ROLE OF LIPIDS IN STABILIZING RED CELLS IN RATS

Sueko Sagawa and Keizo Shiraki
Department of Nutrition, School of Medicine, Tokushima University, Kuramoto-cho, Tokushima 770, Japan
(Received June 13, 1977)

Summary The causes of osmotic fragility of red cells were studied in rats. Osmotic fragility of red cells in vivo changed after removal of the spleen or induction of experimental splenomegaly by repeated intraperitoneal injections of methyl cellulose (MC): in splenectomized rats, the red cells showed reduced osmotic fragility and an increase in diameter as well as in contents of phospholipids and cholesterol. Conversely in rats with splenomegaly, the cells showed increased osmotic fragility and a decrease in diameter and in lipid contents.
Results confirmed that increase in the phospholipid content resulted in decreased fragility and that increase in the cholesterol content brought about decreased spherocytosis. The activity of lecithin cholesterol acyltransferase (LCAT) in the plasma varied inversely with the cholesterol content of the red cells.
The above results show that the fragility of red cells is influenced by their lipid content and shape, and that LCAT activity in the plasma influences the membrane content of cholesterol and spherocytosis.

Considerable changes have been observed in the osmotic fragility of red cells of anemic patients with protein calorie malnutrition (PCM) (11) and experimental animals fed on protein-free diets for a long period (9). The anemia has been attributed to a shortening of the red cell survival time and disturbance of effective hematopoiesis. However, it is uncertain how closely the survival time is related to the osmotic fragility of the red cells because increased osmotic fragility does not always result in shortening of the survival time of red cells in PCM (18).

Fragility of red cells is influenced by many factors, such as changes in the structure and composition of the cell membrane, the composition of the plasma, and splenic function. Previously we reported a close relation between splenic function and fragility of red cells: decreased fragility and an increased survival time of red cells were observed in splenectomized rats (9).
The red cell membrane is composed of complexes of protein and lipid. The
protein seems to be a stable structural component, but the membrane lipids are in equilibrium with lipoproteins in the plasma (4, 16). Therefore, the lipid content of the red cell membrane is affected by that of the plasma. Cooper and Jandle (1) observed that the red cells of patients with obstructive jaundice were flat ("target cells") and osmotically resistant and that their cholesterol content was increased. McBride and Jacob (12) observed an increase in the cholesterol content of red cell membranes with decrease in their osmotic fragility in several syndromes with formation of spur cells. From clinical observations, these investigators speculated that accumulation of red cell cholesterol might be associated with conformational changes of the structural lipoproteins of red cells leading to decreased osmotic fragility of the cells.

In the present study, we examined the factors causing osmotic fragility of rat red cells. Red cells with different osmotic fragilities were obtained from rats with splenectomy or splenomegaly. Splenomegaly was induced by repeated intraperitoneal injections of methyl cellulose (MC), and both intact and splenectomized rats were treated with MC (MC-treated rats). Then the osmotic fragilities, lipid contents, and diameters of the red cells and the lecithin cholesterol acyltransferase (LCAT) activities in the plasma of these four groups (i.e., intact and splenectomized rats with and without MC-treatment) were compared.

MATERIALS AND METHODS

1. Animals. Twenty-five male Wistar rats, weighing 250 to 300 g, were used. They were housed in individual cages with a temperature maintained at 24°C and fed on synthetic standard diet (20% casein). The animals were divided into four groups and their respective treatments and subsequent experimental protocols are shown in Fig. 1. Splenectomy was performed under ether anesthesia seven days before the first injection of MC. Rats were given intraperitoneal injections of 2 ml of 2.5% MC twice a week for five weeks by the method of Palmer et al. (15). Three days after the last injection, all animals were killed by heart

Fig. 1. Experimental schedules. Asterisks indicate the days of intraperitoneal injections of the methyl cellulose (50 mg/day). Numbers in parentheses are numbers of rats examined.
puncture under ether anesthesia and their liver and spleen were rapidly removed and weighed.

2. **Osmotic fragility of the red cells.** Osmotic fragility of red cells was measured by a modification (9) of the method of DACIE and VAUGHAM (2). Results were expressed as the concentration of NaCl giving 50% hemolysis, here designated the half hemolysis rate (HHR).

3. **Measurement of the diameter and spherical index of the red cells.** The diameter of the red cells was measured with an ocular screw micrometer in preparations stained with Wright's solution. At least 100 cells were measured in each preparation and the mean value was taken as the diameter of the cells.

The mean corpuscular thickness of the cells was estimated from the following formula, assuming that each cell was a short cylinder (21):

\[
\text{Mean corpuscular thickness} = \frac{\text{Mean corpuscular volume}}{\pi \left(\frac{\text{Mean cell diameter}}{2}\right)^2}
\]

The mean corpuscular volume was calculated by dividing the hematocrit value by the red cell count. The ratio of the mean corpuscular thickness to the mean cell diameter was named the spherical index of the red cells in estimating spherocytosis. Other routine measurements of the blood were carried out by standard methods.

