Abnormal Phospholipid Metabolism in Spur Cell Anemia: Decreased Fatty Acid Incorporation Into Phosphatidylethanolamine and Increased Incorporation Into Acylcarnitine in Spur Cell Anemia Erythrocytes

By David W. Allen and Nancy Manning

Spur cell anemia is a hemolytic anemia seen in severe alcoholic cirrhosis that is characterized by unusual morphology and a decreased ratio of phospholipids to cholesterol in the erythrocyte membrane. We hypothesized that defective phospholipid repair may contribute to the red blood cell (RBC) phospholipid abnormalities of spur cell anemia. Therefore, we compared RBCs from normal control subjects with RBCs from spur cell anemia patients. The incorporation of \[^{14}C\] arachidonic acid into the phospholipids and acylcarnitine (acyl-Cn) of spur cells and normal RBCs was analyzed by a direct-phase high performance liquid chromatography column to separate both the phospholipids and acyl-Cn.

There was less uptake of the \[^{14}C\] arachidonate into phosphatidylethanolamine of spur cell RBCs (12.9% ± 1.0%) compared with normal RBCs (20.5% ± 2.8%; \(P = .0245\)). However, more arachidonate was incorporated into the acyl-Cn of spur cells (spur cell acyl-Cn [24.5% ± 2.9%] vs normal control acyl-Cn [10.1% ± 1.9%]; \(P = .0018\)). We conclude that phospholipid biosynthesis is inhibited and that acyl-Cn formation is spared in spur cell anemia RBCs. These metabolic changes may help account for the lipid abnormalities seen in spur cell anemia RBCs and contribute to the hemolytic process. This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Chemicals. Fatty acids, phospholipids, phospholipases, defatted bovine serum albumin (BSA), carnitine, and palmitoylcarnitine were obtained from Sigma (St Louis, MO). Peracetic acid was from Aldrich (Milwaukee, WI). Thin-layer chromatography plates were supplied by Analtech (Newark, DE). \[^{14}C\] Arachidonic, \[^{14}C\] palmitic acid (hexadecanoic acid), \[^{3}H\] palmitic acid, \[^{14}C\] stearic acid (octadecanoic acid), \[^{14}C\] 1-palmitoyl-sn-glycero-3-phosphocholine, and \[^{14}C\] palmitoylcarnitine were products of NEN-Dupont (Boston, MA).

Incubation of erythrocytes. Blood was obtained aseptically from normal volunteers, patients with obstructive jaundice but without liver disease, patients with reticulocytosis, and eight patients with spur cell anemia who had given informed written consent according to a protocol approved by the institutional review board. Complete blood counts and liver function tests were performed by the clinical laboratories of the Minneapolis Veterans Affairs Medical Center. Reduced GSH was determined by the method of Beutler. White blood cells and platelets were removed by filtration through cellulose columns. Peracetic-buffered isotonic saline with 8 mmol/L glucose, pH 7.4 (PBS), with defatted albumin (0.8 g/dL; PBS-BSA) was used an incubation medium for RBCs and to wash RBCs after incubation. For \[^{14}C\] arachidonate or \[^{14}C\] palmitic incorporation experiments, PBS-BSA was sterilized by filtration, deoxygenated with nitrogen and the \[^{14}C\] fatty acid, diluted with \[^{14}C\] fatty acid in ethanol, and added drop-wise with mixing. Ethanol was removed from the mixture with a stream of nitrogen for 5 to 30 minutes and the labeled PBS-BSA was incubated for 24 to 48 hours additionally.
in the dark at room temperature. RBCs were then incubated with 50 μmol/L radiolabeled fatty acids in PBS-BSA with 8 mmol/L glucose, penicillin (100 U/mL), and streptomycin (100 μg/mL) (pH 7.4, 20 hours 37°C). Initial kinetic experiments with normal control RBCs showed that the fixed incubation time of 20 hours gave maximal uptake and reproducible relative uptake of fatty acids into phospholipid and acyl-Cn. Acyl carnitines labeled more rapidly than phospholipids, with acyl-Cn uptake at 4 hours being 54% ± 2% of uptake at 20 hours, whereas phosphatidylcholine (PC) uptake at 4 hours was only 33% ± 1% of uptake at 20 hours. Surface fatty acids on spur cell RBCs extractable by PBS-BSA after washing the RBCs with PBS were 20.2% of total RBC counts. Control surface fatty acids measured similarly were 21.2% of total RBC counts (range, 18.2% to 26.7%). In some experiments, RBCs were preincubated in PBS alone (control) or in 100 μmol/L peracetic acid in PBS at 25°C. After 4 hours, the RBCs were washed four times with saline to avoid direct contact between the oxidant and labile, unsaturated fatty acid.

**Lipid analysis.** Lipids were extracted from the RBCs with iso-propanol and chloroform and analyzed by HPLC on 0.5 × 30 cm µ Porasil columns (Waters, Milford, MA) eluted with hexane/2-propanol/H₂O gradient (6:8:0.75 [vol/vol]) to 6:8:1.4 [vol/vol]. The collected fractions were identified by chromatography with known standards and analyzed by UV spectroscopy and phosphate analysis. Fatty acid composition was determined by gas-liquid chromatography. The HPLC fractions were monitored at 206 and 234 nm by UV spectrophotometers in series.

