ERYTHROCYTE MEMBRANE CHARACTERISTICS OF THE HEREDITARY STOMATOCYTOSIS SYNDROME IN THE ALASKAN MALAMUTE

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ABSTRACT

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LITERATURE REVIEW

The erythrocyte is the most popular target of membrane research. The advantages it offers are its availability, ease of separation from other blood components with minimal contamination, relative homogeneity with no subcellular organelles or nucleus, and a basic structure generally representative of most cell membranes. [47, 49] The disadvantages of using erythrocyte membranes to extrapolate information to other membrane systems are that glycoproteins differ among membrane types, the erythrocyte membrane has higher amounts of sialic acid than most cells, and there are no cytoplasmic organelles or nucleus involved with erythrocyte transmembrane control, as there are with other cells.[12] Most membrane studies use human erythrocytes, however, anomalous red cells from other species have been suggested as animal models for the similar dyscrasias in man.[9]

Scanning electron microscopy (SEM), polyacrylamide gel electrophoresis (PAGE), protein phosphorylation measurement, selective protein extractability and ektacytometry are useful techniques for making specific conclusions concerning membrane structure and function. Through these methods the composition of membranes is being identified, the location of subunits specified, the effects of various techniques more clearly pictured and the functional roles of membrane components made more understandable.

The erythrocyte is a very dense solution of hemoglobin. Normal erythrocyte oncotic pressure is maintained by the hemoglobin and relatively impermeable organophosphates. Hemoglobin does not significantly alter the properties of intracellular water or anions. While not a component of the erythrocyte membrane, hemoglobin is critical to cell fluidity, rigidity and blood viscosity.

Properties of cells are related to membrane organization. The major

components of the erythrocyte membrane are protein (50%) and lipid (40%), with carbohydrate comprising the remainder.[47] In the human and canine erythrocyte membranes there are eight major bands identified by molecular weight by PAGE that account for over 2/3 of total membrane protein.[8] Reports of several investigators have substantiated that differences among the major protein bands of man and dogs are not significant.[19,21] Membrane proteins are divided into 2 groups, extrinsic and intrinsic, according to the relative ease with which they are eluted from red cell membranes. Bands I, II, IV.1, IV.2, V and VI are extrinsic proteins, make up 40-50% of total membrane protein and are extracted with low ionic strength agents. They are found at the cytoplasmic surface. Bands I and II have been named spectrin,[8] band V is an actin and band VI is glyceraldehyde-3-phosphate dehydrogenase, an enzyme in the erythrocyte glycolytic pathway.[11]

Intrinsic proteins are eluted from red cell membranes with lipid solvents and are collectively reciprocal to the extrinsic proteins. They include bands III, VII and all the glycoproteins which form numerous minor bands. The glycoproteins are all located at the exterior of the membrane where they provide receptor sites for antibodies and enzyme control.[31,33] The two primary intrinsic proteins, band III and periodic acid Schiff-1 (PAS-1), penetrate the width of the membrane and are involved in transmembrane control.[23] Band VI and IV.2 are bound to band III and elute with the intrinsic and extrinsic proteins. The roles of bands IV.2 and VII are poorly understood, however, it is known that they are asymmetrically located at the cytoplasmic side of the membrane and that band VII is resistant to disruption from its site by either low ionic or lipid-solubilizing agents, perhaps because of strong ionic and lipid association.[51]

Spectrin (bands I and II) and its associated membrane component actin

(band V) and glycophorin (band III) have received increased attention over the last several years.[17] The name "spectrin" comes from the Greek word "spectros" which means ghost.[27] It is applied to a protein component in high concentration (25-35% of membrane protein) derived from the erythrocyte ghost membrane. It polymerizes in the presence of divalent cations to form coiled filaments visible by electron microscopy.

In 1970 the properties of spectrin were poorly understood.[23,26] One group of investigators extracted spectrin and stated that is was a dimer of molecular weight of approximately 460,000, made up of two monomeric species with respective molecular weights of 220,000 and 240,000. They noted that the monomeric forms may be made up of subunits not easily disrupted by ordinary separation methods.[37] The other group solubilized and purified by gel filtration a monomeric protein of molecular weight 140,000-150,000.[23] A comparative study in 1970 revealed that the percent of spectrin in man, guinea pig, horse, sheep and rabbit is consistent, and the amino acid composition and electrophoretic patterns of spectrin are nearly identical.[23] A study published in 1971 supported the 220,00-240,000 molecular weight determinations and referred to the 2 monomeric components of spectrin as the alpha and alpha' parts of "Tektin A."[3] Attempts to fractionate the alpha and alpha' bands were unsuccessful. It was concluded that spectrin is highly hydrophobic, aggregates whenever a small fraction of its charged groups are blocked and has sites for tight binding of manovalent and, especially, divalent cations.

The location of spectrin at the inner surface of the human red cell membrane was defined in 1971.[23] A study of thermal effects at pH 7.4 on human erythrocyte ghosts in 1973 showed four well-defined thermal transitions, each of which was irreversible and represented the denaturing of protein.[16] Erythrocytes were shown to sphere suddenly when heated at 48-50 C, corresponding

to the first thermal transition peak observed and the temperature at which spectrin alone denatures. In 1974 all groups agreed that the molecular weight of spectrin appears in the 200,000 range.[25] Studies in 1976 with deoxycholate, a disaggregator of spectrin, concluded that specrin has only 2 polypeptide chains, A and B, neither of which is dominant in number, and that its hydrodynamic properties in deoxycholate are very similar to those of muscle myosin.[43]

Band V is identical to muscle actin in molecular weight, net charge and ability to polymerize into filaments. It solubilizes with spectrin in the presence of 0.1 mM ethylenediaminetetraacetic acid (EDTA). Erythrocyte actin and spectrin associate to form an anastomosing network at the inside of the membrane that imparts flexibility to the erythrocyte.[49]

As knowledge of cell membranes has increased the once-labeled passive role of lipid in membranes has changed.[45] Lipid and protein are in equal amounts by weight in the membrane. Phospholipids and cholesterol make up 95% of membrane lipid and are in a molar cholesterol:phospholipid ratio of 0.8-1.0:1.0.[5,45,46] The distribution of the phospholipids is asymmetrical.[41] The aminophosphatides, phosphatidyl ethanolamine and phosphatidyl serine are primarily on the cytoplasmic half of the lipid bilayer, and the choline derivatives, phoshatidyl choline and sphingomyelin, are primarily on the external half of the lipid bilayer. No definite biologic explanation for asymmetric lipid distribution has been offered.

Phosphatidyl choline and sphingomyelin make up more than 90% of plasma phospholipid.[5] The slightly different structure of sphingomyelin is less conducive to many exchange and renewal reactions, leaving the majority of plasma-membrane phospholipid exchange to phosphatidyl choline.[5]

Cholesterol is the other major lipid membrane component, and membrane

flexibility is inversely proportional to the amount of cholesterol present.[5] Cholesterol increases the efficiency of packing of certain phospholipids in artifical membranes. Also, the interaction of membrane cholesterol and phospholipids may result in decreased mobility of the bilipid leaflet nearest to the surface, but it is thought that in the deeper hydrophobic region fluidity is increased. Plasma cholesterol is in 2 forms, esterified and unesterified. Membrane cholesterol is in passive equilibrium with unesterified plasma cholesterol, the levels of which are determined by the activity of the plasma enzyme, lecithin-cholesterol acyl transferase, on esterified plasma cholesterol.[45]

The carbohydrate component of the erythrocyte membrane has been referred to as the "glycocalyx" or "cell coat" because, despite comprising less than 10% of the total membrane mass, its exterior position can be identified through special stains. Membrane carbohydrate is located exclusively at the exterior of the cell surface combined in the form of glycoprotein and glycolipid.[26, 51] Membrane carbohydrate may be critical to membrane structure because the carbohydrate serves to anchor membrane protein into position. In addition the oligosaccharide side-chains of the carbohydrate are complex and are likely to serve as points of attachment or as regulating sites for cell function.

