Atypical Autohemolysis in Hereditary Spherocytosis as a Reflection of Two Cell Populations: Relationship of Cell Lipids to Conditioning by the Spleen

By G. R. Langley and C. H. Felderhof

HEREDITARY SPHEROCYTOSIS is characterized by the occurrence of a hemolytic anemia in at least two family members, the presence of spherocytes in the peripheral blood, increased osmotic fragilities of fresh and incubated blood, an increased autohemolysis partially corrected by supplementary glucose and complete clinical remission following splenectomy. Several cases lacking one of these major features of the disease have been recorded. Included among these variants of typical hereditary spherocytosis are patients without an affected parent, with normal osmotic fragility of fresh blood and failure of glucose to reduce the rate of spontaneous autohemolysis during incubation for 48 hours.

The two patients with chronic spherocytosis and a negative family history reported by Young et al. in whom, before splenectomy, glucose did not improve autohemolysis had a complete clinical remission after splenectomy. Following splenectomy the response to glucose during in vitro incubation of blood was similar to patients with typical hereditary spherocytosis. Dacie has made similar observations in three patients in different families. Only one of these patients had a positive family history.

This report describes observations on two patients in whom, before splenectomy, glucose did not reduce the degree of autohemolysis. No other affected individual was identified in incomplete family studies of one patient while the erythrocytes of an affected son of the second patient gave a typical response to glucose during incubation. Following splenectomy, which resulted in a complete clinical remission in both patients, glucose reduced the degree of autohemolysis. Studies into the mechanism by which glucose failed to partially correct autohemolysis suggested a population of cells were present in the peripheral blood which were unable to withstand prolonged in vitro incubation in spite of supplementary glucose. Following splenectomy this population of cells disappeared or at least were less frequent in the peripheral blood.

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Table 1.—Summary of Hematological Findings Before and After Splenectomy

<table>
<thead>
<tr>
<th>AGE</th>
<th>HEMOGLOBIN Gm./100 ml.</th>
<th>HEMATOCRIT %</th>
<th>RED CELL COUNT millions/mm.³</th>
<th>RETICULOCYTE COUNT %</th>
<th>MCV* µ³</th>
<th>MCHC %</th>
<th>MCH µg/m³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>23</td>
<td>10.6</td>
<td>14.3</td>
<td>Before</td>
<td>28</td>
<td>After</td>
<td>38.5</td>
</tr>
<tr>
<td>Case 2</td>
<td>71</td>
<td>11.2</td>
<td>15.6</td>
<td>Before</td>
<td>31.5</td>
<td>After</td>
<td>42.5</td>
</tr>
<tr>
<td>Case 3</td>
<td>32</td>
<td>14.7</td>
<td>—</td>
<td>Before</td>
<td>38.3</td>
<td>After</td>
<td>49</td>
</tr>
</tbody>
</table>

*Normal values for MCV with electronic counting in this laboratory are mean 96 µ³, range 87-105 µ³.
ATYPICAL AUTOHEMOLYSIS

Table 2.—Per Cent Autohemolysis After 48 Hours Incubation

<table>
<thead>
<tr>
<th></th>
<th>Without added glucose</th>
<th>Glucose 0.03M*</th>
<th>Adenosine triphosphate 0.03M*</th>
<th>Adenosine 0.03M*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before splenectomy</td>
<td>21</td>
<td>34.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days after</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>splenectomy</td>
<td>22.4</td>
<td>27.5</td>
<td>21.7</td>
<td>26.3</td>
</tr>
<tr>
<td>3 months after</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>splenectomy</td>
<td>10.4</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>April 11, 1965</td>
<td>23.3</td>
<td>39.1</td>
<td>24.4</td>
<td>21.0</td>
</tr>
<tr>
<td>April 21</td>
<td>19.5</td>
<td>30.0</td>
<td>23.6</td>
<td></td>
</tr>
<tr>
<td>April 26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Splenectomy May 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>May 13</td>
<td>27.5</td>
<td>10.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>August 8</td>
<td>33.8</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before splenectomy</td>
<td>23.5</td>
<td>12.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(son of Case 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After splenectomy</td>
<td>26.9</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Final concentration in whole blood.

