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ORIGINAL

# RED CELL ABNORMALITIES IN A KINDRED WITH AN UNCOMMON FORM OF HEREDITARY SPHEROCYTOSIS

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## SUMMARY

Red cell acetylcholinesterase (AChE) and Na,$^+$ K $^+$- adenosinetriphosphatase (ATPase) activities, cell 2,3 - diphosphoglycerate (2,3 - DPG) and adenosinetriphosphate (ATP) content and filterability ratio were studied in two children (with moderate hemolytic anemia and marked spherocytosis) and their parents. Patients' parents have no medical problem but evidenced discrete spherocytosis on peripheral smear. Except some increased apparent red cell rigidity detected in the father, all the parameters studied in both parents were found to be normal, as compared to healthy controls. In contrast, red cell rigidity, 2,3-DPG and ATP levels and Na,$^+$ K $^+$ ATPase activity were increased in both children, whereas AChE activity was similar to values of normal subjects. These observations suggest that both affected patients suffered from homozygous hereditary spherocytosis linked to an apparently recessively inherited red cell membrane defect.

## RESUMO

**Anomalias eritrocitárias em crianças portadoras de uma variedade rara de esferocitose hereditária**

Em duas crianças (com anemia hemolítica moderada e esferocitose acentuada) e seus pais foi determinada a actividade da acetilcolinesterase (AChE) e adenosinotrifosfatase (ATP-ase) - Na,$^+$ K $^+$ - - dependente da membrana eritrocitária, conteúdo globular em 2,3 - difosfoglicerato (2,3 - DPG) e adenosinotrifosfato (ATP) e índice de filtrabilidade eritrocitária. Nenhum dos progenitores evidenciava alterações patológicas ou anomalias naqueles parametros laboratoriais, além da esferocitose ligeira em ambos e aumento aparente da rigidez globular no pai. Pelo contrário, ambos os doentes, além de sinais clínicos marcados, evidenciavam filtrabilidade eritrocitária nula a par do aumento da concentração globular do 2,3 - DPG e ATP; a actividade de ATPase Na,$^+$ K $^+$ - dependente era superior ao normal apenas em um dos doentes, sendo a actividade da AChE equivalente à dos controlos normais em ambos. Os resultados obtidos parecem evidenciar uma forma rara de esferocitose hereditária em ambos os doentes eventualmente dependente de um defeito da membrana hereditária com características recessivas.

## INTRODUCTION

Hereditary spherocytosis (HS) is a heterogenous disorder, either clinically or in terms of the molecular defects, in apparent association with a primary abnormality of the red cell membrane.[1, 2] Although in the majority of the cases the biochemical defect is still uncertain,[3] in a small group of patients exhibiting marked spherocytosis skeletal protein changes involving the spectrin molecule have been described.[4, 5] These abnormalities might result in membrane instability, red cell fragmentation under circulatory stress and/or loss of erythrocyte deformability.

The increased permeability to sodium (Na) ions observed in intact erythrocytes is another functional deficiency of HS cells, perhaps due to structural changes in the membrane.[6] In addition to the differences reported in the several classes of red cell membrane ATPase,[7, 8] there is also some evidence that the profile of acetylcholinesterase (AChE) activity from HS patients is clearly distint from other hemolytic anemias.[9]

In the present paper we focus attention on red cell membrane Na,$^+$ K $^+$ - ATPase and acetylcholinesterase (AChE) activities, in association to the index of erythrocyte filterability (IF), adenosinetriphosphate (ATP) and 2,3 - - diphosphoglycerate (2,3 - DPG) concentrations, from two siblings (the sons of related but normal parents) affected with inherited spherocytosis.

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## MATERIAL AND METHODS

*Case reports and routine evaluation* - A suggestive form of hereditary spherocytosis was detected in a twelve-year-old white boy (patient 1) first seen by us for clear anemia, skeletal abnormalities and hepatosplenomegaly. A similar clinical situation was soon observed in patient 2, a five-year-old brother of patient 1. Both patients, still non-splenectomized at the moment, showed hemolytic anemia characterized by hemoglobin values ranging from 7 to 8 g/dl, elevated reticulocyte count, spherocytes on peripheral smear and mild elevation of unconjugated bilirubin. The most important routine laboratory findings of both siblings and their parents (fourth degree cousins) are summarised in Table 1.

