Normal Cations and Abnormal Membrane Lipids in the Red Blood Cells of Dogs With Familial Stomatocytosis-Hypertrophic Gastritis

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Examination of the red blood cells (RBCs) of eight dogs with familial stomatocytosis-hypertrophic gastritis (FS-HG), a multiorgan disease associated with hemolytic anemia, hereditary stomatocytosis (HSt), and hypertrophic gastritis resembling Ménétrier’s disease in man, showed abnormal osmotic fragility, normal mean corpuscular volume, slightly increased cell water, and normal cation content and cation fluxes. Cholesterol was decreased in RBC and increased in plasma. In both RBCs and plasma, total phospholipid (PL) was normal, phosphatidylcholine (PC) decreased, and sphingomyelin increased. The palmitic acid content of PC was increased, and the stearic acid content of PC was decreased. Sodium dodecyl sulfate electrophoresis of RBC membrane proteins was normal. These findings have not been described previously in HSt. They suggest that in FS-HG, abnormal composition of the PL in RBCs secondary to abnormal PL in plasma causes defective membrane function and stomatocytic shape-change. This conclusion was supported by a shortened half-life of $^{59}$Cr-labeled RBCs from normal dogs after transfusion in dogs with FS-HG. It was concluded that not all hereditary forms of stomatocytosis are necessarily associated with an intrinsic structural defect of the RBC membrane, but that the change in shape of RBC may also be induced by abnormal composition of the plasma; that stomatocytosis may be caused by loss of membrane surface area, rather than by the increased cation uptake such as has been shown in some human kindreds with HSt, that FS-HG is a disorder of lipid metabolism, and by consequence, that abnormal lipid metabolism might be involved in the pathogenesis of Ménétrier’s disease.

HEREDITARY STOMATOCYTOSIS (HSt) is a heterogeneous group of familial disorders in man characterized by the presence of cup- or bowl-shaped red blood cells (RBCs) (stomatocytes) and chronic hemolytic anemia. Stomatocytosis has also been described as a pleiotropic effect in two hereditary diseases in dogs. The pathogenesis of the stomatocyte formation has been ascribed to an increase in cation, and hence, water content of the RBCs caused by abnormal membrane permeability. Although high cation content of RBCs has been shown in most human families with HSt and in affected dogs, HSt with normal (Na + K) content of RBCs has also been reported. In vitro experiments have shown that stomatocytoses also can be produced by altering the surface charge of the RBC.

When HSt is associated with increased cation content there is a deficiency of an integral band 7 membrane protein, but in other cases of the disease, the nature of the membrane defect is unclear. The lipid composition of the RBCs in HSt has been reported as normal, though abnormal in a number of HSt-allied disorders.

Familial stomatocytosis-hypertrophic gastritis (FS-HG) is a hereditary multiorgan disease in dogs, inherited as an autosomal recessive trait. Its pathogenesis is unknown. Apart from HSt, dogs with FS-HG consistently have hypertrophic gastritis resembling Ménétrier’s disease in man. The etiology and pathogenesis of Ménétrier’s disease is unknown, but a hereditary defect has been suspected.

Knowledge of the pathobiologic properties of RBCs in dogs with FS-HG may help to elucidate the pathogenesis of FS-HG, HSt, and Ménétrier’s disease. Therefore, we performed hematologic and biochemical studies in eight dogs with FS-HG. This showed a normal RBC volume and cation content, but a unique alteration in the lipid composition of the RBC membrane and the plasma.

MATERIALS AND METHODS

The dogs with FS-HG consisted of three females and five males, 3 to 22 (median 8) months of age, from two families of the Drentse patroijshond breed, and were privately owned. Pedigrees and clinical details have been published. Routine hematology was performed as described previously. Hemoglobin (Hb) was measured as cyanmethemoglobin. RBCs were counted electronically (Coulter counter, model B; Coulter Electronics, Hialeah, FL). Packed cell volume (PCV) was determined in microhematocrit capillary tubes filled to 85% of capacity and centrifuged for 5 minutes at 10,000g. A fixed correction of 3% was made for trapped plasma. Mean corpuscular Hb (MCH), MCH concentration (MCHC), and mean corpuscular volume (MCV) were calculated from PCV, Hb, and the RBC count.