The major glycoprotein of the human erythrocyte membrane, glycophorin, is a PAS positive, water soluble component. This glycoprotein has a molecular weight of 50,000 and is 60% carbohydrate and 40% protein.[24] Its terminal end, the part that bears the carbohydrate, is located at the outer surface of the membrane with the protein towards the inner surface. Glycophorin carries some of the blood group antigens, viral receptor sites and most of the sialic acid of the erythrocyte.[29] It provides a convenient method for distinguishing

the exterior from the interior membrane surface. Glycoproteins of erythrocyte membranes are completely different from those of hepatocyte membranes or renal brush border membranes.[12]

Normal Functioning of the Erythrocyte Membrane.

In order for the erythrocyte to fulfill its various functions it must maintain the flexibility to withstand the stresses of the microcirculation and provide for the exchange of oxygen and carbon dioxide, monovalent and divalent cations and the transport of glucose. In dogs and man, normal erythrocytes are biconcave discs. As they swell their volume increases without a surface area increase until a spherical shape is reached, at which time, hemolysis occurs.[42]

The deformability of erythrocytes is affected by several factors. Any decrease or increase in cell volume alters the normal optimum ratio of surface area to cell volume, thus altering the deformability. Normal human erythrocytes can swell to 175% of their original volume before lysing. Tests with the human erythrocyte have shown that the membrane will deform upon the application of only 4 mm negative water pressure, while normal tissue culture cells require 20-50 mm negative water pressure. A drop in pH from 7.4 to 6.8 increases cell rigidity two-fold and a decrease in oxygen tension to values below 25 mm Hg increases cell rigidity. Fourthly, adenosine triphosphate (ATP) is essential to maintenance of the biconcave shape.[20] ATP increases in response to increases in intracellular calcium and prevents calcium from binding to the membrane and decreasing cell deformability.[42]

Cations and anions do not traverse the erythrocyte membrane by the same route.[30] The membrane is relatively permeable to water and anions, but impermeable to cations. Passage of bicarbonate is a passive process and

facilitates intracellular exchange of oxygen and carbon dioxide.[42] Cations assume a regulatory role for cell volume at a relatively low energy output. The precise mechanism of cation control is unknown. It may be that the route is through effects on sulfhydryl groups or through localized cation specific structural changes of membrane lipid protein complexes.[30]

The sodium and potassium ion content of canine erythrocytes is nearly the reverse of that of man.[1] In the high potassium erythrocytes of man, active sodium-potassium ion transport across the cell membrane is part of a tightly linked "pump" system that is activated when sodium ion levels rise above 8 meq/l.[39] ATP-ase is an integral component of this system in intact human erythrocytes and only ATP can be utilized.[39] This "pump" is inhibited by ouabain or strophanthin. In mature canine erythrocytes no "pump" or ATP-ase exists.[34] When erythrocytes were removed from dogs and incubated in supranormal quantities of sodium ion and reinjected into the dogs, the cells corrected their status over a 24-48 hour period. The mechanism of correction is unknown, but does demonstrate an in vivo ability. When canine red cells are shrunken osmotically, membrane permeability to sodium is increased and to potassium is decreased, and, when swollen, the permeabilities are reversed.[35] Sodium ion flux across the canine erythrocyte membrane increases in the presence of ATP.[35] ATP has stereospecific sites on the membrane and its effect is only on cations. It does not alter membrane shape, however, a parallel study of ATP on ascites tumor cells suggested that alteration of membrane contractile protein may be the effect of ATP activity.[15] Erythrocytes incubated in ATP and sucrose, an osmotic shrinking agent, showed a sodium flux nearly equivalent to the sum of the individual fluxes.[6,7] Consequently the routes of sodium transport in the presence of ATP and sucrose may be independent and parallel.

The absence of glucose from an incubating medium results in rapid erythrocyte swelling and lysis.[34] Glucose does not require the presence of insulin to enter the erythrocyte and, when presented to a glucose-deficient cell, it traverses the membrane more rapidly than a simple diffusion process would account for.[18] It appears that a carrier-mediated transport system is involved. Stereospecific recognition of the glucose molecule takes place, accomodates the glucose, and either the glucose molecule crosses alone or the carrier-glucose complex shifts glucose to the interior and the carrier returns to the exterior. Chemical identification of such a carrier is yet to be accomplished.

Hereditary Stomatocytosis.

In man stomatocytes are cup-shaped erythrocytes seen in certain hemolytic anemias.[2,22,28,29,31,53,54] In air-dried films the central palor is elongated and appears as a slit. Stomatocytes can be induced from normal erythrocytes in vitro by decreasing pH. In addition, some cationic compounds can intercalate with the lipid of the interior half of the bilayer forming stomatocytes.[44]

Hereditary stomatocytosis was first described in man in 1961.[22] Additional reports have confirmed it role in some human hereditary hemolytic disorders.[22,28,29,31,53,54] The consistent findings in man are abnormal erythrocyte osmotic fragility, the presence of stomatocytes in peripheral blood and shortened red cell survival.[22,28,29,31,53,54] The inconsistent findings in man, in order of decreasing frequency, are an absolute decrease of hemoglobin,[28,29,31,53,54] increased monovalent cation permeability,[28,29,31,53,54] reticulocytosis,[28,29,53,54] increased cation "pump" activity (from 2 to 30 times normal),[28,29,53,54] normal glycolytic intermediates,[28,31] splenomegaly,

[28,53,54] decreased severity of anemia following splenectomy,[28,54] normal lipid in membranes,[28,31] increased lipid primarily in the form of phosphatidyl choline,[53] decreased mean corpuscular hemoglobin concentration (MCHC),[28,31,54] increased MCHC,[53] normal glutathione,[22,31] increased glutathione,[53] increased mean corpuscular volume (MCV),[28,29,31, 54] and decreased spectrin phosphorylation to 20% of normal.[28] Decreased protein kinase activity has also been reported in patients with hereditary spherocytosis[13,50] and in patients with sickle cell disease.[14]

The variation of findings reflects the limited ability of the red cell membrane to manifest alteration to an insult, resulting in a heterogeneous assortment of anomalies all named stomatocytosis. Splenectomy usually improves the anemia of stomatocytosis as the decreased oxygen tension and lowered pH of the splenic environment results in sequestration of stomatocytes.