**Enzyme assay.** Acyl-CoA:1-palmitoyl-sn-glycero-3-phosphocholine acyl transferase was assayed using [¹⁴C]1-palmitoyl-lyso-phosphocholine and either palmitoyl-CoA or arachidonoyl-CoA as previously described.

**Statistics.** Statistical analysis used the software program True Epistat from Epistat Services (Richardson, TX). Results are expressed as means ± standard error of the mean (SEM). Ninety-five percent confidence intervals (CI) are the mean ± twice the SEM. In some experiments, one-tailed paired t-tests were performed because a specific null hypothesis was available.

**RESULTS**

We consistently observe a radioactive peak after labeling both normal and spur cell RBCs with radioactive fatty acids (palmitic, stearic, and arachidonic), with a retention time between 40 and 45 minutes on HPLC, just slightly after sphingomyelin. Gas-liquid chromatography of this peak from RBC membranes after hydrolysis and derivatization showed a mixture of fatty acids similar in composition to normal RBC membrane phospholipid fatty acid composition. There was no corresponding increase in absorption at 206 or 234 nm. Phosphate analysis of this peak showed no detectable phosphate above background. Radioactive palmitic acid was largely used in subsequent identification because more palmitoyl derivatives were available commercially and this saturated fatty acid is reasonably stable. The radioactive peak isolated from labeled RBC membranes, although saponifiable, was resistant to pancreatic phospholipase, phospholipase C, and phospholipase A₂. Unlike acyl-CoA, this peak was not converted to the acyl hydroxamate by neutral hydroxylamine, although it was susceptible to alkaline hydroxylamine. A review of likely acyl oxy-esters suggested that the peak was acyl-Cn. Cochromatography with standard palmitoylcarnitine reagent (Sigma) in a sufficient amount (0.459 μmol) to be observed at 206 nm overloaded and broadened both the radioactive and reagent peaks that were congruent.
Table 1. Clinical Data of Spur Cell Patients

<table>
<thead>
<tr>
<th></th>
<th>Normal Values</th>
<th>Patients</th>
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</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>12-17</td>
<td>9.7 (9.1-10.3)</td>
</tr>
<tr>
<td>Reticulocyte (%)</td>
<td>1-2</td>
<td>7.5 (5.5-9.5)</td>
</tr>
<tr>
<td>Spur cell (%)</td>
<td>0</td>
<td>30 (19-41)</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.2-1.4</td>
<td>16.5 (4.6-28.1)</td>
</tr>
<tr>
<td>Prothrombin time (s)</td>
<td>11-13</td>
<td>20.3 (17.3-23.2)</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.9-4.9</td>
<td>3.2 (2.7-3.7)</td>
</tr>
<tr>
<td>Aspartate aminotransferase (IU/L)</td>
<td>14-45</td>
<td>151 (52-249)</td>
</tr>
<tr>
<td>Alanine aminotransferase (IU/L)</td>
<td>7-56</td>
<td>107 (36-178)</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>43-122</td>
<td>184 (106-220)</td>
</tr>
<tr>
<td>Glutathione (μmol/g Hb)</td>
<td>6.7-8.9</td>
<td>4.8 (4.6-4.9)</td>
</tr>
</tbody>
</table>

Values are the mean with the CI (mean ± 2× SEM) in parentheses.

compared with simultaneous controls (33,452 ± 4,372 cpm; P = .0144). There was no difference in cpm incorporated between PE for reticulocyte-rich RBC (10,565 ± 1,804 cpm) and PE of controls (11,199 ± 2,002 cpm; P = .43). Acyl-Cn showed higher incorporation in reticulocyte-rich RBCs (13,773 ± 2,573 cpm) than in controls (7,893 ± 1,449 cpm), but there was no significant difference in the percentage of total counts in acyl-Cn between reticulocyte-rich RBCs and control RBCs (P = .4).

Typical replicate HPLC chromatograms from both control and spur cell RBCs are shown in Fig 2, in which the cpm of [14C] arachidonate are plotted against the retention time. Points from the individual duplicate chromatograms are indicated; the lines are the average of the points for control or spur cell RBCs at the same retention time. There was no consistent difference in either the phospholipid content or absorbance at 234 nm absorption between the control and spur cell RBCs. We also measured conjugated dienes at 234 nm on the effluent from the HPLC column. Absorption at 234 nm was increased at 19 minutes of retention time in spur cell RBCs, but this proved to be a function of hyperbilirubinemia, because absorption at 19 minutes was also increased, proportional to the bilirubin, in RBCs from patients with obstructive jaundice but with no cirrhosis and no spur cells. Patients with obstructive jaundice showed no abnormality of fatty acid incorporation. Although there was decreased incorporation in the duplicate determinations of the phospholipids of the spur cell patient, more of the label was incorporated into neutral lipids, free fatty acids, and acyl-Cn.