METHODS

The hematologic methods used were as described by Dacie and Lewis. Red blood cell counts were performed by electronic counter. Cultures were taken from all incubations and failed to show bacterial growth in thiglycolate broth. Erythrocyte lipids were extracted under nitrogen from duplicate 3 ml. aliquots of red cells with chloroform and methanol as described by Reed et al. Lipid extracts, washed with methanol, chloroform and KC1 as described by Ways and Hanahan, were dried to constant weight, brought to known volume with benzene and aliquots analyzed for phosphorus and cholesterol. Iron was administered intravenously in the citrate form in a dose of 5 μg. Radioactivity of hemolysates was measured in a well type scintillation counter. Red cell survival was studied with radioactive sodium chromate as described.

CASE REPORTS

Case 1

A white Canadian housewife aged 23 years was referred to the Victoria General Hospital for investigation of anemia detected in the 9th month of her first pregnancy three months previously. She complained of mild fatigue. Jaundice had occurred at age 8. During the 5 years previous to this admission when she had been employed as a clerk in a pharmacy, a physician commented on several occasions that her sclera were icteric.

Examination revealed an apparently healthy young lady with scleral icterus. The spleen was firm and descended 4 cm. below the left costal margin on inspiration. Because of a paternity problem, only a few family members were available for study. Those studied were found to have normal hematologic findings. The laboratory studies on this patient are shown in Tables 1 and 2 and Figures 1, 2 and 3. At the time of admission, the hemoglobin was 10.6 Gm. per 100 ml.; the hematocrit 28 per cent and the reticulocyte count 30 per cent. The red cells were normochromic and many microspherocytes were present on the blood smear. The bone marrow was extremely hypercellular with a myeloid erythroid ratio of 0.5:1. Erythropoiesis was normoblastic and the marrow hemosiderin was increased. The serum bilirubin was 1.9 mg. per 100 ml.; the direct bilirubin 0.2 mg. per 100 ml. The Coombs' direct antiglobulin test was negative, the serum folic acid was 10.5 μg. per ml. Fresh and incubated osmotic fragility curves showed decreased resistance to hypotonic saline. Spontaneous autohemolysis was increased and was not reduced with supplementary glucose. The spleen was removed and was found to weigh 500 Gms. The
spleen was congested and the red cells were confined to Billroths cords. The sinuses were rather empty. Following splenectomy the hemoglobin increased to 14.3 Gm. per 100 ml. and the reticulocyte count fell to 0.6 per cent. At this time there were few spherocytes in the peripheral smear and the autohemolysis was largely corrected by glucose. She has maintained a normal hemoglobin and reticulocyte count during the two years since splenectomy.

**Case II**

A 71 year old farmer was admitted to the Victoria General Hospital because of difficulty in voiding. This symptom had first occurred two years previously and had been increasing in severity. Recently he had noticed mild exertional dyspnoea.

Thirty years previously, a physician commented that the patient had an enlarged spleen and on a few occasions subsequently this finding had been confirmed. The patient knew his family were susceptible to "splenic anemia." A brother in 1922 and a sister in 1923 had required splenectomy in Toronto for familial hemolytic anemia* and were said to be well since. The patient had 2 children, one of whom was well. A 32 year old son was suspected by the family as being affected since they had observed scleral icterus on many occasions and episodes of marked fatigue had occurred.

Examination showed an elderly pale man with mild scleral icterus. The spleen was firm and extended 7 cm. below the left costal margin. He had bilateral hydroceles and an enlarged smooth prostate.

The laboratory studies are recorded in Tables 1 and 2 and Figures 1, 2 and 3. On admission, the hemoglobin was 11.2 Gm. per cent, the hematocrit was 31.5 per cent, the reticulocyte count was 15.6 per cent. Many spherocytes were present on the blood smear. The white cell count was 15,000/mm.³ with a shift to the left in the differential count. The bone marrow showed extreme hypercellularity with normoblastic erythropoiesis. The myeloid erythroid ratio was 1:1.5. Marrow hemosiderin was markedly increased. The Coombs' direct antiglobulin test was negative. The serum bilirubin was 3.4 mg. per 100 ml.; the direct bilirubin was 0.4 mg. per 100 ml. The urine urobilinogen was positive in a dilution of 1:40. Fresh and incubated osmotic fragility curves showed decreased resistance to hypotonic saline. Autohemolysis was increased and was not corrected by glucose.