Studies of RBC morphology by stereoscopic electron microscopy (SEM) (Philips, Eindhoven, The Netherlands) were performed according to routine methods. Sodium dodecyl sulfate (SDS) gel electrophoresis of membrane proteins was performed after preparation of RBC ghosts and extraction of the proteins with alkali. In vivo apparent half-life of $^{59}$Cr-labeled RBC ($^{59}$CrT) was measured for (1) autologous RBCs in dogs with FS-HG, (2) RBCs from dogs with FS-HG in dogs with blood group C, (3) RBCs from normal CEA, negative canine donors in dogs with FS-HG, and (4) autologous RBCs in normal dogs. Before and after RBC survival studies, cross-reactivity between serum and RBCs of donor and recipient was checked by direct agglutination and lysis tests and Coombs’ antiglobulin tests.

Cholesterol was assayed and in the lipids extracted from leucocyte-free packed cells. RBC cholesterol content was calculated from the cholesterol concentration of the packed cells using the formula:

$$\text{RBC}_{\text{cholesterol}} = (\text{cholesterol/liter RBC}) \times (\text{MCH/}\text{Hb})$$

Phospholipids in plasma and RBC were extracted, separated by
two-dimensional silica thin-layer chromatography,25 and quantitated by phosphorus determination.26 The phospholipid (PL) fatty acid composition was determined by gas-liquid chromatography.27 Cell water and cell nonwater content of RBC were determined in duplicate by weighing packed cells before and after drying at 110°C to constant weight. The volume of cell water was computed, assuming that the volume of nonwater constituents of canine RBCs equals 0.7412 L/kg dry weight.38

To avoid exchange of cations between the medium and the RBCs from a possible unknown cation gradient, Na and K concentrations in RBCs were estimated as follows: Blood was anticoagulated with lithium heparin and freed of leucocytes by aspirating and discarding the buffy coat after each of three centrifugations. The buffy coat after each of three centrifugations was used to resuspend the RBCs to a concentration of about 0.7412 mL/wk dry weight.38 Radioactivity (disintegrations/L/h) was measured in a well-type liquid scintillation counter (Nuclear Chicago, Chicago, IL). Counting was extended sufficiently to give standard deviations of less than 1% and corrections were made for dilution, for decay of the isotope, and for background activity. Corrections for hemolysis were not necessary, because less than 1% of the Hb was released during incubation. The pH of the incubation medium varied from 7.05 to 7.28 during the experiments. RBC tracer uptake followed a well-defined linear time course in both controls and patients, except for Na uptake during the first 10 to 20 minutes in two dogs, which was ascribed to an initial difference between the intracellular and extracellular tonicity. The steady state influx of Na and K was calculated from the ratio of the slope of the regression line fitted to the calculated cell activity at various times. For sodium, the data from the first 20 minutes were neglected. Cation uptake (mmol/Lh) was calculated from this by multiplying the figures for tracer uptake (net counts/L/h) with the specific activity of Na and K.

RESULTS

Light microscopy of routinely stained blood smears showed 14% to 38% RBC with a slit-like central pallor characteristic of stomatocytes.1 Almost all RBC were found to be cup shaped when examined by SEM (Fig 1). The osmotic fragility was increased in all dogs with FS-HG. Osmotic fragility curves of normal dogs and dogs with FS-HG did not overlap, indicating that all RBCs are affected in FS-HG (Fig 2).

RBC water content in normal dogs was in agreement with published data.23 In dogs with FS-HG, RBC water was 6% higher (Table 1). In our reference dogs, the K concentration was 5.5 ± 0.6 (M ± SD) mmol/L RBCs or 7.7 mmol/L cell water, in agreement with published data.23 The Na concentration of RBCs in normal dogs has been reported to be from 121 to 162 mmol/L cell water.23 In the present study, sodium in normal dogs was 102.5 (SD = 1.7) mmol/L RBCs or 145 mmol/L cell water. The reliability of this is supported by the fact that the sum of Na and K concentrations was slightly less in the cell water (152.7 mmol/L) than in plasma water (168.1 mmol/L). In dogs, Na and K concentrations in RBCs and plasma are nearly in Donnan equilibrium.40

In RBCs of dogs with FS-HG, Na, and the sum of Na and K concentrations were normal, but K concentration was increased (Table 1).

In the reference dogs, Na and K uptake (Table 1) were consistent with reported data for cation uptake by canine RBCs containing 65% water at 37°C.41,42 In dogs with FS-HG, 48K influx was the same as in the reference dogs, but 22Na influx was four times less (Table 1).

