for both proband and control cells.

SDS-PAGE patterns of the proband, parents, and normal canine erythrocyte membrane are shown in Fig. 4. Band 4.1 was absent in membranes from the proband. The parents had decreased levels of band 4.1 when compared to normal erythrocytes, with band 3 as a reference (Table 2).

The deformability index of the proband was 84% of that observed in normal erythrocytes. His membranes fragmented at a faster rate than normal membranes when they were subjected to 600 dyne/sq cm shear stress (Fig. 5). Fragmentation time for 4 normal canine ghosts was 132 sec (SD = 20.5) and for elliptocytic ghosts was 31 sec.

DISCUSSION

Canine elliptocytosis with protein band 4.1 deficiency is similar to the human counterpart.¹¹ Both are inherited as autosomal recessive traits with heterozygotes having about 50% band 4.1. Both have altered erythrocyte morphology, including elliptocytosis, membrane fragmentation, and poikilocytosis. Neither has increased the thermal fragmentation characteristic

Table 1. Hematologic Investigations

Investigation	Unit	Control Mean ± 1 SD (n = 6)	Sire	Dam	Proband
RBC	× 10 ⁶ μl	6.91 ± .74	6.80	7.14	8.13
PCV	%	44.6 ± 4.33	42.0	46.8	42.7
Hb	g/dl	16.0 ± 1.68	15.2	16.6	15.4
MCV	f1	65.3 ± 2.16	62.0	66.0	53*
MCHC	%	35.5 ± .85	35.9	35.1	35.7
MCH	pg	23.4 ± 1.16	22.5	23.4	19.0*
Reticulocyte	%	1.3 ± .34	1.3	0.7	3.0*
GSH	μmole/g Hb	7.89 ± 0.550	7.88	7.49	6.38*

*Significant difference from normal ($p < 0.05$).

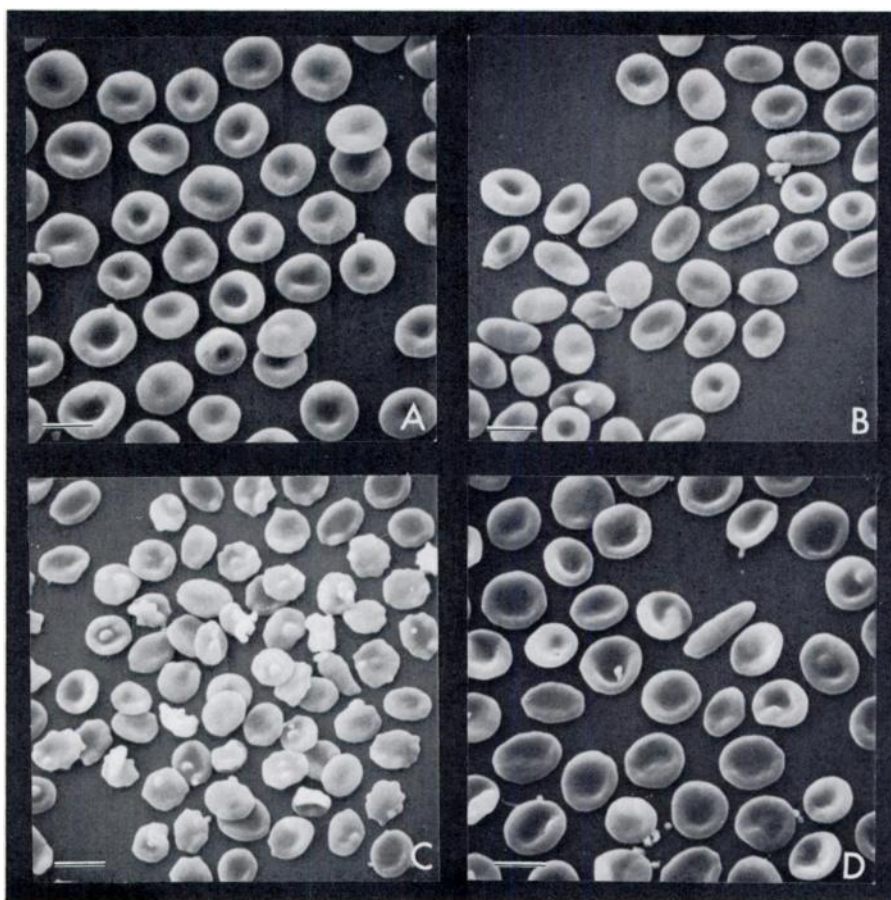


Fig. 2. Scanning electron micrographs of erythrocytes from a control (A), the proband with canine hereditary elliptocytosis (B), his dam (C), and his sire (D). Bar is equal to 5 μ m.