A transurethral resection of the prostate was performed on April 12, 1965. There was a transient fall in the reticulocyte count during the first five post operative days (Table 2). The hemoglobin fell from 11.2 to 8.9 Gm. per 100 ml. during this period. This did not seem to be due to the minimal blood loss and was attributed to a mild suppression of erythropoiesis. Splenectomy was performed on May 5, 1965, and the spleen was found to weigh 1010 Gm. There was some congestion of dilated sinusoids but the cords were filled with blood. Following splenectomy, the reticulocytosis subsided and the hemoglobin rose to normal. Autohemolysis was increased in the post operative period but was now corrected by glucose. When seen again 3 months and 2 years later, he had a normal hemoglobin and reticulocyte count and only a few microspherocytes were present on the blood smear. The hematologic findings in a 33 year old son of this patient was normal.

**Case III**

Case III was the 32 year old son of Case II. He was initially examined at the time of admission of his father to hospital and found to have scleral icterus and a spleen palpable 4 cm. below the left costal margin. Laboratory studies at this time disclosed a hemoglobin of 14.2 Gm. per 100 ml. and a reticulocyte count of 10.6 per cent. Many spherocytes were present on the peripheral blood smear; the fresh and incubated osmotic fragility curves showed decreased resistance to hypotonic saline. Autohemolysis was increased and was partially corrected by glucose.

One year later this patient was referred by his family physician because his icterus was clinically more intense and a recent episode of marked fatigue had occurred from which he had recovered. On this occasion, the hemoglobin was 15.6 Gm. per 100 ml.; the

*Information kindly provided by Dr. Barbara Hazlett.
hematocrit 40.5 per cent; the reticulocyte count 30.5 per cent. The white cell count was 12,800/mm.$^3$ with a left shift in the differential count. Many spherocytes were present on the peripheral blood smear. Splenectomy was performed and the spleen was found to weigh 779 Gm. There was marked congestion of the pulp and some congestion of the sinusoids. Following splenectomy the reticulocytosis subsided and only occasional microspherocytes were present on the blood smear. When seen 1 year later, the hematocrit and reticulocyte count were normal.

**Results**

**Osmotic Fragility**

Both patients in whom glucose failed to partially correct autohemolysis had a large number of cells which were extremely susceptible to osmotic hemolysis. Between 40 to 50 per cent of their cells hemolized in concentrations of NaCl greater than 0.6 per cent. Case III had a smaller proportion of his cells which hemolized in this range (Figure 1). When the per cent increment in hemolysis was plotted against the concentration of NaCl bimodal curves were obtained (Figure 2). Following splenectomy, the population of cells which displayed greater sensitivity to osmotic lysis had largely disappeared.

**Autohemolysis**

In both Case I and Case II, autohemolysis was not improved by supplementary glucose and in both patients glucose seemed to result in increased lysis. Neither adenosine triphosphate or adenosine had a corrective effect on hemolysis. When the plasma supplemented with glucose was exchanged every 12 hours in Case I, hemolysis was not increased over that observed with non supplemented incubations. In one experiment in Case II, the pH of the serum after 48 hours incubation with supplementary glucose added, was 6.7. Lactic acid, added to fresh whole blood without added glucose to produce a similar pH, resulted in a 38 per cent reduction in autohemolysis after 48 hours of incubation.
Fig. 2.—Increment hemolysis curves before (A) and after (B) splenectomy. The extremely fragile population of erythrocytes in Cases I and II and to a lesser extent in Case III which hemolized between 0.85 and 0.65 per cent NaCl have largely disappeared after splenectomy.

Following transurethral resection of the prostate in Case II the hemoglobin fell over a 5 day period from 11.2 Gm. per 100 ml. to 8.9 Gm. per 100 ml. and there was a fall in the reticulocyte count. Thereafter, the hemoglobin rose spontaneously to 11.5 Gm. per 100 ml. During this period, several studies showed that although glucose failed to correct autohemolysis the previously observed increased hemolysis with glucose was not as marked. Glucose did not partially correct autohemolysis 2 days post splenectomy in Case I but by 8 days in Case II a corrective effect was apparent. In both patients a typical response was obtained when the blood was studied 3 months after splenectomy. The results of autohemolysis in the son of Case II were considered typical of hereditary spherocytosis before and after splenectomy.