4. **Measurement of lipids.** Heparinized blood was centrifuged at 1,500 g for 10 min at 5°C and then the red cells were washed three times with cold isotonic phosphate buffer (pH 7.4) to remove white cells and platelets. The washed red cells were resuspended in 0.9% NaCl at a density corresponding to the original hematocrit value, and samples were taken to measure the packed cell volume and lipid content. Lipids were extracted from the red cells with a mixture of two volumes of chloroform and one volume of methyl alcohol (5), and portions of the extracts were used to measure total cholesterol, free cholesterol (22), and total phospholipid (17). Values for cell lipids were expressed on the basis of the volume (in ml) of packed cells.

5. **Measurement of plasma LCAT activity.** LCAT activity in the plasma was measured with labeled substrate by the method of GLOMSET and WRIGHT (7). A mixture of 7-α-³H-cholesterol-albumin (Radiochemical Centre, England: specific activity of 32.7 mCi/mg), heated plasma, and fresh plasma (1: 8: 1, v/v) was incubated for 5 hr at 37°C. Then free and esterified cholesterol were separated by thin-layer chromatography, and the radioactivity of each was counted in a liquid scintillation spectrometer (Packard, Tricarb 3300, U.S.A.) to estimate the cholesterol-esterifying activity in the plasma.

LCAT activity was calculated by multiplying the plasma concentration of free cholesterol by the cholesterol-esterifying activity, and results are expressed as µg cholesterol ester produced per ml plasma per hour.
RESULTS

The body and organ weights of control and MC-treated rats are shown in Table 1. Treatment with MC increased the average weight of the spleen to about four times the control value, and also increased the weight of the liver.

Table 1. Body and organ weights of control and MC-treated rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Condition</th>
<th>No. of rats</th>
<th>Initial wt. (g)</th>
<th>Final wt. (g)</th>
<th>Food intake (g/day)</th>
<th>Spleen % body wt.</th>
<th>Liver % body wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>5</td>
<td>177.8</td>
<td>288.1</td>
<td>14.4</td>
<td>0.18</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>intact</td>
<td></td>
<td>±12.1</td>
<td>±15.0</td>
<td>±0.5</td>
<td>±0.01</td>
<td>±0.15</td>
</tr>
<tr>
<td>2.</td>
<td>Control</td>
<td>5</td>
<td>185.2</td>
<td>279.9</td>
<td>12.5</td>
<td>—</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td>splenex</td>
<td></td>
<td>±5.9</td>
<td>±14.8</td>
<td>±1.2</td>
<td>±0.11</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>MC-treated</td>
<td>9</td>
<td>198.0</td>
<td>256.7</td>
<td>14.3</td>
<td>0.79***</td>
<td>3.22***</td>
</tr>
<tr>
<td></td>
<td>intact</td>
<td></td>
<td>±9.5</td>
<td>±19.4</td>
<td>±2.6</td>
<td>±0.30</td>
<td>±0.17</td>
</tr>
<tr>
<td>4.</td>
<td>MC-treated</td>
<td>6</td>
<td>193.7</td>
<td>268.2</td>
<td>14.4</td>
<td>—</td>
<td>3.10***</td>
</tr>
<tr>
<td></td>
<td>splenex</td>
<td></td>
<td>±8.5</td>
<td>±8.0</td>
<td>±0.8</td>
<td>±0.13</td>
<td></td>
</tr>
</tbody>
</table>

Values are means±S.D. Levels of significance of difference: **, p<0.005; *** , p<0.001 from control intact group, and ¡¡¡, p<0.001 from control splenectomized group.

Hematological data on the rats are shown in Table 2. Significant decreases in the red cell count, hemoglobin concentration, and hematocrit value and increase in reticulocytes were observed in MC-treated intact rats. These hematological changes after MC-treatment were less marked in splenectomized rats.

Figure 2 shows the relation of the osmotic fragility of red cells to the weight of the spleen in all groups. The fragility of red cells increased with increase in the weight of the spleen, reaching a plateau at an HHR of 0.48.

The average osmotic fragilities and lipid contents of red cells in the four groups are shown in Fig. 3. The fragility of red cells was greatly decreased in splenectomized rats. Injections of MC resulted in a significant increase in osmotic fragility of red cells in intact rats, but not in splenectomized rats. Specifically,
Fig. 2. Relation of fragility of red blood cells to the weight of the spleen. HHR means the concentration of NaCl for 50% hemolysis. For details, see text.

Fig. 3. Osmotic fragility and lipid contents of red blood cells. Results are means±S.D. HHR means the concentration of NaCl for 50% hemolysis. Level of significance of difference: ***, p<0.001 from control intact group, and +++, p<0.001 from corresponding intact group.

MC had no influence on the fragility of red cells in splenectomized rats. Splenectomy increased the total phospholipid content of red cells significantly (p<0.001), and conversely injection of MC into intact rats reduced the phospholipid content significantly (p<0.001). Cholesterol in the red cells of splenectomized rats was significantly increased in both control and MC-treated rats; the cholesterol level in red cells of intact rats was not affected by MC-treatment.