Hereditary stomatocytosis in the Alaskan Malamute was first described in 1972,[37] and the erythrocyte syndrome has been proposed as an animal model of human disease.[9] The stomatocytosis syndrome in the Malamute accompanies chondrodysplasia.[10,38,48] The blood picture is very similar to many reported cases in humans with stomatocytosis: decreased hemoglobin, low MCHC, high MCV, reticulocytosis, altered osmotic fragility and shortened red cell life span.[38] In the Malamutes the glycolytic intermediates are normal, glutathione is deficient and the anemia is not improved by splenectomy in one patient.[38] Stomatocytes are present in peripheral smears (1-4%), a considerably smaller percentage than usually seen in man (up to 75%).[28]

The primary goal of hematologic research with Alaskan Malamutes is to determine the cause of the red cell anomaly. Secondly, a test to differentiate heterozygous from normal dogs would solve the most severe problem facing the breed today.

REFERENCES

1. Bernstein, R E: Potassium and Sodium Balance in Mammalian Red Cells. Science, 120, (1954): 459-460.

2. Bessis, M, Weed, R I, and LeBlond, P F: Red Cell Shape. Springer-Verlag, New York, (1973): 3-9.

3. Clarke, M: Isolation and Characterization of a Water Soluble Protein from Bovine Erythrocyte Membranes. Biochem Biophys Res Comm, 45, (1971): 1063-1070.

4. Conn, E E, and Stumpf, P K: Outines of Biochemistry. John Wiley and Son, New York, (1972): 59-63.

5. Cooper, R D: Abnormalities of Cell Membrane Fluidity in the Pathogenesis of Disease. New Engl J Med, 297, (1977): 371-377.

6. Elford, B C: Independent Routes for Na^+ Transport Across Red Cell Membranes. Nature, 256, (1975): 580-582.

7. Elford, B C, and Solomon, A K: Factors Influencing Na^+ Transport in Dog Red Cells. Biochem Biophys Acta, 373, (1974): 253-264.

8. Fairbanks, G, Steck, T L, and Wallach, D F H: Electrophoretic Analysis of the Major Polypeptides of the Human Erythrocyte Membrane. Biochem, 10, (1973): 2606-2617.

9. Fletch, S M, and Pinkerton, P H: Congenital Hemolytic Anemia: Animal Model of Human Disease. Am J Path, 71, (1973): 477-480.

10. Fletch, S M, Smart, M E, Pennock, P W, and Subden, R E: Clinical and Pathologic Features of Chondrodysplasia(Dwarfism) in the Alaskan Malamute. J Am Vet Med Assoc, 162, (1973): 357-361.

11. Girotti, A W: Glyceraldehyde-3-Phosphate Dehydrogenase in the Isolated Human Erythrocyte Membrane: Selective Displacement by Bilirubin. Arch Biochem Biophys, 173, (1976): 210.

12. Glossman, H, and Neville, D M, Jr: Glycoproteins of Cell Surfaces. J Biol Chem, 246, (1976): 6339-6346.

13. Greenquist, A C, and Shohet, S B: Phosphorylation in Erythrocyte Membranes from Abnormally Shaped Cells. Blood, 46, (1976): 877-886.

14. Hayashi, R J, Koomoto, R, Yano, A, Ishigami, S, Tsujino, G, Saeki, S, and Tanaka, T: Abnormality in a Specific Protein of the Erythrocyte Membrane in Hereditary Spherocytosis. Biochem Biophys Res Comm, 57, (1974): 1038-1044.

15. Hempling, H G, Stewart, C C, and Gasic, G: The Effects of Exogenous ATP on the Electrolyte Content of TA_3 Tumor Cells. J Cell Physiol, 73, (1969): 133-140.

16. Jackson, W M, Kostyla, J, Nordin, J H, and Brandts, J F: Calorimetric Study of Protein Transitions in Human Erythrocyte Ghosts. Biochem, 12, (1973): 3362-3667.

17. Jacob, H S: Tightening Red Cell Membranes. New Engl J Med, 294, (1976): 22.

18. Jung, C Y: Carrier-Mediated Glucose Transport. In The Red Blood Cell. Edited by D M Surgenor. Academic Press, New York (1975): 706-708.

19. Kobylka, D, Khetry, A, Shin, B C, and Carraway, K L: Proteins and Glycoproteins of the Erythrocyte Membrane. Arch Biochem Biophys, 148, (1972): 457-487.

20. LaCelle, P L: Alteration of Membrane Deformability in Hemolytic Anemias. Semin Hematol, 7, (1970): 355-371.

21. Lenard, J: Protein Components of Erythrocyte Membranes from Different Animal Species. Biochem, 9, (1970): 5037-5040.

22. Lock, S P, Smith, R S, and Hardisty, R M: Stomatocytosis: Hereditary Red Cell Anomaly Associated with Hemolytic Anemia. Biochem, 15, (1976): 303-314.

23. Marchesi, S L, Marchesi, V T, and Tillack, T W: Physical and Chemical Properties of a Protein from Red Cell Membranes. Biochem, 9, (1970): 50-57.

24. Marchesi, V T: Chemical Characterization and Surface Orientation of the Major Glycoproteins of the Human Erythrocyte Membrane. Proc Nat Acad Sci, USA, 69, (1972): 1445-1449.

25. Marchesi, V T: Isolation of Spectrin from Erythrocyte Membranes. Meth in Enzymol, 32B, (1974): 275-277.

26. Marchesi, V T: Molecular Orientation of Proteins in Membranes. MTP International Rev Sci, Series One, Vol 2. Butterworths, London, (1975).

27. Marchesi, V T, and Steers, E, Jr: Selective Solubilization of a Protein Component of the Red Cell Membrane. Science, 159, (1968): 203-204.

28. Metzner, W C, Jr, Smith, W B, Goldstone, J, and Shohet, S B: Hereditary Stomatocytosis: Membrane and Metabolism Studies. Blood, 46, (1975): 659-669.

29. Miller, D R, Rickles, F R, Lichman, M A, LaCelle, P L, Bates, J and Weed, R I: A New Variant of Hemolytic Anemia with Stomatocytosis and Erythrocyte Cation Abnormality. Blood, 38, (1971): 184-204.

30. Nystrom, R A: Membrane Physiology, First Edition, Prentice Hall, Englewood, New Jersey (1973): 104-113.

31. Oski, F A, Naiman, J L, Blum, S F, Zarawshi, H S, Whaun, J, Shohet, S B, Green, A, and Nathan, D G: Congenital Hemolytic Anemia with High-Sodium, Low-Potassium Red Cells. New Engl J Med, 280, (1969): 909-916.

32. Parker, J C: Dog Red Blood Cells--Adjustment of Density in vivo. J Gen Physiol, 61, (1973): 146-157.

33. Parker, J C: Dog Red Blood Cells--Adjustment of Salt and Water Content in vitro. J Gen Physiol, 62, (1973): 147-156.

34. Parker, J C, and Hoffman, J F: Interdependence of Cation Permeability, Cell Volume and Metabolism in Dog Red Cells. Fed Proc, 24, (1965): 589.

35. Parker, J C, and Snow, R L: Influence of External ATP on Permeability and Metabolism of Dog Red Blood Cells. Am J Med, 223, (1972): 888-893.

36. Pinder, J C, Tidmarsh, S, and Gratzer, W E: Integrity of Polypeptide Chains of Spectrin from Erythrocytes. Arch Biochem Biophys, 172, (1976): 654-660.

37. Pinkerton, P H, and Fletch, S M: Inherited Hemolytic Anemia with Dwarfism. Blood, 40, (1972): 963.

38. Pinkerton, P H, Fletch, S M, Brueckner, P J, and Miller, D M: Hereditary Stomatocytosis with Hemolytic Anemia in the Dog. Blood, 44, (1974): 557-567.