The shape of the osmotic fragility and increment hemolysis curves indicated both Case I and II had a large population of cells which were extremely susceptible to hypotonic saline. To study these two populations further, 5 μc of 59Fe citrate was administered intravenously to the second patient on April 16th, four days after the transurethral resection of the prostate. This was at the end of the period when a transient mild suppression of erythropoiesis was suspected. The specific activity of the hemoglobin of whole blood five days later was 3126. One volume of these cells was hemolized in
ATYPICAL AUTOHEMOLYSIS

Table 3.—Autohemolysis of Erythrocytes Resistant to Hemolysis in 0.55 per cent NaCl* compared with autohemolysis of Whole Blood After 48 Hours Incubation

<table>
<thead>
<tr>
<th></th>
<th>Per cent autohemolysis of erythrocytes resistant to hemolysis in 0.55 % NaCl</th>
<th>Per Cent autohemolysis of whole blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without added glucose</td>
<td>15.8</td>
<td>28.5</td>
</tr>
<tr>
<td>With added glucose (0.03M)</td>
<td>8.0</td>
<td>30</td>
</tr>
</tbody>
</table>

*One volume of fresh whole blood was added to 20 volumes of 0.55 per cent NaCl and incubated at 37 C. for 30 minutes. 26 per cent hemolysis occurred under these circumstances. The supernatant was removed after centrifugation and the remaining cells resuspended in their own serum to a hematocrit equal to whole blood and incubated at 37 C. for 48 hours.

20 volumes of 0.55 per cent NaCl. The supernatant was removed after centrifugation, an aliquot was counted for radioactivity and the remaining cells were suspended in their own serum to a similar hematocrit as whole blood and incubated 48 hours with and without added glucose. Whole blood incubations were carried out simultaneously. There was substantially less hemolysis in the more osmotically resistant cells and glucose had a corrective effect (Table 3). The hemolysis of whole blood incubated without glucose was 28.5 per cent in this experiment. Since only 15 per cent hemolysis occurred in the 74 per cent of cells which were osmotically less fragile (Table 3), 68 per cent of the most osmotically fragile cells must have autohemolized during incubation of whole blood.* In whole blood incubations with added glucose however, 90 per cent of the more fragile population of cells must have autohemolized.

The hemoglobin of intact erythrocytes recovered after hemolysis in 0.55 per cent NaCl had a higher specific activity indicating the more resistant cells were a younger population (Table 4). The specific activity of the hemoglobin from erythrocytes which had hemolyzed after 48 hours incubation of whole blood without added glucose was similar to the specific activity of the hemoglobin of the most fragile erythrocyte population suggesting that during incubation an older and more fragile population of cells were undergoing lysis. Although slightly more hemolysis occurred with glucose, the hemoglobin had a lower specific activity than that of incubations without glucose. This suggested that the increased hemolysis observed with glucose was due to more hemolysis in an older cell population.

*Per cent autohemolysis in whole blood of the most osmotically fragile population

\[ H_w \times \frac{(r - H_r)}{H_r} \]

Where

- \( H_w \) = per cent autohemolysis whole blood;
- \( r \) = per cent of cells remaining after exposure to 0.55 per cent NaCl;
- \( H_r \) = per cent autohemolysis of cells recovered from 0.55 per cent NaCl;
- \( H_r \) = per cent of cells which hemolized in 0.55 per cent NaCl.
Table 4.—Specific Activity of Fe59 Hemoglobin in Osmotically Resistant and Fragile Erythrocytes and in Erythrocytes Which Autohemolized

<table>
<thead>
<tr>
<th>SPECIFIC ACTIVITY cpm/Gm. Hemoglobin</th>
<th>PER CENT Autohemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td></td>
</tr>
<tr>
<td>Serum from 48 hour incubations</td>
<td></td>
</tr>
<tr>
<td>without added glucose</td>
<td>1988</td>
</tr>
<tr>
<td>with added glucose</td>
<td>1629</td>
</tr>
<tr>
<td>Erythrocytes recovered after*</td>
<td>3475</td>
</tr>
<tr>
<td>hemolysis in 0.55% NaCl.</td>
<td></td>
</tr>
<tr>
<td>Supernatent of erythrocytes*</td>
<td>2078</td>
</tr>
<tr>
<td>which hemolized in 0.55% NaCl.</td>
<td></td>
</tr>
</tbody>
</table>

*1 volume fresh whole blood added to 20 volumes 0.55 per cent NaCl and incubated 30 minutes at 37 C. Aliquots of the erythrocytes which hemolized and those which remained intact after this exposure counted for radioactivity. 26 per cent of erythrocytes hemolized under these circumstances. See also Table 3.