In dogs with FS-HG, lipid composition was abnormal in plasma and RBCs (Table 2). Cholesterol was increased in plasma and decreased in RBCs. In both plasma and RBC, total PL was essentially normal, but phosphatidylcholine (PC) was decreased and sphingomyelin (SM) proportionally increased.

The fatty acid composition of the main plasma and RBC PLs measured in five dogs with FS-HG varied but consistently significant changes were observed in PC (Table 3).

SDS gel electrophoresis showed no abnormalities of the total and integral membrane proteins in two dogs with FS-HG.

In dogs with FS-HG, a much higher percentage of stomatocytes was found by SEM than by routine light microscopy. This discrepancy has been observed before in patients with HS-St.22 Possibly the glutaraldehyde fixation used in SEM evokes the stomatocytic shape change in predisposed RBC.43

The change from a normal "discocyte" RBC into a stomatocyte implies an increase in the cell volume/surface area ratio. Osmotic fragility is a function of this ratio,43 which explains the increased osmotic fragility in dogs with FS-HG. It has been postulated that increased permeability of the RBC membrane to cations plays an important role in the pathogenesis of stomatocytes in HS-St in man.1 A stomatocytic shape change may occur when inward sodium leak prevails over outward potassium leak.44 This results in an increase in the intracellular Na/K ratio and the total cation content. Because net water and cation uptake are proportional, the sum of Na and K concentrations remains constant, but cell water, MCV, and consequently, osmotic fragility increase. Remarkably, stomatocytosis with high potassium and low sodium has also been reported in dogs,2 even though in normal canine RBCs a Na/K pump is missing and Na and K concentrations are almost the same in RBCs and plasma.42

In our dogs with FS-HG, the pathogenesis of stomatocytosis can only be explained by a decrease of RBC surface area by loss or contraction of membrane constituents. Osmotic fragility was consistently highly increased even though MCV was normal and cell water only 6% increased, i.e., not in proportion to the increase reported in Malamute dogs2 and human patients1 with high sodium and low potassium HSt. The (Na + K) content was normal. The Na/K ratio was
decreased rather than increased and even studies of the $^{24}\text{Na}$ and $^{40}\text{K}$ fluxes showed no cation leakage. K flux was the same, and Na flux four times lower, than that in controls. This is consistent with the slightly increased hydration of the RBC in dogs with FS-HG. Inward Na flux rapidly decreases when cell water content increases from $+55\%$ to $70\%$, whereas the K flux hardly changes over that range.\textsuperscript{42}

The K concentration in RBC of dogs with FS-HG was increased. This should be ascribed to the young age of the RBC population\textsuperscript{44} rather than to a membrane defect.

Frank stomatocytosis without membrane leakage of cations and with normal contents of intracellular electrolytes has been reported before,\textsuperscript{5,9} but without a satisfactory explanation for its pathogenesis in vivo. In vitro experiments suggest that altered membrane charges and/or conformational changes in the membrane lipids or proteins rather than the cell contents may be responsible for changes in the red cell shape.\textsuperscript{10,45} The shape of red cell membranes made permanently leaky, and therefore, unable to support an osmotic gradient, may be changed instantaneously and reversibly into stomatocytes by manipulation of ionic strength.\textsuperscript{45} Even slight changes in the fatty acid composition of PC in the RBC membrane may cause shape changes and enhance osmotic fragility.\textsuperscript{46}
In dogs with FS-HG, abnormal composition of the PLs may have caused membrane contraction or membrane loss. The relative composition of the PLs of the RBCs was abnormal and possibly associated with the abnormal composition of the plasma lipids. RBCs continuously exchange cholesterol and phospholipids, especially lyso-PC, PC, and SM, with the environment. In dogs with FS-HG, the relative amounts of PC and SM were altered in the same direction in RBC and plasma and qualitative changes in the fatty-acyl constituents of PC were also similar. It is true, that plasma cholesterol was low and RBC cholesterol was high, but the RBC cholesterol is in free equilibrium with unesterified cholesterol in plasma alone, not with the esterified molecules, and we have not measured the esterification of the cholesterol in the plasma and RBC.

Absence of the band 7.2b integral membrane protein seems to be the most unifying feature of HSt in humans, and suggests that the stomatocytic shape of the RBC is based on a primary defect of the cytoskeleton. Electrophoresis of the RBC membrane proteins in two dogs with FS-HG was normal. This is consistent with our suggestion that the shape change of the RBC may also result from other membrane components such as the abnormal lipid composition.