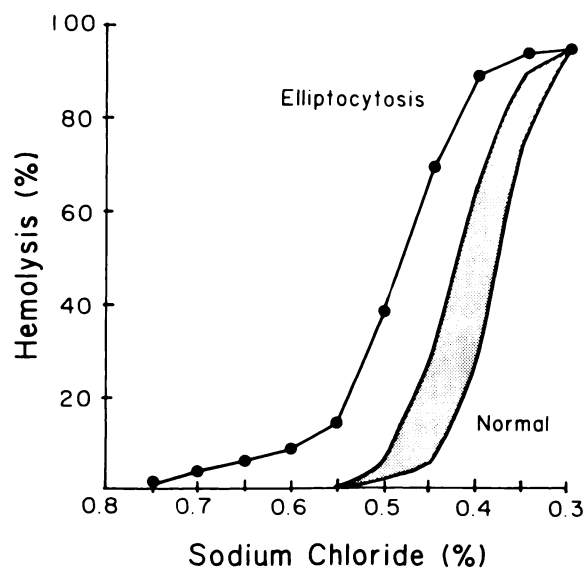


Fig. 3. Osmotic fragility of the proband and control ($n = 4$) erythrocytes.

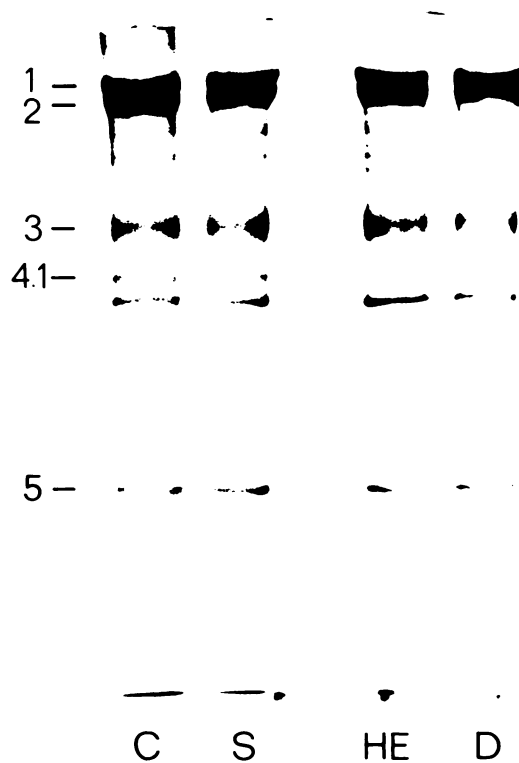


Fig. 4. Electrophoretic patterns of erythrocyte membranes from a control (C), hereditary elliptocytosis (HE), his dam (D), and his sire (S).

Table 2. Comparison of Erythrocyte Membrane Protein Band 4.1 Levels

	Band 4.1/Band 3
Proband	0
Sire	8.7%
Dam	11.2%
Controls (6)	21.3% ± 3.43% (SD)

of pyropoikilocytosis. Their erythrocytes are smaller, have an increased osmotic fragility, and are less deformable than normal erythrocytes of the respective species.

Mechanical instability of erythrocyte membrane, seen in both species, emphasizes the importance of band 4.1 in maintaining erythrocyte integrity. Loss of band 4.1's modulating influence on the spectrin-actin interaction must be important in resisting the fluid stresses of microcirculation.

Band 4.1 deficiency in the dog differs in some respects from the human disorder. In human patients the anemia is severe before splenectomy. Patients require multiple transfusions because their erythrocyte lifespan is too short to be adequately compensated by increased erythropoiesis. After splenectomy, their anemia becomes well compensated, with hemoglobin concentrations nearly normal. Although we do not know the degree of anemia during the growing period in canine elliptocytosis, it must not have been life-threatening.

The erythrocyte morphology of heterozygotes is less striking than that seen in the human counterpart. The mechanism for that difference is unexplained.

The marginally low glutathione level is probably secondary. Dogs with shortened erythrocyte lifespan and reticulocytosis usually increase the erythrocyte glutathione. Explanation of the low glutathione is not apparent.

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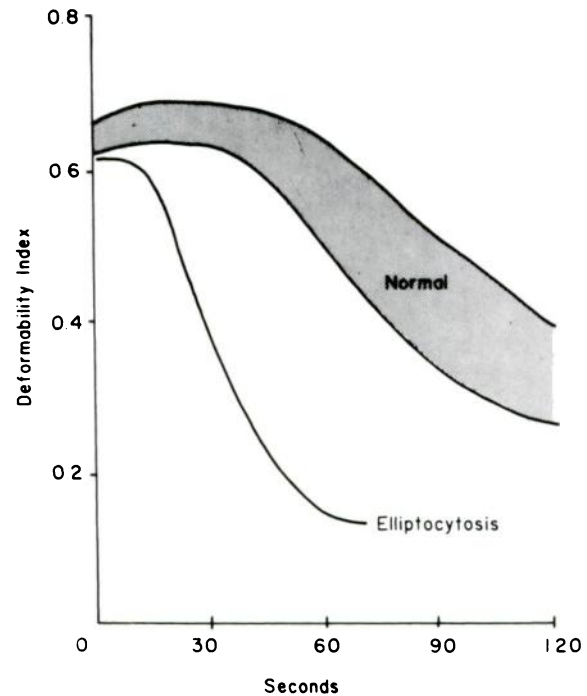


Fig. 5. Fragmentation curves for resealed pink ghosts from the proband with canine hereditary elliptocytosis and control dogs ($n = 4$).

Canine elliptocytosis with protein band 4.1 deficiency provides another animal model for studying the relationship of erythrocyte membrane proteins to cellular integrity.

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