Erythrocyte Life Span and Splenic Sequestration

The survival and sites of sequestration of 51Cr labeled erythrocytes from Cases I and II are shown in Figure 3. An initial phase of rapid destruction is evident from both curves. Approximately 50 per cent of the cells in both patients were removed during this initial period of rapid destruction. The cells were sequestrated in the spleen and no appreciable uptake occurred in the liver.

Erythrocyte Lipids

The lipid content of erythrocytes of the patients before and after splenectomy and the effect on erythrocyte lipid composition following splenectomy of 24 hours of incubation without added glucose is shown in Table 5. For comparison similar data for three patients who were considered typical hereditary spherocytosis1 is shown. Before splenectomy, the total lipid, the lipid phosphorus and cholesterol content of the cells were decreased in spite of reticulocytosis in all patients. After splenectomy, the total lipid content of the erythrocytes had increased to within the normal range although the cell cholesterol was slightly above normal. When normal erythrocytes were incubated for 24 hours without additive there was no change in lipid phosphorus content but a 9.2 per cent loss of cholesterol occurred. This decrease of cholesterol was significant (p < 0.01). During a similar period of incubation, hereditary spherocytosis erythrocytes lost 16 per cent of their total lipid and lipid phosphorus and 19 per cent of their cholesterol. The pattern of lipid loss observed in Case I and II with the atypical autohemolysis response before splenectomy is similar to that seen in Case III who demonstrated the usual response. The pattern of lipid loss in all three patients are similar to that observed in the three patients with typical hereditary spherocytosis.

In Case I cell lipids were determined on the 60 per cent of red cells which survived hemolysis in 0.60 per cent NaCl and are shown in Table 5. The
osmotically resistant cells had 6 per cent more lipid phosphorus and cholesterol. These osmotically resistant cells had a slightly lower mean cell hemoglobin.

**DISCUSSION**

The two patients described in whom glucose failed to correct autohemolysis demonstrated several of the other features of hereditary spherocytosis including spherocytosis of the peripheral blood, increased osmotic fragility of fresh and incubated blood and complete clinical remission after splenectomy. No other affected family member was identified in incomplete family studies of one patient although this is not a rare finding in patients with otherwise typical hereditary spherocytosis. An affected son of the second patient had an autohemolysis pattern similar to typical hereditary spherocytosis. The lipid loss pattern observed in these patients after splenectomy, while not unique to erythrocytes from patients with hereditary spherocytosis, was similar in degree to that seen in this disease.

The significant difference between the two patients (Cases I and II)
Table 5.—Erythrocyte Lipids Before and After Splenectomy and After Incubation for 24 Hours Without Added Glucose

<table>
<thead>
<tr>
<th></th>
<th>BEFORE SPLENECTOMY ERYTHROCYTE LIPID</th>
<th>AFTER SPLENECTOMY ERYTHROCYTE LIPID</th>
<th>AFTER SPLENECTOMY ERYTHROCYTE LIPID INCUBATED 24 HOURS WITHOUT ADDITIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TOTAL LIPID mg. x 10⁻⁶ per cell</td>
<td>LIPID PHOSPHORUS mg. x 10⁻⁶ per cell</td>
<td>CHOLESTEROL mg. x 10⁻⁶ per cell</td>
</tr>
<tr>
<td>Case I</td>
<td>3.92</td>
<td>2.45</td>
<td>0.74</td>
</tr>
<tr>
<td>Case II</td>
<td>3.60</td>
<td>10.12</td>
<td>0.83</td>
</tr>
<tr>
<td>Case III</td>
<td>3.52</td>
<td>9.15</td>
<td>0.81</td>
</tr>
<tr>
<td>Typical H.S.</td>
<td>—</td>
<td>5.67</td>
<td>12.18</td>
</tr>
<tr>
<td>Typical H.S.</td>
<td>5.52</td>
<td>12.14</td>
<td>1.52</td>
</tr>
<tr>
<td>Normal</td>
<td>5.05</td>
<td>12.79</td>
<td>1.09</td>
</tr>
<tr>
<td>N = 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>±0.24</td>
<td>±1.3</td>
<td>±0.08</td>
</tr>
</tbody>
</table>