This is supported by the results of the RBC survival studies in vivo. The slightly reduced $[^{14}C]_{23}$ of normal RBC after

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**Table 2. Lipid Composition of Plasma and RBCs in Dogs With FS-HG**

<table>
<thead>
<tr>
<th>Reference Values</th>
<th>Dogs With FS-HG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Plasma</td>
<td>Cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Total PL (mmol/L)</td>
<td>3.45</td>
</tr>
<tr>
<td>SM (%)</td>
<td>8.1</td>
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<tr>
<td>Phosphatidylcholine (%)</td>
<td>81.2</td>
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<tr>
<td>Lyso-phosphatidylcholine (%)</td>
<td>6.6</td>
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<tr>
<td>Phosphatidylserine (%)</td>
<td>2.2</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (%)</td>
<td>2.0</td>
</tr>
<tr>
<td>L/S ratio</td>
<td>10.07</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>Cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Total PL (mmol/L)</td>
<td>238</td>
</tr>
<tr>
<td>SM (%)</td>
<td>11.7</td>
</tr>
<tr>
<td>Phosphatidylcholine (%)</td>
<td>41.6</td>
</tr>
<tr>
<td>Lyso-phosphatidylcholine (%)</td>
<td>2.4</td>
</tr>
<tr>
<td>Phosphatidylserine (%)</td>
<td>18.4</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (%)</td>
<td>26.5</td>
</tr>
<tr>
<td>L/S ratio</td>
<td>3.71</td>
</tr>
</tbody>
</table>

L/S ratio (lecithin/sphingomyelin ratio), phosphatidylcholine/sphingomyelin ratio.

* P < .05 (two-tailed t-test).
† P < .0001 (two-tailed t-test).
‡ P < .00001 (two-tailed t-test).
§ P < .01 (two-tailed t-test).

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**Table 3. Fatty Acid Composition of Phosphatidylcholine (PC) in RBCs and Plasma of Dogs With FS-HG**

<table>
<thead>
<tr>
<th></th>
<th>References</th>
<th>Dogs With FS-HG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>RBC PC</td>
<td>16:0</td>
<td>26.2</td>
</tr>
<tr>
<td>18:0</td>
<td>24.6</td>
<td>2.6</td>
</tr>
<tr>
<td>18:1</td>
<td>17.7</td>
<td>3.8</td>
</tr>
<tr>
<td>18:2</td>
<td>16.7</td>
<td>2.3</td>
</tr>
<tr>
<td>20:4</td>
<td>14.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Plasma PC</td>
<td>16:0</td>
<td>14.6</td>
</tr>
<tr>
<td>18:0</td>
<td>29.6</td>
<td>2.0</td>
</tr>
<tr>
<td>18:1</td>
<td>12.7</td>
<td>4.0</td>
</tr>
<tr>
<td>18:2</td>
<td>16.5</td>
<td>3.2</td>
</tr>
<tr>
<td>20:4</td>
<td>21.9</td>
<td>4.1</td>
</tr>
<tr>
<td>22:6</td>
<td>3.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Unknown | 1.2 | 0.0 | 2 | 2.6 | 0.9 | 2 |

* P < .001 (two-tailed t-test).
† P < .0001 (two-tailed t-test).
‡ P < .00001 (two-tailed t-test).
§ P < .05 (two-tailed t-test).
transfusion in dogs with FS-HG probably indicates loss of viability of the RBC because of the uptake of the abnormal plasma PLs of the host (Table 4).

$^{51}$Cr$^3+$ of RBCs of dogs with FS-HG was less in normal recipients than in the affected dogs themselves. This may indicate that in dogs with FS-HG, membrane function is not just disturbed in RBC, but also in phagocytic cells. It is unlikely that $^{51}$Cr-RBC survival studies were hampered by iso- or allo-antibodies because no cross-reactivity could be shown between the serum and the RBC of the donors and recipients before and after the transfusion experiments.

Abnormal lipid composition of RBC associated with stomatocytosis has been described before but not in the form presented here. Our findings support the theory that the sto- matocytosis syndrome has been described before and not in the form presented here. Our findings support the theory that the stomatocytic shape change is a nonspecific expression of membrane dysfunction and that HSt is a heterogeneous group of diseases, both in man and dogs. Stomatocytes may develop not only to the dysfunction of the membrane but also to the pathology of other organ systems involved in that disease, including the hypertrophic gastritis.

ACKNOWLEDGMENT

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REFERENCES

lipid. II. Ion permeability and transport abnormalities. Blood 42:1, 1973
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