39. Post, R L, and Jolly,P C: The Linkage of Sodium, Potassium and Ammonium Across the Erythrocyte Membrane. Biochim Biophys Acta, 25, (1957):118.

40. Post, R L, Merritt, C R, Kinsolving, C R, and Albright, C D: Membrane Adenosine Triphosphate as a Participant in the Transport of Sodium and Potassium in the Human Erythrocyte. J Biol Chem, 235, (1960): 1796-1802.

41. Rothman, J E, and Lenard, J: Membrane Asymmetry. Science, 105, (1977): 743-753.

42. Sachs, J R, Knauf, P A, and Dunham, P B: Transport Through Red Cell Membranes. In The Red Blood Cell. Vol I. Edited by D M Surgenor, Academic Press, New York (1975): 665-670.

43. Schecter, N M, Reynolds, J A, and Tanford, C: Erythrocyte Spectrin: Purification of Deoxycholate and Preliminary Characterization. Biochem, 15, (1976): 1897-1904.

44. Sheetz, M P, and Singer, S J: Biological Membranes as Bilayer Couples. A Molecular Mechanism of Drug Erythrocyte Interactions. Proc Nat Acad Sci, USA, 71, (1974): 4457-4461.

45. Shohet, S B: Hemolysis and Changes in Erythrocyte Membrane Lipids. New Engl J Med, 11, (1972): 577-583 and 12, (1972): 628-644.

46. Shohet, S B, and Ness, P M: Hemolytic Anemia: Failure of Red Cell Membranes. Med Clin N Amer, 60, (1976): 913-932.

47. Steck, T L: The Organization of Proteins in the Erythrocyte Membrane. J Cell Biol, 62, (1974): 1-19.

48. Subden, R E, Fletch, S M, Smart, M A, and Brown, R G: Genetics of the Alaskan Malamute Chondrodysplasia Syndrome. J Hered, 63, (1972): 149-152.

49. Tilney, L G: Actin in Erythrocyte Ghosts and its Association with Spectrin. J Cell Biol, 66, (1975): 508-520.

50. Yawata, Y, Koresawa, S, Yamada, O, and Shibada, S: Defective Membrane Phosphorylation in Red Cells of a Patient with Hereditary Spherocytosis. Acta Hem Jap, 38, (1975): 311-314.

51. Yu, J, Fishman, D A, and Steck, T L: Selective Solubilization of Proteins and Phospholipids from Red Blood Cell Membranes by Nonionic Detergents. J Supramol Structure, 1, (1973): 233-247.

52. Weed, R I: The Importance of Erythrocyte Deformity. Am J Med, 49, (1970): 147-150.

53. Wiley, J S, Ellory, J C, Shuman, M A, Shaller, C C, and Cooper, R A: Characteristics of the Membrane Defect in the Hereditary Stomatocytosis Syndrome. Blood, 46, (1975): 337-356.

54. Zarkowsky, H S, Oski, F A, Sha'afi, R, Shohet, S B, and Nathan, D G: Congenital Hemolytic Anemia with High Sodium, Low Potassium Red Cells. New Engl J Med, 278, (1968): 573-581.

PAPER 1. ERYTHROCYTE MEMBRANE CHARACTERISTICS OF THE HEREDITARY STOMATOCYTOSIS SYNDROME IN THE ALASKAN MALAMUTE

SUMMARY AND CONCLUSIONS

Erythroctye stomatocytosis in the Alasmkan Malamute occurs with chondrodysplasia as an autosomal recessive inherited condition (gene symbol: dan). Erythrocyte shape abnormalities suggested alterations in activity or absence of membrane components. Blood was collected from 10 adult dogs: 4 dan/dan Alaskan Malamutes, 3 dan/ + Alaskan Malamutes, and 3 + / + normal dogs, 2 of which were Alaskan Malamutes. Significant differences ($P<0.05$) between carriers and normals were found for hemoglobin and mean copuscular hemoglobin concentration (MCHC). Polyacrylamide gel electrophoresis (PAGE) was performed on isolated erythrocyte membranes and showed no quantitative differences in membrane proteins. Selective protein extractability tests with ethylenediaminetetraacetic acid (EDTA) failed to demonstrate significant differences among the 3 groups. Selective protein extractability tests with Triton-X demonstrated highly significant($P<0.01$) differences between carriers and normals. Protein kinase activity with ^{32}P-ATP on isolated membranes did not differ significantly among the 3 groups. Studies with an ektacytometer at 3 different forces detected no deformability differences among the 3 groups.

INTRODUCTION

The erythrocytes of hereditary stomatocytosis in the Alaskan Malamute are morphologically similar to those of hereditary stomatocytosis in humans. The condition has been proposed as an animal model of human disease.[9,23] The defect in Alaskan Malamutes is inherited as an autosomal recessive[14] and is accompanied by chondrodysplasia. Currently, test breeding is the only reliable method for identifying heterozygous Malamutes. The process is time consuming and the destruction of litters is emotionally and financially draining to owners and breeders.

Erythrocyte membrane shape abnormalities have often been associated with alterations in membrane composition or membrane protein phosphorylation. Four of 15 human patients with hereditary spherocytosis showed a complete absence of band IV.2.[11] Protein phosphorylation *in vitro* of the intact erythrocytes or prepared membranes provide similar results,[25] with band II incorporating most of the labeled phosphorus.[1] One patient with hereditary stomatocytosis had normal membrane protein but decreased spectrin kinase activity.[20] Protein kinase activity is also decreased in patients with hereditary spherocytosis.[10,28] Petients with sickle cell disease have decreased erythrocyte kinase activity[13] and alterations in the spectrin-actin lattice.[19]

Advancements in techniques allow investigators to focus on specific erythrocyte parameters of membrane analysis and metabolic requirement. Specifically, PAGE methods fractionate membrane components into 8 major bands that account for over 2/3 of membrane protein.[8] EDTA elutes loosely attached proteins extrinsic to the bilipid membrane core, Triton-X elutes those intrinsic proteins bound hydrophobically to the bilipid core, as well as those extrinsic proteins bound ionically to intrinsic proteins, and protein kinase activity measures membrane protein phosphorylation.[8,14,20,29]

Abnormally shaped cells are frequently less deformable. When these "rigid" cells reach the splenic environment, they are sequestered. In man, splenectomy decreases erythrocyte destruction and diminishes the anemia in many cases of hereditary stomatocytosis.[21,30]

This study investigated possible differences among 3 groups of Alaskan Malamutes: those that exhibit stomatocytosis, those that are heterozygous for the defect and a third group of normal dogs.