*The normal values are for erythrocytes from normal individuals with intact spleens incubated 24 hours without additive, 18, 19
Table 6.—Lipids of Whole Blood and the Osmotically Resistant Red Cell Population

<table>
<thead>
<tr>
<th></th>
<th>RETICULOCYTES</th>
<th>MCH</th>
<th>LIPID PHOSPHORUS</th>
<th>CHOLESTEROL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per Cent*</td>
<td>mgm. /cell</td>
<td>µgm. x 10^{-6}/cell</td>
<td>mg. x 10^{-6}/cell</td>
</tr>
<tr>
<td>Whole blood</td>
<td>30</td>
<td>31.5</td>
<td>9.35</td>
<td>0.84</td>
</tr>
<tr>
<td>Cells resistant to 0.60% NaCl</td>
<td>45</td>
<td>29.3</td>
<td>10.03</td>
<td>0.90</td>
</tr>
</tbody>
</table>

12 cc of fresh whole blood was suspended in 300 cc of 0.6 per cent NaCl and hemolysis allowed to occur. The cells which failed to hemolize were then recovered. 40 per cent hemolysis occurred under these circumstances.

*The reticulocyte count for whole blood was performed on defibrinated blood in serum, all other values were obtained on washed cells. The reticulocyte count for washed whole blood was inadvertently omitted.

and patients with typical hereditary spherocytosis was the inability of glucose to correct autohemolysis before splenectomy. The studies reported here suggest that this was due to the behaviour of one of two populations of erythrocytes in the peripheral blood. Two populations of erythrocytes in both patients were demonstrated in several of the studies carried out. In both patients the erythrocyte survival curves showed a population of cells which were rapidly removed from the circulation and a second population with a more prolonged survival. The increment hemolysis curves gave a bimodal pattern separating the erythrocytes into almost equal numbers of extremely fragile and a less fragile population. This more fragile cell population appeared to be somewhat older. The more osmotically resistant population on the other hand appeared to be younger and displayed the autohemolysis response seen in classical hereditary spherocytosis. Following splenectomy the more osmotically fragile population of cells could no longer be demonstrated and the autohemolysis response was similar to hereditary spherocytosis. Griggs et al. employed 59Fe cohort labeling of red cells before splenectomy to identify the age of the most osmotically fragile population. They observed that the younger cells were less fragile and after a period of about 10 days the osmotic fragility of the 59Fe labeled population approximated that of whole blood.

Two cell populations are frequently demonstrable both in the peripheral blood and the spleen in patients with typical hereditary spherocytosis. Thus, Dacie and coworkers recovered red cells from the spleen of two patients and retransfused them after splenectomy. There was an initial rapid loss of cells from the circulation, the remaining cells having a considerably longer survival. The pattern of survival of the cells recovered from
the spleen in their patients was similar to that observed with Chromium\textsuperscript{51} labeled peripheral blood cells in the two patients studied here. Emerson et al. observed that following splenectomy, the very fragile population of cells was reduced in the peripheral blood and increment hemolysis curves gave a unimodal distribution. The accumulation of this fragile population of cells in the peripheral blood prior to splenectomy was felt to be due to the continued trapping and release of these cells by the spleen.\textsuperscript{31} The degree to which this "conditioned" fragile red cell population is present in the peripheral blood in hereditary spherocytosis appears to vary from patient to patient. Dacie\textsuperscript{2} has observed, however, that individuals with hereditary spherocytosis seem to retain their own characteristic type of osmotic fragility curve for long periods and affected family members often have the same type of curve.

Autohemolysis studies in patients with typical hereditary spherocytosis indicate that glucose is able to reduce the amount of hemolysis. The fact that substantial degrees of hemolysis often persist however, in spite of supplementary glucose, is evidence that some cells are not able to withstand the prolonged incubation. In the two patients reported here it appears that a larger population of cells which were unable to survive in vitro incubations and which had extreme osmotic fragility have accumulated in the peripheral blood. In both patients hemolysis was very slightly increased by supplementary glucose. The reason for this increase also observed by Young and colleagues\textsuperscript{15} is not clear. It did not appear to be due to the low pH. since lactic acid added to incubations to simulate this effect actually decreased autohemolysis.

