Recurrent Acute Splenic Sequestration Crisis Due to Interacting Genetic Defects: Hemoglobin SC Disease and Hereditary Spherocytosis

By Theodore E. Warkentin, Ronald D. Barr, Mahmoud A.M. Ali, and Narla Mohandas

A 14-year-old boy with hemoglobin SC disease and α-thalassemia-2 experienced five episodes of acute splenic sequestration crisis (ASSC), while two of his siblings with identical globin genotypes (SC and -α/αα) had no such experience. To determine if an additional red blood cell (RBC) defect was responsible for the unusual occurrence of frequent ASSCs, we performed detailed rheologic characterization and membrane protein analysis on RBCs from the proband and other members of his family. Reduced surface area, increased mechanical instability, and decreased spectrin content of the membrane, distinguishing features of RBCs in hereditary spherocytosis, were observed in cells from the proband and his mother, but not in cells from other family members. These findings are consistent with the dominant inheritance of spherocytosis by the proband. We suggest that the combined effects of SC disease and spherocytosis in the proband resulted in decreased RBC deformability and led to increased splenic trapping, intrasplenic sickling, and, consequently, recurrent sequestration crisis. Marked clinical and hematologic improvement occurred from splenectomy. Thus, inheritance of interacting genetic defects, sickling hemoglobinopathy, and hereditary spherocytosis appear to be responsible for the unusual clinical manifestation of recurrent ASSC in this patient.

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nylpyrrolidone (PVP) from Sigma Chemical Co, St Louis, MO; and dextran from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents were of the highest grade available.

**Hematologic data.** Complete blood counts and RBC indices were determined using a Coulter S-Plus instrument (Coulter Electronics, Hialeah, FL). Identification of mutant hemoglobins was accomplished by starch gel electrophoresis at pH 8.9 and citrate-agar gel electrophoresis at pH 6.2.\textsuperscript{1,11} Quantitation of the percentages of mutant hemoglobins was achieved using high pressure liquid chromatography.\textsuperscript{12} Reticulocytes were enumerated after staining with New Methylene Blue (J.T. Baker Co, Phillipsburg, NJ).

**Determination of α-globin genotype.** The α-globin genotypes were determined by Southern blot hybridization and restriction endonuclease mapping of genomic DNA extracted from peripheral blood leukocytes.\textsuperscript{13} For these analyses, the restriction endonuclease Bgl II and the α-globin probe JW 101 were used.

**Measurement of cellular deformability.** Deformability of intact RBCs was measured using osmotic gradient ektacytometry, an assay in which whole-cell deformability is measured as a continuous function of suspending medium osmolality.\textsuperscript{14} For these measurements, we prepared gradients from two solutions of 4% PVP in phosphate-buffered saline, one adjusted to 50 mmOsm/kg and the other to 900 mmOsm/kg. The gradients were mixed in the first stage of the three-stage mixing chamber of a Beckman gradient former (Beckman Instrument Co, Palo Alto, CA), Packed RBCs (70% to 80% hematocrit) were pumped into the second stage of the chamber by a Harvard infusion pump (model no. 802, South Natick, MA) and mixed with the gradient to a final hematocrit of 0.2%. Thorough mixing was ensured by passage of the cell suspension through the third stage of the mixing chamber. The suspension was then pumped through a Wescan conductivity meter (Wescan Instruments, Santa Clara, CA) to continuously monitor its conductivity, and finally into the ektacytometer for measurement of cellular deformability, at a constant shear stress of 170 dynes/cm². The osmolality at which the deformability index (DI) reaches a minimum in the hypotonic region of the gradient has been shown to be the same as the osmolality at which 50% of the cells will hemolyze in a standard osmotic fragility test.\textsuperscript{14} This point provides a measure of the average surface area-to-volume (SA/V) ratio of the population of cells studied. Cells attain their maximally deformed state at the physiologically relevant osmolality of 290 mmOsm. In the presence of normal membrane deformability, this maximum value of the DI has previously been shown to be related quantitatively to the membrane surface area.\textsuperscript{14} The hypertonic region of the curves provides information on the state of cell hydration.