MATERIALS AND METHODS

Ten adult dogs were utilized during the research project. All, except one normal, were purebred Alaskan Malamutes of known ancestry. Blood was collected in sterile syringes containing EDTA as an anticoagulant. Total red cell counts were determined with an electronic particle counter,[a] hemoglobin as the cyanomethemoglobin derivative,[12] packed cell volume (PCV) by micro-hematocrit and glutathione (GSH) as the yellow 5,5' dithiobis (2-nitro-benzoic acid) anion.[4]

Polyacrylamide gel electrophoresis-- The procedure followed that of Fairbanks et al.,[8] except that 5% polyacrylamide gel was used rather than 5.6%, and final SDS concentration was 0.1% rather than 1.%. Whole blood was filtered through washed cotton balls to remove the leukocytes,[5] washed 3 times in 5mM phosphate buffered saline at pH 8, centrifuging each time at 3,000 x g for 4 min. Membranes were prepared by hypotonic lysis[7] using 5 mM phosphate buffer at pH 8. Membranes were spun at 30,000 x g for 10 minutes between each 10 minute lysing period and the procedure was repeated until they appeared free of hemoglobin, usually 3 times. Protein quantity was determined using bovine serum albumin as a standard.[18]

Membranes (40 µg /gel) for electrophoresis were placed in an incubation medium with 2% SDS and 100 mM dithiothreitol(DDT)[b]. Samples were run at 8 mAmp/gel.[8] Gels were stained with 0.05% Coomassie blue solution in a 60 C water bath for 2 hours and destained with 10% acetic acid. Observations for band difference were done visually and with a gel scanner[c] at 550 nm using standard nomenclature for band identification.[8]

Protein Extractability-- Fresh plasma membranes were diluted 1:10 with 0.1 mM EDTA (pH 8), sampled while incubating 15 minutes at 37 C, centrifuged

at 30,000 x g for 20 minutes and sampled again. Another group of fresh membranes was diluted 1:6 in 56 mM borate buffer (pH 8), sampled while incubating 20 minutes at 4 C, centrifuged at 30,000 x g for 20 minutes and sampled again.[29]

Protein quantity was determined using bovine serum albumin as a standard. Blank, standard and undiluted triplicate samples (0.02 ml) were placed in 1.05 % SDS (1.38 ml). Triplicate EDTA samples (0.12 ml) were placed in 1.431% SDS (0.28 ml), and triplicate Triton-X samples (0.05 ml) were placed in 1.143 % SDS (0.35 ml).

Protein Kinase--Spectrin kinase was assayed using casein as a substrate.[2] Membranes (25 µl) were incubated in 0.175 ml reaction mixture (14.5 ml of 100 mM Tris HCl pH 7.4, 0.5 M KCl, 25 mM $MgCl_2$ with 2.9 ml 1% casein). Gamma-^{32}P ATP was prepared enzymatically using phosphoglycerate kinase.[6] Samples were incubated at 37 C for 15 minutes. Gamma-^{32}P-ATP (0.2 mM, 50 µl) was added and the reaction stopped after 5 minutes with 1 ml 10% trichloroacetic acid (TCA). Samples were centrifuged at 900 x g for 10 minutes, the pellet washed twice with 2 ml TCA, once with 6 ml TCA and centrifuged at 900 x g for 5 minutes. Pellets were resuspended, dissolved in 0.4 ml 1 N NaOH and transferred to scintillation vials with 9.6 ml water. Activity was counted using Cerenkov radiation.[d]

Membrane Deformability-- Deformability studies were conducted with an ektacytometer.[3] Measurements of erythrocyte length and width were done at a resting state and three levels of stress: 100, 330, and 600 dynes/cm^2. Photographs were taken and measurements (in mm) made from the negatives.

Statistical Analysis-- Data collected was analyzed statistically by analysis of variance[e] among three groups of dogs and by the Student-Newman-Keuls test to locate differences.[24]

RESULTS

The erythrocyte parameter results (Table 1) showed significant differences ($P<0.05$)for red cell counts and GSH between dwarfs and normals, hemoglobin and GSH between dwarfs and carriers and hemoglobin and mean corpuscular hemoglobin concentration (MCHC) between carriers and normals. Highly significant differences ($P<0.01$) were found for hemoglobin, mean corpuscular volume (MCV) and MCHC between dwarfs and normals and MCV and MCHC between dwarfs and carriers. There were no highly significant differences ($P<0.01$) between carriers and normals for any of the parameters measured.

The bands formed during gel electrophoresis in the Malamutes correspond to those identified previously in dogs.[15,16] No differences in protein band position or quantity were visible grossly or following analysis of the electrophoretic patterns measured by the gel scanner.

The extractability results (Table 2) represent the percent of protein extracted by each procedure. The EDTA method extracted between 37.6% and 42.8% of membrane protein, while the Triton-X method extracted between 48.8% and 72.6% of membrane protein. The differences among the groups extracted with EDTA were not significant ($P>0.05$). The differences in the Triton-X extractions were significant ($P<0.05$) between dwarfs and normals and highly significant ($P<0.01$) between carriers and normals and carriers and dwarfs.

No significant differences among protein kinase values (Table 3) for the 3 groups were detected. In addition the results of the deformability studies (Table 4)showed all groups to be statistically similar.

DISCUSSION

The figures in Table 1 are generally consistent with those previously reported in 1972.[22] However, no differences were found between carriers and

normals for MCV ($P>0.05$), a value previously reported as highly significant.

A quantitative difference in protein is not a contributing factor to the stomatocytes of Alaskan Malamutes. This is the more common situation with protein content of stomatocytes in man.

The highly significant differences particularly between carriers and normals under the effects of Triton-X indicate decreased Triton-X extractability peculiar to the heterozygous Malamutes. Decreased extraction could indicate altered chemical composition resulting in decreased protein solubilization. As the quantity of protein is not different in membranes among the three groups then altered chemical binding within the membrane may be responsible for the decreased extractability.

The use of casein as a substrate only allows for evaluation of spectrin kinase activity. In the Malamutes, with normal spectrin levels, spectrin kinase activity values indicate similar protein phosphorylation. It is possible that alteration in actual site of spectrin phosphorylation may be discovered as different among the three groups.

The erythrocytes of dwarf Malamutes should measure smaller than those of the heterozygous and normal Malamutes, as their MCVs are larger. Dwarf erythrocyte deformability under stress should have been lower than the other two groups. In this trial no significant differences were observed in response to mechanical stress. It is possible that the population of stomatocytes (1-3%) in dwarf Malamutes is too low to detect a difference among the three groups. However, the MCV of erythrocytes from dwarf Malamutes is significantly elevated ($P<0.01$). Since these cells showed no reduced deformability the increased MCV is of minor importance to survival of the erythrocyte. Once the stomatocytic shape occurs then sequestration by the spleen may be enhanced.

REFERENCES

1. Avruch, J, and Fairbanks, G: Phosphorylation of Endogenous Substrates by Erythrocyte Membrane Protein Kinase. I. A Monovalent Cation-Stimulated Reaction. Biochem, 13, (1974): 5507-5514.

2. Avruch, J, Fairbanks, G, and Crapo, L M: Regulation of Plasma Membrane Protein Phosphorylation in Two Mammalian Cell Types. J Cell Physiol, 89, (1976): 815-826.

3. Bessis, M, and Mohandas, N: A Diffractometric Method for the Measurement of Cellular Deformability. Blood Cells, 1, (1975): 307-313.

4. Beutler, E: Drug-Induced Hemolytic Anemia. Pharmacol Rev, 21, (1969): 73-103.

5. Beutler, E: A Manual of Biochemical Methods. 2nd Edition. Grune and Stratton, New York (1975): 10.

6. Beutler, E, and Guinto, E: A Simple Rapid Technique for the Preparation of Gamma ^{32}P-Labeled Guanosine Triphosphate (GTP) and Adenosine Triphosphate (ATP). J Lab Clin Med, 88, (1976): 520-524.