An explanation for the accumulation of such a large population of fragile cells in the circulation of these patients is not apparent. The lesion in hereditary spherocytosis could have been more severe in these patients. However, if so, the more severely damaged cells were not removed by the spleen and accumulated in the blood. It is known that although the spleen enlarges in response to the work load, the process appears to be self limited.\textsuperscript{33} In these two patients, the spleen appeared not to have the capacity to handle the work, in the form of damaged erythrocytes, imposed upon it. This resulted in large numbers of extremely fragile cells, of the type usually found primarily in the spleen, being detected in the peripheral blood. The fact that spleen did not remove all of these severely damaged cells may have imposed a limit on the degree of hemolysis and on the severity of the anemia.

In addition to their greatly increased osmotic fragility and inability to withstand incubation, these conditioned cells were characterized by a decreased content of surface lipid. This decreased lipid content of the red cells reflected a decrease in both cholesterol and lipid phosphorus when related to normal cells or their own cells after splenectomy. If the decreased lipid content of the erythrocytes occurred as they were conditioned by the spleen, it would appear that cholesterol loss was proportionately greater...
than that of lipid phosphorus, assuming the lipid was lost from cells with a cholesterol and lipid phosphorus content similar to their own cells after splenectomy. The erythrocyte population presplenectomy however was characterized by an increased reticulocyte count. Hallinan and Eden\textsuperscript{34} observed a proportionately greater increase in phospholipid in rat reticulocytes, due they felt, to an increase of intracellular phospholipid. It is therefore possible that a significant proportion of the cell lipid phosphorus presplenectomy was intracellular. The decreased cell surface lipid pattern could therefore have reflected either proportionate losses of cholesterol and phospholipid or greater losses of the latter. It was shown by Griggs et al.\textsuperscript{27} and supported by these studies that the more osmotically resistant red cell population is younger. These osmotically resistant (younger) cells had more lipid and cholesterol than the total cell population which contained both resistant and fragile cells. The decrease in cell lipid was due to a proportionate decrease in both lipid phosphorus and cholesterol.

These conditioned, fragile, lipid depleted cells were smaller with a mean cell volume 18 per cent below mean normal in spite of reticulocytosis, findings similar to those reported by Guest.\textsuperscript{35} On the basis of Guest's measurements the surface area of the erythrocytes in hereditary spherocytosis was 16 per cent less than normal cells. In spite of the substantial decrease in surface lipids, decrease in volume and surface area in the patients erythrocytes before splenectomy intracellular hemoglobin was within the normal range although slightly lower than that observed in their own cells after splenectomy. The osmotically resistant cells with greater cell lipid content did not have more intracellular hemoglobin perhaps because in this high reticulocyte population, intracellular hemoglobin synthesis had not been completed. The decrease in cell lipid in the whole cell population compared with the younger, osmotically resistant cells, occurred however without a decrease in intracellular hemoglobin. This suggested some cell lipid loss in vivo in hereditary spherocytosis, may occur without a corresponding loss of hemoglobin molecules. Large losses of cholesterol\textsuperscript{26} or lipid phosphorus\textsuperscript{37} or both\textsuperscript{19,21,22} have been observed from erythrocytes in other situations without recognizable change in other major structural components or intracellular hemoglobin. The incubated post splenectomy erythrocyte lost slightly more cholesterol than lipid phosphorus. Dissociation of the relative proportions of cholesterol and lipid phosphorus lost from the hereditary spherocyte after splenectomy has been observed in some\textsuperscript{26,38} but not all in vitro studies.\textsuperscript{11,20} This variability in in vitro results may be due to the conditions under which cells are incubated.\textsuperscript{36}

The relationship of this erythrocyte lipid loss to the in vivo survival of the cell and the fundamental defect in hereditary spherocytosis is of interest. The lipid content of cells after splenectomy when hemolysis had subsided was not decreased. In spite of this, the cells were abnormal as reflected by the persistence of an increased osmotic fragility. This persistence of increased osmotic fragility after splenectomy, has been repeatedly, though
not invariably observed in hereditary spherocytosis, in spite of the fact that these post splenectomy cells do not have a quantitative decrease in membrane lipid or protein content. These findings suggest therefore that lipid loss follows increased osmotic fragility and is a later manifestation of an underlying membrane defect. The increased osmotic fragility after splenectomy apparently does not reflect an increase in total intracellular sodium plus potassium. It may be noted however that Weed and Bowdler found the intracellular sodium content of the post splenectomy hereditary spherocyte to be 20 per cent greater and the potassium content 9 per cent less in 5 patients compared with 10 normal subjects although the difference was not significant (p > 0.2). In view of the finding of increased membrane permeability to sodium and the correlation of increased intracellular sodium with decreased survival, this difference, although not statistically significant, is of interest. Guest observed a decreased critical hemolytic volume in hereditary spherocytosis erythrocytes before splenectomy. He found that the critical hemolytic volume, decreased in one patient before splenectomy, increased to within the normal range after splenectomy while Weed and Bowdler found decreased critical hemolytic volume in their patients after splenectomy.