**Mechanical stability measurements.** Resealed membranes for stability measurements were prepared by a procedure adopted from Johnson.\textsuperscript{15} RBCs were washed three times in 5 mmol/L Tris, 140 mmol/L NaCl (pH 7.4), and then lysed in 40 vol/vol of 5 mmol/L Tris, 7 mmol/L NaCl (pH 7.4). Membranes were then pelleted by centrifugation, resuspended in 10 vol/vol of 5 mmol/L Tris, 140 mmol/L NaCl (pH 7.4), and incubated for 30 minutes at 37°C for rescaling.

For mechanical stability measurements, ressealed membranes were pelleted by centrifugation and 100 μL of 40% membrane suspension was mixed with 3 mL dextran (40,000 molecular weight (mol wt), 35 g/100 mL in 10 mmol/L phosphate buffer, pH 7.4, viscosity 95 centipoise), and subjected continuously to 750 dynes/cm² in the ektacytometer. Under this stress the membranes progressively fragment, generating undeformable spheres. This process is detected as a time-dependent decrease in the DI. The time required for the DI to decrease to 60% of its maximum value is termed T₆₀ and is taken as a measure of membrane stability.\textsuperscript{16}

**RBC membrane protein analysis.** RBC membranes were prepared by lysing 1 vol of thrice-washed packed RBCs with 40 vol of lysing buffer (5 mmol/L phosphate, 0.2 mmol/L DFP, pH 8.0). The resulting membrane pellet was washed three times with the lysing buffer. Washed membranes were dissolved in sodium dodecyl sulphate (SDS) and electrophoresed through 3.5% to 17% polyacrylamide slab gels using the Fairbanks buffer system.\textsuperscript{17,18} Slabs were stained with Coomassie Brilliant Blue R256 and destained until the background was colorless. Bands corresponding to spectrin and band 3 were excised, the dye was eluted in 25% (vol/vol) pyridine in water, and the absorbance of the spectrin eluate and the band 3 eluate were measured at 605 nm.\textsuperscript{17,18} The ratio of the absorbances (spectrin/band 3 ratio) for various patient membrane samples were normalized with those obtained for control membranes prepared at the same time and electrophoresed in alternate lanes of the same gels.

**RESULTS**

Relevant hematologic data obtained on each family member and on the proband are shown in Table 1. Examination of the hemoglobin and reticulocyte values reveals that the proband and his mother suffer from hemolysis of greater severity than the other family members. Moreover, the data imply that the severity of hemolysis in the proband is not related solely to his globin genotype (SC and −α/α), because hemolysis in two of his siblings with identical globin

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>α-genotypes</th>
<th>Hemoglobin Composition</th>
<th>Hemoglobin (g/L)</th>
<th>Reticulocytes (× 10⁶%)</th>
<th>MCV (fl)</th>
<th>MCHC (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>β</td>
<td>% A</td>
<td>% S</td>
<td>% C</td>
<td>(normal &lt;100) (Adult)</td>
</tr>
<tr>
<td>Father</td>
<td>47</td>
<td>αα/αα</td>
<td>β β</td>
<td>61</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Mother</td>
<td>38</td>
<td>−α/αα</td>
<td>β β</td>
<td>64</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Proband</td>
<td>14</td>
<td>−α/αα</td>
<td>β β</td>
<td>0</td>
<td>56</td>
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<td>Baseline</td>
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<tr>
<td>ASSC</td>
<td></td>
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<tr>
<td>Postsplenectomy</td>
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<td>120</td>
</tr>
<tr>
<td>Brother</td>
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<td>−α/αα</td>
<td>β β</td>
<td>0</td>
<td>52</td>
<td>43</td>
</tr>
<tr>
<td>Sister</td>
<td>12</td>
<td>−α/αα</td>
<td>β β</td>
<td>0</td>
<td>53</td>
<td>44</td>
</tr>
<tr>
<td>Sister</td>
<td>16</td>
<td>−α/αα</td>
<td>β β</td>
<td>67</td>
<td>0</td>
<td>31</td>
</tr>
</tbody>
</table>

The α-thalassemia-2 genotype was demonstrated by gene mapping studies to consist of the 3.7-kb "rightward deletion."
genotypes is less severe. Normal values for serum iron, iron binding capacity, and ferritin, as well as a negative direct antiglobulin (Coombs) test, ruled out iron deficiency and an immune hemolytic process as causes for the greater severity of anemia in the proband (data not shown).