As shown in Fig. 4, the content of phospholipid had a highly significant correlation with the fragility of red cells in all the groups of rats (r=−0.918, p<0.001).

The diameter and spherical index of red cells are summarized in Fig. 5. The diameter corresponded well with the level of cholesterol in the red cells, while the spherical index decreased with increase in the cholesterol content and diameter of the cells. Therefore, increase in cholesterol caused decrease in spherocytosis. The cholesterol content of red cells was linearly related with the diameter of the red cells, as shown in Fig. 6, the correlation coefficient being 0.672 (p<0.001).
Fig. 4. Relationship between the content of phospholipids and osmotic fragility of red blood cells in all rats. HHR means the concentration of NaCl for 50% hemolysis.

Fig. 5. Changes in diameter and spherical index of red blood cells. Results are means±S.D. Level of significance of difference: +++ p<0.001 from corresponding intact group.

Fig. 6. Relationship between the cholesterol content and diameter of red blood cells.

Fig. 7. Relationship between the plasma LCAT activity and the cholesterol content of red blood cells.

In Fig. 7, the cholesterol contents of the red cells of all the rats examined are plotted against the corresponding plasma LCAT activities; the cholesterol content
LIPIDS AND STABILIZING RED CELLS IN RATS

of red cells is negatively correlated with plasma LCAT activity ($r = -0.743$, $p < 0.001$).

DISCUSSION

Methyl cellulose (MC), like other non-metabolizable macromolecules, is taken up by the reticuloendothelial cells of the spleen and induces splenomegaly in rats (15).

The present work showed that increase in the size of the spleen was closely related to increase in fragility of the red cells. We did not measure red cell survival in the present study, but Giblett et al. (6) reported a reduced T/2-Rbc, measured with $^{51}$Cr-labeled red cells, in MC-treated rats. Our present work shows that the reduced number of red cells in circulation in rats with an enlarged spleen is partly due to increased susceptibility of the cells to lysis.

It is unknown why the fragility of red cells increases in the rats with an enlarged spleen: stasis of red cells in enlarged sinuses may be one contributing factor, and change in the lipid content of the red cell membrane due to change in the plasma content may be another.

Some of the lipids in the red cell membrane are in exchange equilibrium with lipoproteins in the plasma. Cholesterol in the membranes, which is entirely unesterified, exchanges rather quickly with free cholesterol in the plasma, reaching equilibrium in 8 to 12 hr either in vivo or in vitro (4). Phospholipids in the membranes exchange more slowly, lecithin having the highest turnover of less than 10% in 12 hr (16). Free cholesterol in plasma is believed to be esterified by the action of LCAT (8). Norum and Gjone (13) observed cholesterol-rich red cells in patients with marked inborn deficiency of LCAT. Esterification of cholesterol causes a movement of free cholesterol from the red cell membrane to plasma lipoproteins, and so a decrease in LCAT activity will result in an increased cholesterol content in the red cells, as shown in Fig. 7. The liver is known to synthesize and liberate LCAT into the circulation (14), and this suggests that some impairment of liver function associated with MC-induced splenomegaly decreases the plasma LCAT activity, as observed in patients with liver disease (19).

Cooper and Jandle (1) demonstrated that loss of membrane cholesterol reduced the surface area of the red cells, and that reduction in the cholesterol content of red cells resulted in the increase in osmotic fragility in agreement with our results.

The present study confirmed that the membrane content of cholesterol was correlated with the diameter of red cells and that increase in spherocytosis was proportional to decrease in the cholesterol content of red cell membrane.

Donaldson et al. (3) reported a great increase in the sequestration of red cells from circulation when the spleen was enlarged. Stasis of blood in an enlarged spleen probably increases the susceptibility of red cells to osmotic lysis in rats because Wennberg (20) observed a high hematocrit value and low glucose level.
and pH value in sinuses of the spleen, and studies in this laboratory have shown that at low pH values the cholesterol content of red cells decreases and their osmotic fragility increases (Sagawa, unpublished data), thus we may speculate that the stasis of red blood cells in a low pH condition, likewise in splenic sinuses, is one of the reasons why cholesterol is lost by red cell membrane.

Present results show that the molar ratio of cholesterol to phospholipids of red blood cells (0.87 in control group, and 0.94 in MC-treated group) did not change when the osmotic fragility was decreased by splenectomy because the contents of cholesterol and phospholipids increased at the same rate. KUIPER et al. (10) reported that the increased osmotic fragility of red cells of heat-exposed hamsters was correlated with lower cholesterol/phospholipids ratio. The discrepancy between the two observations may be caused by the difference of stimulation in changing osmotic fragility.

From the present results, we conclude that increase in phospholipid will result in decreased fragility and increase in cholesterol will bring about a stabilization of cell membrane by decreasing spherocytosis. These two effects, working together, will reduce the susceptibility of red cells to lysis and prevent their destruction in rats, whereas a decrease in phospholipids and cholesterol will have the opposite effect.

REFERENCES