In contrast with the physical and hematologic abnormalities found in both siblings, either parent has had any clinical problem and both had normal physical and routine laboratory tests. However, a small percentage of spherocytes as well as poiquilocytes and increased reticulocyte count (3-4, 5 %) were observed in blood smears of both parents.

In all members of this family there was no abnormal hemoglobin detected, $HbA_2$ ande HbF levels were not raised, Coombs' tests (direct and indirect) were negative and enzyme activities of red cell pyruvate-kinase and glucose 6-phosphate dehydrogenase were found to be comparable to those of normal subjects.

*Procedures* - Blood from each patient and both parents was drawn into vacutainer tubes containing heparin or acid citrate dextrose as anticoagulants, and immediately subdivided in aliquots before being processed further.

Red cell morphology, blood cell counts, immediate osmotic fragility, autohemolysis in vitro, direct and indirect Coombs' tests, hemoglobin electrophoresis and total and unconjugated bilirubin were performed by routine laboratory techniques.

Red cell 2,3 - diphosphoglycerate concentration was determined in 8 % trichloroacetic acid extracts from one blood aliquot, according to the method of Rose and Liebowitz.[10] The erythrocyte adenosinetriphosphate content was measured in the 10 % perchloric acid extract of each blood specimen by the method of Jaworek and Coworkers.[11]

Red cell acetylcholinesterase activity was measured colorimetrically by the method of Kapland,[12] in a prepared 50 % suspension three times saline-washed erythrocytes and using acetylthiocholine iodide as substracte.

Hemoglobin-free erythrocyte ghosts were prepared by hypotonic lysis, as described by Cha et al.[13] Protein content was measured by the method of Lowry.[14] Membrane $Na,^+$ $K,^+$ ATPase activity was determined as the release of inorganic phosphate during incubation of ghosts with ATP, according to the technique of Tausshy et al.[15]

The erythrocyte filterability index was carried out by a modification of the method of Reid et al,[16] using Nucleopore polycarbonate sieves (Nucleopore Corp, Pleasanton, U.S.A.), with a pore diameter of 5 $\mu$; erythrocytes suspended in Tris-albumin buffer (pH 7.4) at a hematocrit of 8 % were used for testing and compared to the filtration time of the solvent; the calculated index of filtration incorporated a correction for measured hematocrit.[17]

## RESULTS

The results for the red cell studied parameters in all members of the family are shown in Table 2. Red cell AChE activity of patients and their parents failed to show any consistent variation from the normal pattern. Membrane $Na,^+$ $K^+$ - ATPase activity from the parents showed a quite normal value, whereas raised activity levels were remarkable in their sons. A similar feature was observed with

TABLE 1 **Standard hematologic data on members of the family studied**

| | Patient 1 | Patient 2 | Mother | Father |
|---|---|---|---|---|
| Hemoglobin (g/dl) | 7.6 | 7.1 | 13.7 | 16.6 |
| Hematocrit (1/l) | 0.22 | 0.22 | 0.45 | 0.52 |
| Red cell count (x $10^{12}$ /l) | 2.7 | 2.6 | 5.0 | 5.6 |
| Mean corpuscular volume (fl) | 81.5 | 84.6 | 90.0 | 93.6 |
| Mean corpucular hemoglobin concentration (g/dl) | 34.5 | 32.3 | 30.4 | 31.7 |
| Reticulocytes (%) | 7 | 18.5 | 3.5 | 4.2 |
| Leucocyte count (x $10^9$ /l) | 9.7 | 5.9 | 6.3 | 6.0 |
| Platelet count (x $10^9$ /l) | 300 | 360 | 300 | 280 |
| Total bilirubin (mg/dl) | 2.5 | — | 0.43 | 0.9 |
| Unconjugated bilirubin (mg/dl) | 1.9 | — | — | 0.8 |
| Serum iron (mg/dl) | 87 | 128 | 141 | 121 |
| Total iron binding capacity (mg/dl) | 187 | — | — | — |
| Coombs tests (direct and indirect) | negative | negative | — | — |
| Hemoglobin F (%) | 2.5 | 2.0 | — | — |
| Hemoglobin $A_2$ (%) | 3.5 | 3.0 | — | — |
| Osmotic fragility (g/l NaCl) | | | | |
| hemolysis begins | 5.0 | 5.0 | 5.5 | 4.5 |
| 50 % hemolysis (normal range: 4.0-4.45) | 4.65 | 4.45 | 4.35 | 4.15 |
| Autohemolysis 37°C (%) | | | | |
| 48 h, without added glucose (normal range: 0.2-4.5) | 6.0 | 4.9 | 5.1 | 2.8 |
| 48 h, with added glucose (normal range: 0-0.5) | 1.21 | 0.95 | 0.73 | 0.80 |
| Blood peripheral smear * | | | | |
| spherocytic RBC | + + | + + | + | + |
| nucleated RBC | + + | + | — | — |
| poikilocytosis and fragmented RBC | + + | + + | + | + |
| anisocytosis | + + + | + + + | — | — |
| achantocytic RBC | — | — | ± | — |