7. Dodge, J T, Mitchell, C, and Hanahan, D J: Preparation and Chemical Characteristics of Hemoglobin-free Ghosts of Human Erythrocytes. Arch Biochem Biophys, 110, (1963): 119-130.

8. Fairbanks , G, Steck, T, and Wallach, D F H: Electrophoretic Analysis of the Major Polypeptides of the Human Erythrocyte Membrane. Biochem, 10, (1971): 2606-2617.

9. Fletch, S M, and Pinkerton, P H: Congenital Hemolytic Anemia. Am J Path, 71, (1973): 477-480.

10. Greenquist, A C, and Shohet, S B: Phosphorylation in Erythrocyte Membranes from Abnormally Shaped Cells. Blood 46, (1976): 877-886.

11. Hayashi, R J, Koomoto, R, Yano, A, Ishigami, S, Tsujino, G, Saeki, S, and Tanaka, T: Abnormality in a Specific Protein of the Erythrocyte Membrane in Hereditary Spherocytosis. Biochem Biophys Res Comm, 57, (1974): 1038-1044.

12. Henry, R J: Clinical Chemistry. 2nd Edition. Harper and Row, New York (1974): 1131-1135.

13. Hosey, M M: Altered Erythrocyte Membrane Phosphorylation in Sickle Cell Disease. Nature, 263, (1976): 424-425.

14. Hosey, M M, and Tao, M: An Analysis of the Autophosphorylation of Rabbit and Human Erythrocyte Membranes. Biochem, 15, (1976): 1561-1568.

15. Kobylka, D, Khetyr, A, Shin, B C, and Carraway, K L: Proteins and Glycoproteins of the Erythrocyte Membrane. Arch Biochem Biophys, 148, (1972): 457-487.

16. Lenard, J: Protein Components of Erythrocyte Membranes from Different Animal Species. Biochem, 9, (1970): 5037-5040.

17. Lock, S P, Smith, R S, and Hardisty, R M: Stomatocytosis: Hereditary Red Cell Anomaly Associated with Hemolytic Anemia. Brit J Hematol, 7, (1961): 303-314.

18. Lowry, O H, Roberts, N R, Leiner, K Y, Wu, M L and Farr, A L: Quantitative Histochemistry of Brain. I. Chemical Methods. J Biol Chem, 207, (1954): 1-17.

19. Lux, S E, and John, K M: The Membrane Defect in Irreversibly Sickled Cells (ISCs): Irreversible Deformation of the Spectrin-Actin Lattice. Blood 46, (1975): 1052.

20. Mentzer, W C Jr, Smith, W B, Goldstone, J, and Shohet, S B: Hereditary Stomatocytosis: Membrane and Metabolism Studies. Blood, 46, (1975): 659-669.

21. Oski, F A, Naiman, J L, Blum, S F, Zarkowsky, H S, Whaun, J, Shohet, S B, Green, A, and Nathan, D G: Congenital Hemolytic Anemia with High-Sodium, Low-Potassium Red Cells. New Engl J Med, 280, (1969): 909-916.

22. Pinkerton, P H, and Fletch, S M: Inherited Hemolytic Anemia with Dwarfism (Gene Symbol: dan) in the Dog. Blood (Abs), 40, (1972): 963.

23. Pinkerton, P H, Fletch, S M, Brueckner, P J, and Miller, D M: Hereditary Stomatocytosis with Hemolytic Anemia in the Dog. Blood, 44, (1974): 557-567.

24. Sokal, R R, and Rohlf, F J: Biometry. 1st Edition. Freeman, San Francisco (1969); 240-245.

25. Shapiro, D L, and Marchesi, V T: Phosphorylation in Membranes of Intact Human Erythrocytes. J Biol Chem, 252, (1977): 508-517.

26. Subden, R E, Fletch, S M, Smart, M A, and Brown, R G: Genetics of the Alaskan Malamute Chondrodysplasia Syndrome. J Hered, 63, (1972): 149-152.

27. Wiley, J S, Ellory, J C, Shuman, M A, Shaller, C C, and Cooper, R A: Characteristics of the Membrane Defect in the Hereditary Stomatocytosis Syndrome. Blood, 46, (1975): 337-356.

28. Yawata, Y, Koresawa, S, Yamada, O, and Shibata, S: Defective Membrane Phosphorylation in Red Cells of a Patient with Hereditary Spherocytosis. Acta Hem Jap, 38, (1975): 311-314.

29. Yu, J, Fischman, D A, and Steck, T L: Selective Solubilization of Proteins and Phospholipids from Red Blood Cell Membranes by Nonionic Detergents. J Supramol Structure, 1, (1976): 233-247.

30. Zarowsky, H S, Oski, F A, Sha'afi, R, Shohet, S B, and Nathan, D G: Congenital Hemolytic Anemia with High Sodium, Low Potassium Red Cells. New Engl J Med, 278, (1968): 573-581.

TABLE 1--Screening Trial Results From Dwarf, Carrier, and Normal Alaskan Malamutes

Erythrocyte Parameter	Mean ± SEM		
	Dwarf dan/dan n=4	Carrier dan/+ n=3	Normal +/+ n=3
red blood cell count (10^{12}/l)	5.07 ± 0.40	6.21 ± 0.60	7.16 ± 0.34
PCV	47.3 ± 1.61	44.3 ± 1.61	48.7 ± 2.19
hemoglobin (g/dl)	12.3 ± 0.55	14.4 ± 0.42	17.2 ± 0.85
MCV (fl)	94.3 ± 3.96	72.5 ± 5.43	68.3 ± 3.61
MCH (pg)	24.5 ± 1.56	23.6 ± 1.85	24.1 ± 1.47
MCHC (g/dl)	25.8 ± 0.82	32.5 ± 0.23	35.4 ± 0.21
GSH*	1.51 ± 0.05	1.96 ± 0.10	2.12 ± 0.23

* GSH values were calculated from data on 3 dwarfs, 3 carriers, and 2 normals (units are mg/gm Hb)

Level of Significance for Comparison Among the Three Groups of Alaskan Malamutes.

Erythrocyte Parameter	Dwarf vs Carrier	Dwarf vs Normal	Carrier vs Normal
red blood cell count	---	$P<0.05$	---
PCV	---	---	---
Hb	$P<0.05$	$P<0.01$	$P<0.05$
MCV	$P<0.01$	$P<0.01$	---
MCH	---	---	---
MCHC	$P<0.01$	$P<0.01$	$P<0.05$
GSH	$P<0.05$	$P<0.05$	---

TABLE 2--Protein Extractability. Numbers Represent the Percent of Protein Extracted by Each of the Methods Employed.

Extractability Method	Mean ± SEM		
	Dwarf dan/dan	Carrier dan/+	Normal +/+
0.1 mM EDTA	n=4 42.8 ± 1.88	n=3 37.6 ± 0.43	n=3 42.2 ± 0.77
Triton-X	n=4 72.6 ± 3.46	n=2 48.8 ± 0.38	n=7* 64.0 ± 2.65

* 2 of the 7 dogs were Alaskan Malamutes

TABLE 3--Spectrin Kinase Following Membrane Incubation in the Presence of γ-^{32}P-ATP.