Comparison of these studies on the hereditary spherocyte before and after splenectomy suggests that an indication of early membrane change as observed in fresh cells after splenectomy is increased osmotic fragility unaccompanied by any change in total Na+ plus K+ or quantitative decrease in surface lipid or protein. At this time also, there is increased membrane permeability to sodium, a decreased critical hemolytic volume and in some patients an increase in cell cholesterol is apparent. Later lipid loss from the cell membrane occurs and sodium and potassium permeability becomes markedly abnormal. The fundamental defect in the membrane which is manifested by increased osmotic fragility, decreased critical hemolytic volume, increased membrane permeability and a propensity for the cell to lose surface lipid and sequester in the spleen is unknown. A major structural component of the membrane is protein. Although quantitatively the protein does not appear to be decreased in hereditary spherocytosis erythrocytes after splenectomy when osmotic fragility is increased a qualitative abnormality in protein is possible. Whatever the underlying defect in this cell, it appears that the mechanism by which survival is impaired probably is due to the changes observed in membrane function and integrity.

**Summary**

Two patients with hereditary spherocytosis had increased autohemolysis not corrected by supplementary glucose, while an affected son of the second patient had a typical autohemolytic response with glucose. Almost one half of the circulating red cells in both patients had markedly increased osmotic fragility. When these were eliminated in vitro, by selective osmotic hemolysis, or in vivo by splenectomy, the autohemolysis response was corrected.
ATYPICAL AUTOHEMOLYSIS

by glucose. The loss of this large population of extremely fragile cells after splenectomy suggested they were the product of conditioning by the spleen.

In addition to having decreased osmotic resistance, an autohemolysis not corrected by glucose, and a short in vivo survival, these conditioned cells were smaller and had a considerable decrease in cell lipid. After splenectomy, with the loss of the most fragile cell population, cell size and lipid content increased. However in spite of no decrease in cell lipid after splenectomy, osmotic fragility remained abnormal. Increased osmotic fragility may be one of the earliest manifestations of a membrane abnormality in hereditary spherocytosis.

SUMMARIO IN INTERLINGUA

Duo patientes con spherocytosis hereditari manifestava evidentia de un augmentate autohemolyse que non esseva corrigite per glucosa supplementari, durante que un equalmente affice filio del secunde del duo patientes monstrava un typic responsa autohemolytic a glucosa. Quasi un medietate del erythrocytos circulante in ambe le patientes possedeva marcamente augmentate fragilitates osmotic. Quando iste erythrocytos esseva eliminate in vitro per medio de un selective hemolyse osmotic o in vivo per medio de splenectomy, le responsa autohemolytic esseva corrigite per glucosa. Le perdita de iste population de extrememente fragile cellulas post splenectomy suggestionava que iste cellulas esseva el producto de un conditionamento per le splen.

A parte le facto que iste conditionate cellulas habeva un reduce resistentia osmotic, un responsa autohemolytic non corrigite per glucosa, e un breve supervivientia in vivo, illos etiam esseva plus micre e habeva un considerabilemente reducece contento de lipid cellular. Post splenectomy, resultante in le perdita del plus fragile population cellular, le dimensiones cellular e le contento lipidic del celular accresceva. Tamen, in especto del facto que de splenectomy resultava in nulle declino in le contento lipidic del celular, le fragilitate osmotic remaneva abnormal. Un augmentate fragilitate osmotic es possibilemente un del plus precoce manifestationes de un abnormalitate membranal in spherocytosis hereditari.

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REFERENCES

34. Hallinan, T., and Eden, E.: The Structure and Composition of Rat reticuloocytes. II. Phospholipid and total cholesterol


Atypical Autohemolysis in Hereditary Spherocytosis as a Reflection of Two Cell Populations: Relationship of Cell Lipids to Conditioning by the Spleen

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