* Semi-quantitative results in a range of 0-4+; RBC = red blood cells.

the 2,3 - DPG concentration. No clear variation on ATP levels was detected in all members of the family. As far as the index of red cell filtration is concerned, the increased values observed in both patients and, also in their father, might indicate a loss of deformability, in contrast to the normal value detected in the mother.

## DISCUSSION

Hereditary spherocytosis seems to be the result of structural defect of erythrocyte membrane, yet to be defined in the most frequent form.[1-3] Usually this disorder occurs with discreet clinical symptoms and mild anaemia, in apparent dependence of a mendelian autosomal trait, perhaps with variable penetrance; no cases of homozygoty have been described.[18, 19]

The presence of spherocytic cells in all members of the family we have studied appears as an uncommon event. Although no clinical manifestations were apparent in the (distantly related) patients' parents, mild spherocytosis and other signs of presumed hemolytic compensation were detected on blood smear. In contrast, both siblings showed remarkable spherocytosis and other red cell abnormalities (Table 1), in association with clear clinical symptomatology.

The obvious poikilocytosis and the normal values of mean corpuscular hemoglobin concentration, as observed on these cases, are unusual laboratory events in HS. Although some confusion may arise with other spherocytic hemolytic anemias, it is unsafe to assume that one is not dealing with HS. In fact, the combination of anemia, increased reticulocytosis (particularly evident in patient 2), slight elevation of serum-indirect reacting bilirubin (in patient 1), spherocytosis, osmotic fragility (in patient 1), autohemolysis (in both), splenomegaly and skeleton abnormalities, as observed, support the diagnosis of HS.

Meanwhile, the reported negative Coombs' test and corrected autohemolysis by glucose excludes the diagnosis of immune spherocytosis; the differentiation from the various causes of splenomegaly and hypersplenism offered no doubt by the physical examination; the inexistence of red cell glucose 6-phosphate dehydrogenase and pyruvate-kinase deficiencies, or the absence of sickle cell trait and other hemoglobinopathies also distinguished the presenting cases from congenital non-spherocytic hemolytic anemias.

Such appearance may suggest that the patients are homozygous for a recessive trait, responsible for a congenital hemolytic anemia with the main characteristics of hereditary spherocytosis.

In addition, both patients clearly exhibited decreased erythrocyte deformability, also observed in a slightest degree in their father. The reduced deformability, documented by various methods as a characteristic abnormality of HS erythrocytes,[20] might be in the presenting cases a consequence of diminished surface area/volume ratio [21] and/or reduced mechanical stability of spherocytic membranes.[22]

As a consequence of a reduced deformability, progressive membrane loss and cell fragmentation occurs in the circulation and might be related to the severity of hemolysis and resulting anemia in HS patients.[19, 20] In fact, the abnormal osmotic properties [23] and decreased lifespan of HS erythrocytes [21] have been attributed to a diminished surface area/volume ratio [23] also suggested as the primary factor leading to decreased deformability. In addition, the reduced surface area/volume ratio appears to be correlated with the degree of spherocyte rigidity.[24]

Although our patients' parents also presented spherocytic cells, the percentage of their expected undeformable cells was not sufficient to produce the hematologic pattern of typical HS, but a discreet reduced erythrocyte deformability in the father.

For the majority of HS patients, the red cells have a normal discocytic shape and about normal osmotic and membrane properties; only a variable subpopulation of truly spherocytic cells accounts for the difference in deformability and the degree of the anemia.[24] So far, the moderate anemia and osmotic fragility showed in both siblings might be explained by a remarkable percentage of underformable spherocytes, as observed.