	Mean ± SEM		
	Dwarfs dan/dan n=4	Carriers dan/+ n=3	Normals +/+ n=3
Spectrin kinase*	3.77 ± 0.88	4.12 ± 0.59	2.77 ± 0.66

* nmol casein phosphoylated mg^{-1} min^{-1}

TABLE 4--Deformability Measurements (mm) of the Diffraction Ring on Dwarf, Carrier and Normal Dog Erythrocytes.

Shear Stress ($dynes/cm^2$)	Mean ± SEM length x width		
	Dwarf dan/dan n=4	Carrier dan/+ n=3	Normal +/+ n=4
0	5.00 ± 0.08 x 5.00 ± 0.08	4.83 ± 0.09 x 4.83 ± 0.09	4.83 ± 0.18 x 4.83 ± 0.09
100	10.9 ± 0.24 x 3.38 ± 0.08	9.60 ± 0.66 x 3.17 ± 0.09	10.3 ± 0.44 x 3.10 ± 0.04
330	11.9 ± 0.38 x 3.20 ± 0.11	11.7 ± 0.7 x 2.90 ± 0.10	12.0 ± 0.36 x 2.93 ± 0.05
600	13.0 ± 0.36 x 2.88 ± 0.05	13.5 ± 0.8 x 2.63 ± 0.22	13.3 ± 0.3 x 2.73 ± 0.05

PAPER 2. THE EFFECTS OF ADENOSINE TRIPHOSPHATE ON THE ERYTHROCYTES OF ALASKAN MALAMUTES

SUMMARY AND CONCLUSIONS

Erythrocyte stomatocytosis in the Alaskan Malamute occurs with chondrodysplasia as an autosomal recessive inherited condition (gene symbol: dan). Erythrocytes incubated in buffered adenosine triphosphate (ATP) solution swell with increased intracellular Na^+ and water. Sodium ion flux into the canine erythrocyte is also increased in the presence of sucrose, an osmotic shrinking agent. Blood was collected from 32 adult dogs : 4 dan/dan Malamutes, 3 dan/+ Malamutes, 10 +/+ Malamutes and 15 clinically normal mongrel dogs. Washed red cells were incubated in a series of buffers with and without ATP and sucrose to attempt to differentiate normal from heterozygous Malamutes by measuring hemolysis spectrophotometrically at 412 nm. Early trial results indicated significant differences ($P<0.05$) among the three groups of dogs. Additional tests with normal mongrels and normal Malamutes resulted in a wide range of values that overlapped heterozygous and dwarf Malamute values.

INTRODUCTION

The erythrocytes of hereditary stomatocytosis in the Alaskan Malamute are morphologically similar to those of humans with hereditary stomatocytosis.[8,9] The condition has been proposed as an animal model of human disease.[3] The defect in the Alaskan Malamute is inherited as an autosomal recessive and is accompanied by chondrodysplasia, or dwarfism.[11] Currently test breeding is the only reliable method for identifying heterozygous dogs. The process is time consuming and the destruction of litters is emotionally and financially draining to owners and breeders.

Canine red cells respond to small changes in volume by large changes in cation permeability.[6] The permeability to shrunken canine cells is increased to Na^+ but is decreased to K^+, while the permeability of swollen cells is

decreased to Na^+ and is increased to K^+.[5] The absence of glucose in an incubation medium with canine erythrocytes results in rapid cellular swelling and lysis.[6]

Stereospecific sites on the canine erythrocyte membrane bind ATP causing increased Na^+ permeability.[7] The increased membrane activity is specific for ATP and cations. However, ATP does not alter cell shape. Sucrose can also increase the Na^+ movement across the red cell membrane. When erythrocytes are incubated with both ATP and sucrose, the flux was nearly the sum of the individual fluxes, suggesting independent and parallel routes of Na^+ transport in the presence of ATP and sucrose.

Differences between carrier and normal Malamutes have been reported as highly significant (P 0.01) for mean corpuscular volume (MCV) and red cell Na^+.[9] The purpose of this study was to test the hypothesis that an incubation medium and an ATP concentration could be found in which the amount of hemolysis between carriers and normals from swelling in an ATP solution would be significantly different.

MATERIALS AND METHODS

Thirty-two dogs were utilized during the research project: 4 dwarf Malamutes, 3 carrier Malamutes, 10 normal Malamutes and 15 normal mongrels. The ancestry of the Malamutes was known. Blood was collected in sterile syringes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant.

The packed cells from centrifugation were washed three times in phosphate buffered saline (5mM, pH 8.0) removing the buffy coat and upper layer of cells each time. Centrifugation was at 3,000 x g for 10 minutes. Washed red cells were added to 2.2 ml of buffer (Table 1) with and without ATP to achieve a packed cell volume of approximately 10. The solutions were incubated in a 37 C water bath. Samples were mixed gently, a 0.25 ml aliquot was removed,

placed in micropolypropylene test tubes[a] and spun in a microcentrifuge[b] for 2 minutes. Supernatant (0.1ml) was removed and added to 1.9 ml water and read spectrophotometrically at 412 nm against a water blank.

Buffer E (Table 1) was used during the initial ATP trial, which included 4 dwarf Malamutes, 3 carrier Malamutes and 3 normal dogs, 2 of which were Malamutes. Samples were incubated 2.5 hours in 2.7 mM ATP. To expand the base on normal values red cells from 8 mongrel dogs were used. Following the first attempts with mongrel dogs, each component of buffer E was evaluated. N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) was replaced with KCl, and albumin was eliminated. Samples were incubated 3.0 hours in 3.0 mM ATP. Buffer K (Table 1) was tested on the red cells of 6 more mongrel dogs and 8 normal Malamutes.

The sucrose trial was run separately. Red cells were added to tubes with and without sucrose (100 mM) and ATP (3 mM), incubated 3 hours, sampled and read spectrophotometrically at 412 nm against a water blank.

Data collected was analyzed statistically by analysis of variance[c] among three groups of dogs and by the Student-Newman-Keuls test to locate differences.[10]

RESULTS

The ATP values for the three groups of Malamutes were compiled over 5 different days. The differences between the carriers and dwarfs and between the normals and dwarfs were highly significant (P 0.01) and the differences between the carriers and normals were significant (P 0.05). Normal and carrier values differed maximally when the ATP concentration was 3 mM and the samples were incubated 3 hours at 37 C. Blood filtered through washed cotton balls gave similar results as did blood stored at 4 C for 24 hours.

When red cells from mongrel dogs were used in the ATP trial (Table 3), the range of values was wide and overlapped the carrier values. No differences were found in tests with and without albumin or by changing TES to KCl. Trials with the new buffer, buffer K (Table 1), were run at fixed concentrations of EDTA. Red cells from 6 mongrel dogs incubated in buffer K produced extensive hemolysis (Table 4). Finally red cells from 8 normal Malamutes (Table 5) also hemolysed in buffer K.

When dwarf Malamute red cells were incubated in sucrose and ATP no differences in hemolysis were observed. The results of the early trials were successful in detecting carrier Malamutes, however, it was not possible to perfect a repeatable method for incubation of Malamute erythrocytes in which carriers would significantly differ from normals.

DISCUSSION

The results in Table 2 indicated that carrier and normal Malamute erythrocytes swell in response to incubation in ATP enough to be significantly different ($P<0.05$). Dog 731 showed an elevated difference compared to the other normal dogs. However a retest of the same dog the following day gave readings compatible with the two other normals.