The proband’s peripheral blood film revealed several “sickle” forms, occasional microspherocytes, and very few target cells (Fig 1). These findings are atypical for hemoglobin SC disease, as fewer sickle forms and more target cells are usually seen. The proband's blood film could not be considered diagnostic for one of the classic membranopathies (hereditary spherocytosis, elliptocytosis, or pyropoikilocytosis). In comparison, the mother’s blood film showed more numerous microspherocytes than the proband (not shown).

Deformability of RBCs from the proband, his parents, and a sibling with identical globin genotype are shown in Fig 2. Deformability profiles for whole blood from these four members of the family were different from those obtained with blood from normal donors. The DI in the hypotonic region ($O_{\text{min}}$) was shifted to lower osmolality for RBCs from the father and brother, indicating that these cells had an increased SA/V ratio (osmotic resistance). The maximally attained DI (maximum height of the curve $-D_{\text{I}_{\text{max}}}$) was in the normal range, indicating that these RBCs had normal membrane surface area. This implies that the increase in SA/V is the result of decreased cell volume. The deformability profile of RBCs from the mother, on the other hand, exhibited a shift in $O_{\text{min}}$ to a higher osmolality and a decreased $D_{\text{I}_{\text{max}}}$. This implies that these cells had a decreased SA/V (increased osmotic fragility) as a result of decreased surface area of the RBCs. In fact, the osmotic deformability profile of RBCs from the mother is diagnostic of hereditary spherocytosis. The deformability profile of RBCs from the proband is different from those of other family members. The $O_{\text{min}}$ was in the normal range implying normal osmotic fragility, while $D_{\text{I}_{\text{max}}}$ was decreased, suggesting reduced membrane surface area. A combination of decreased membrane surface area and decreased cell volume results in normal SA/V ratio for these RBCs. Thus, the characteristics of RBCs from the proband appear to represent a combination of changes seen in hemoglobin SC disease and spherocytosis (see Discussion).

The membrane mechanical stability of the various blood samples was quantitated by monitoring the decrease in the DI, at a constant applied shear stress, as a function of time (Fig 3). Since the DI is a measure of shear-induced membrane ellipticity, it decreases with time as the membrane fragments into nondeformable spheres. The fragmentation pattern of membranes prepared from RBCs obtained from the father and the sibling was the same as that of normal membranes. In contrast, membranes derived from RBCs of the proband and his mother fragmented more rapidly than normal membranes, implying that they exhibit decreased membrane mechanical stability. As the extent of decreased stability noted for these membranes is similar to that documented previously for membranes in hereditary spherocytosis with spectrin deficiency, we quantitated the spectrin content of these membranes (Table 2). The spectrin content of membranes prepared from RBCs of the proband and his mother was indeed decreased to 90% of normal, while that of membranes derived from RBCs of the proband’s father and sibling was normal. This combination of reduced cell surface area, decreased mechanical stability, and spectrin deficiency supports the diagnosis of a dominantly inherited form of hereditary spherocytosis in the proband.

During an episode of ASSC, the proband’s mean corpuscular volume (MCV) increased and mean corpuscular hemoglobin concentration (MCHC) decreased (Table 1), suggesting that a susceptible population of small, dense, RBCs was sequestered preferentially within the spleen.
RECURRENT ACUTE SPLENIC SEQUESTRATION CRISIS

PROBAND

Fig 2. Osmotic deformability profiles of RBCs from the proband, his parents, and a sibling with identical globin genotype (hemoglobin SC and −α/αα). Normal range is indicated by the stippled area.

DISCUSSION

The unusual occurrence of repeated ASSC in a young boy with hemoglobin SC disease and α-thalassemia-2 led us to consider whether other interacting genetic defects could account for this unusual clinical manifestation. Detailed studies of cellular and membrane characteristics enabled us to define hereditary spherocytosis, due to spectrin deficiency, as the additional abnormality. We suggest that the combined deleterious effects of hemoglobin SC and spherocytosis could have been responsible for the recurrent ASSC in this patient. The rationale for invoking a role for interacting genetic defects is based on the following arguments.