As a likely consequence of the red cell fragmentation, the increased 2,3 - DPG content found in both patients could account for reduced oxygen affinity of hemoglobin [25] and hence more oxygen being released to the hypoxic tissues.[26] Our data oppose former results of low 2,3 - DPG levels in HS patients, also with intact spleen,[27] but are in agreement with more recent results of increased red cell 2,3 - DPG content in HS children.[28]

The alteration in red cell 2,3 - DPG levels might be also governed by some other metabolic factors, namely the overall rate of erythrocyte glycolytic pathway.[29]

Although the rate of glycolysis is decreased in human erythrocytes containing high concentrations of 2,3 - DPG under *in vitro* conditions,[29] the concentration of ATP were found to be slightly elevated in *high DPG* cells.[30] Furthermore there is no evidence of a major decrease in the levels of ATP when 2,3 - DPG concentration increases in various hypoxic conditions.[31]

The major cation flux abnormality in HS cells appears to be an excessive sodium permeability.[6] HS cells oppose the

TABLE 2 **Red cell 2,3-diphosphoglycerate and adenosinetriphosphate concentration, filterability ratio, membrane acetylcholinesterase and Na,+ K - adenosinetriphosphate activities**

| | Patient 1 | Patient 2 | Mother | Father | Normal controls (mean; SD) |
|---|---|---|---|---|---|
| 2,3 - diphosphoglycerate (μmoles/gHb) | 18.4 | 21.6 | 11.6 | 12.5 | 12.5; 1.8 |
| Adenosinetriphosphate (μmoles/gHb) | 6.7 | 5.5 | 4.4 | 3.8 | 5.9; 1.1 |
| Filterability ratio | no filtration | | 12.2 | 34.4 | 13.9; 2.2 |
| Acetylcholinesterase (U/min/mgHb) | 232 | 230 | 229 | 280 | 231.7; 52.5 |
| Na,+ K + - ATPase (μmoles phosphate/mg membrane protein) | 0.316 | 0.548 | 0.109 | 0.144 | 0.160; 0.085 |

abnormaly high $Na^+$ influx by increased pumping, dependent of higher ATP generation by glycolysis.[32, 33] The higher than normal Na,+ K+ ATPase activity observed in both siblings, confirming former studies in HS patients,[7, 33] could be a reflection of an excessive sodium permeability in HS cells, being the $Na^+$ pumping sustained by increase glycolysis; thus an elevation in ATP levels (with parallel increase of 2,3 - DPG) might be required to maintain the ion pumping. However, such gain in intracellular ATP was not evident in our study, and seems dissociated of increased 2,3 - DPG levels.

In HS cells, the Na,+ K+ - ATPase was shown in correlation with the rate of sodium efflux;[33] however, none of these parameters seems to influence the red cell survival.[34] In addition, neither the HS cells became spherical due to an increase in total cation and water content [23] nor the spheroidal shape itself is responsible for increased glycolysis;[35] similar abnormalities in cation flux and pumping are also found in some other hemolytic [36] or nonhemolytic disorders.[37]

All these observations suggest that excessive sodium permeability and erythrocyte metabolic adaptation, as well as some other described characteristics of HS cells (e. g. spherodicity, increased osmotic fragility, changes in membrane lipids or abnormalities in membrane proteins and lipid phosphorylation) are secondary to basic structural changes in red cell membrane.[1-3, 19, 20, 37]

This view seems to be also partly supported in a recent study, where the profile for erythrocyte membrane acetylcholinesterase in HS was clearly distint of normal and some other hemolytic anemias;[9] the elevation of AChE activity was detected exclusively by using an ionic substracte and was inapparent with a lipophylic one, perhaps due to a more fully exposition of the enzyme molecules at the outer membrane side; however the AChE activity was not influenced by the spherocytic shape.

Conversely, we were unable to confirm by similar methodology an elevation of erythrocyte AChE activity in our patients or their parents. This discrepancy between our results and those of Streichman et al [9] may be due to changes that particular cytoskeletal membrane interactions may impose to the AChE molecules, in dependence of the transmembrane control of integral membrane protein distribution.[38]

In spite of all spherocytosis is not the same,[1-3, 18, 19] and the underlying molecular abnormality is still undefinied for the majority of HS patients,[39] most data point towards the red cell membrane cytoskeleton as being the site of the primary genetic defect in HS.

In this regard and associated to the heterogeneity in inheritance, the basic lesion in HS might reflect diverse abnormalities of the membrane cytoskeleton, perhaps as final consequence of many distinct biochemical defects, as recently suggested.[40]

The clinical course and uncommon inheritance pattern of our patients may be related to a such still uncovered membrane defect. Further investigations upon the red cell membrane characteristics in this family are now in progress in our laboratory.

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