Extrapolation of this test to normal dogs did not seem feasible following the results attained in Table 3, which suggested that Malamute red cells differ to the point of responding in a specific fashion to an ATP incubation medium. Attempts to find an alternate buffer that could produce greater differences among the 3 groups, especially carriers and normals, led to buffer K. Buffer K was continuously re-evaluated against buffer E until it was decided that buffer K was more promising. However these trials were performed with only one Malamute from each of the 3 groups.

The response of the 6 normal mongrels and 8 normal Malamutes was similar. The amount of hemolysis was greater in general for the normal dogs, especially the Malamutes, in buffer K than buffer E. The true comparative values of these buffers need to be further evaluated against a greater number of dwarf, carrier and normal Malamutes.

REFERENCES

1. Elford, B C: Independent Routes for Na^+ Transport Across Red Cell Membranes. Nature, 256, (1975): 580-582.

2. Elford, B C: Interactions Between Temperature and Tonicity on Cation Transport in Dog Red Cells. J Physiol, 246, (1975): 371-395.

3. Fletch, S M, and Pinkerton, P H: Congenital Hemolytic Anemia: Animal Model of Human Disease. Am J Path, 71, (1973): 477-480.

4. Nystrom, R A: Membrane Physiology. First Edition. Prentice Hall, Englewood, New Jersey (1973): 104-113.

5. Parker, J C: Dog Red Blood Cells--Adjustment of Salt and Water Content in vitro. J Gen Physiol, 62, (1973): 147-156.

6. Parker, J C, and Hoffman, J F: Interdependence of Cation Permeability, Cell Volume and Metabolism in Dog Red Cells. Fed Proc, 24, (1965): 589.

7. Parker, J C, and Snow, R L: Influence of External ATP on Permeability and Metabolism of Dog Red Blood Cells. Am J Physiol, 223, (1972): 888-893.

8. Pinkerton, P H, and Fletch, S M: Inherited Hemolytic Anemia with Dwarfism (Gene Symbol: dan) in the Dog. Blood (Abs), 40, (1972): 963.

9. Pinkerton, P H, Fletch, S M, Brueckner, P J, and Miller, D M: Hereditary Stomatocytosis with Hemolytic Anemia in the Dog. Blood, 44, (1974): 557-567.

10. Sokal, R R, and Rohlf, F J: Biometry. First Edition. Freeman, San Francisco (1969): 240-245.

11. Subden, R E, Fletch, S M, Smart, M A and Brown, R G: Genetics of the Alaskan Malamute Chondrodysplasia Syndrome. J Hered, 63, (1972): 149-152.

TABLE 1--Buffers used in ATP Trials.

Buffer E	mM	Buffer K	mM
NaCl	137.2	NaCl	153.4
TES	5.0	KCl	5.6
Na_2HPO_4	5.2	Na_2HPO_4	5.2
NaH_2PO_4	0.8	NaH_2PO_4	0.8
Glucose	5.0	Glucose	5.0
	153.2		170.0
Adjust pH to 7.4 with KOH. Add 1 g albumin and q.s.to 100 ml with buffer on day of use.		Check pH 7.5.	

TABLE 2--ATP Incubation Trial Results from Alaskan Malamutes. Red Blood Cells Incubated 2.5 Hours at 37 C. Amount of Hemolysis Measured by Absorbance at 412 nm against a Water Blank.

Dog #	Absorbance Difference between samples with and without 2.7 mM ATP	Mean ± SEM
Dwarfs		
263	1.494	
264	0.793	
265	2.731	
266	2.525	1.850 ± 0.450
Carriers		
C-1	0.179	
C-2	0.149	
C-3	0.213	0.180 ± 0.016
Normals		
657	0.028	
658	0.027	
731*	0.222	0.092 ± 0.056

* Mongrel

TABLE 3--Measurement of Hemolysis Following Incubation of Red Blood Cells from Normal Mongrel Dogs in Buffer E for 3 Hours with and without 2.7 mM ATP.

Dog Number	Hemolysis Difference*(Control + ATP - Control)
1	-0.008
77	0.118
80	0.262
92	0.174
157	0.098
1143	0.103
1164	0.626
1193	0.579

* Measured by Absorbance at 412 nm

TABLE 4--Measurement of Hemolysis Following Incubation of Red Blood Cells from Normal Mongrel Dogs in Buffer K for 3 Hours with and without 3 mM ATP.

Dog Number	Hemolysis Difference*(Control + ATP - Control)
93	0.368
94	1.165
218	0.656
281	0.364
291	0.055
724	0.247

* Measured by Absorbance at 412 nm

TABLE 5--Measurement of Hemolysis Following Incubation of Red Blood Cells from Normal Alaskan Malamutes in Buffer K for 3 Hours with and without 3 mM ATP.

Dog Number	Hemolysis Difference*(Control + ATP - Control)
1	2.147
2	0.545
3	0.458
4	1.210
5	1.184
6	2.443
7	0.495
8	0.049

* Measured by Absorbance at 412 nm

APPENDIX

Gel Scanner Results of Erythrocyte Membrane Polyacrylamide Electrophoresis. Numbers Represent Percent Area Occupied by each Band. Read at 560 nm.

Protein Band	Mean ± SEM		
	Dwarf dan/dan n=4	Carrier dan/+ n=3	Normal +/+ n=3
I	18.8 ± 1.9	19.3 ± 0.9	19.0 ± 0.5
II	20.0 ± 0.8	21.3 ± 0.9	19.3 ± 1.1
III	29.8 ± 1.0	31.3 ± 0.7	31.0 ± 0.9
IV.1 & IV.2	13.5 ± 1.3	11.7 ± 0.3	12.7 ± 0.6
V	11.8 ± 0.9	11.7 ± 0.3	12.3 ± 1.2
VI	4.3 ± 0.5	3.7 ± 0.6	4.0 ± 0.6
VII	1.8 ± 0.5	1.3 ± 0.3	2.0 ± 0.6

ERYTHROCYTE MEMBRANE CHARACTERISTICS OF THE HEREDITARY STOMATOCYTOSIS SYNDROME IN THE ALASKAN MALAMUTE

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Hereditary stomatocytosis is associated with dwarfism as a simple autosomal recessive genetic defect (gene symbol: dan) in the Alaskan Malamute. The primary biochemical defect is unknown, however, similarities exist between stomatocytosis in man and in Malamutes.

The erythrocytes of dwarf, carrier and normal Alaskan Malamutes were evaluated using routine red cell parameters, polyacrylamide gel electrophoresis, ethylenediaminetetraacetic acid extraction, Triton-X extraction, protein kinase activity and ektacytometric deformability. In addition, hemolysis studies with adenosine triphosphate and sucrose incubation were conducted to differentiate carrier from normal Malamutes.

Highly significant differences ($P<0.01$) were found for mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and Triton-X extractability between dwarfs and carriers, for hemoglobin, MCV and MCHC between dwarfs and normals, and for Triton-X extractability between carriers and normals. Significant differences ($P<0.05$) were found for hemoglobin and glutathione (GSH) between dwarfs and carriers, for red blood cell count, Triton-X extractability and GSH between dwarfs and normals, and for hemoglobin and MCHC between carriers and normals.

The results support the use of erythrocytes from the Alaskan Malamute as a model for hereditary stomatocytosis in man.