Although ASSC complicating hemoglobin SC disease has been described previously,2-5 such a clinical course, with five episodes occurring in childhood, is unprecedented. Concomitant α-thalassemia needs to be considered as an explanation for the proband’s distinct clinical and hematologic phenotype. While some investigators believe that concomitant α-thalassemia may increase the frequency of ASSC in sickle cell anemia,6-9 this is disputed.1 Moreover, such an association has not been claimed for hemoglobin SC. In fact, α-thalassemia does not appear to modify the clinical features of hemoglobin SC disease.10 In any case, α-thalassemia in conjunction with hemoglobin SC cannot account for our patient’s unusual clinical course because two siblings with identical α- and β-globin genotypes have not experienced ASSC. Recently, several cases of heterozygous β-thalassemia interacting with hereditary spherocytosis were reported, and reduction in the severity of the spherocytic phenotype was noted.22

Coinheritance of sickle cell trait,23-26 sickle cell anemia,27,28 and hemoglobin SC disease29 with hereditary spherocytosis has been reported rarely. Sequestration crisis, such as were seen in our patient, have not been documented in these instances.

The peculiar combination of three distinct disorders in our proband (hemoglobin SC disease, α-thalassemia-2, and hereditary spherocytosis) prompts speculation on how these might interact to produce the observed clinical phenotype of recurrent ASSC. It is known that the intraerythrocytic concentration of hemoglobin S is one of the most important factors in determining sickling.30 In the case of the proband, there are two factors that tend to increase the MCHC (and thereby the concentration of Hb S): hemoglobin SC causes RBC dehydration31,32 and hereditary spherocytosis is characterized by the presence of subpopulations of dense dehydrated cells.33 It is possible that within the splenic microcirculation, in which migration is delayed by spherocytosis,34 the proband’s poorly deformable hemoglobin SC RBCs were at higher risk of undergoing sickling and causing intravascular obstruction.

As indicated by the data in Table 1 and Fig 2, the proband, as compared with his Hb SC and −α/αα siblings, had smaller RBCs containing a higher concentration of Hb S and possessing a normal SA/V ratio. The proband’s increased Hb S concentration could account for the increased numbers of sickle forms seen on his blood film. Similarly, the normal SA/V ratio correlates with the paucity of target cells in his blood film.

The normal SA/V ratio of the proband implies that the conventional diagnostic test for spherocytosis, the documentation of increased osmotic fragility, may not be useful in such patients with coinheritance of spherocytosis and hemoglobin SC disease. This is because the combined effects of loss of cell surface area due to spherocytosis and decreased cell volume due to hemoglobin SC disease result in cells with normal SA/V ratio and, hence, presumably normal osmotic fragility. The osmotic deformability profile, on the other

Table 2. Spectrin Content of Membranes Derived From RBCs of Various Family Members

<table>
<thead>
<tr>
<th>Member</th>
<th>Spectrin to Band 3 Ratio (% of normal)</th>
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<tbody>
<tr>
<td>Proband</td>
<td>88%</td>
</tr>
<tr>
<td>Mother</td>
<td>91%</td>
</tr>
<tr>
<td>Father</td>
<td>100%</td>
</tr>
<tr>
<td>Sibling with identical globin genotype</td>
<td>99%</td>
</tr>
<tr>
<td>Normals</td>
<td>100% ± 3%</td>
</tr>
</tbody>
</table>

Fig 3. Membrane mechanical stability of RBCs from the proband, his parents, and a sibling with identical globin genotype (hemoglobin SC and −α/αα). Normal range is indicated by the stippled area.
hand, is able to distinguish spherocytosis in spite of a normal cell SA/V ratio by its ability to quantitate cell surface area independently. Confirmation of the inheritance of a membrane abnormality can then be established by documenting decreased mechanical stability and altered skeletal protein.

REFERENCES


ACKNOWLEDGMENT

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