Table 2 shows the average of replicate determinations of incorporation of [14C] arachidonate into 0.375 ml. RBCs containing 48.6 ± 0.7 μg lipid phosphorus from each of the seven patients studied during this period, as well as seven age-matched controls, with incorporation studies performed at the same time. Incorporation and percentage incorporation into PE and acyl-Cn are shown for patients and controls. Also shown is the mean ± SEM and the probability from a matched t-test with the null hypotheses that incorporation into PE is not decreased in spur cell anemia and that acyl-Cn is not increased. Average total incorporation in spur cell RBCs (24,284 ± 2,220 cpm) was not greater than control RBCs (28,786 ± 4,097 cpm), as would be expected from the reticulocytosis. PE showed diminished incorporation in the spur cell RBCs when corrected for total radioactivity in the lipid extract and reported as a percentage. On the other hand, both cpm and percentage of the total counts of acyl-Cn are increased in the RBC lipids of spur cell anemia patients to a highly significant extent. Counts in neutral lipids and free fatty acids were increased and counts in PC were decreased in spur cell patients, but the results were not statistically significant.

Decreased incorporation of fatty acids into PE and increased uptake into acyl-Cn does not appear to be confined to incorporation of arachidonate. Figure 3 shows the uptake of [14C] palmitic acid into control and spur cell lipids. As with [14C] arachidonate, there is decreased uptake in PE and increased uptake in Acyl-Cn. Note that the distribution of [14C] palmitic acid is different from [14C] arachidonic acid in both patients and controls, with a greater fraction of the palmitic acid label in the acyl-Cn, as has been previously observed.

To explore the possible role of oxidative stress in these results, we used a previously employed in vitro model. When normal RBCs were exposed to 100 μmol/L peracetic acid (4 hours at 25°C), GSH decreased to 13 μmol/g hemoglobin (CI, 0.9 to 1.7). With this same model, RBCs treated with 100 μmol/L peracetic acid (4 hours) and subsequently incubated with [14C] arachidonate (20 hours) had decreased incorporation into both phospholipids and acyl-Cn; PE was 67% ± 7% of control, PC was 68% ± 13% of control, and acyl-Cn was 71% ± 9% of control. This model was not fully successful because, although phospholipid incorporation decreased as in spur cell RBCs, unlike spur cell RBCs, acyl-Cn incorporation also decreased. A reasonable explanation may be that the increased acyl-Cn of spur cell anemia is, at least in part, a result of the reticulocytosis, obviously not present in the peracetic acid model.
Because uptake of both \[^{14}C\] arachidonic and palmitic acids in spur cell anemia RBC lipids is decreased more into PE than PC, assay of the acyl-CoA lysoPE transferase would be most appropriate to explore possible explanations for the diminished uptake in PE. This has not been performed as yet because the radioactive substrate lysoPE is not available commercially. However, \[^{14}C\] lysoPC may be obtained, permitting study of repair of PC. In previous experiments, using aliquots of RBCs from the same individual, we showed inhibition of acyl-CoA lysoPC acyltransferase by peracetic acid.\(^{15}\) But, in spur cell anemia, we were unable to detect a significant difference in mean arachidonoyl-coenzyme A lysoPC acyltransferase by peracetic acid.\(^{13}\) Palmitoyl-coenzyme A lysoPC acyltransferase likewise was no different in patients (control, 2.86% ± 0.36%/mg; spur cell, 2.62% ± 0.49%/mg).

### DISCUSSION

Although the observations presented here concerning changes in phospholipid metabolism do not show the mechanism of hemolysis in spur cell anemia, they do suggest at least three possible future lines of inquiry in this regard. (1) Spur cells have been shown to have excess cholesterol compared with phospholipid, whereas target cells, with similar RBC cholesterol, have balanced amounts of phospholipid.\(^{2}\) Spur cell anemia may result when RBC phospholipid biosynthesis is inhibited and unable to compensate for the added cholesterol, with the resulting imbalance producing decreased membrane fluidity and resulting in hemolysis.\(^{1}\) (2) Loss of GSH sulfhydryls in spur cell anemia indicates a possible oxidative threat to enzyme thiol groups in enzymes and coenzymes as well as in membrane skeletal proteins. Because fatty acid acyl groups are activated, transported, and metabolized as their thioesters,\(^{5}\) oxidation of thiol groups might modulate fatty acid metabolism. The mechanism of acyl-Cn increase remains to be determined. Because body Cn stores are in part dietary\(^{22}\) and malnourished cirrhotics are deficient in Cn,\(^{23}\) it is likely that the increased biosynthesis of acyl-Cn is not explained by acylation of excess stores of Cn. Because acyl-Cn labeling appears to be increased in presumably normal reticulocytes after blood loss or in autoimmune hemolytic anemia, the pattern of lipid incorporation in spur cell anemia RBCs may result from inhibition of uptake into PE (possibly oxidant-induced; cf, the effect of peracetic acid\(^{15}\) and the increased uptake into acyl-Cn by reticulocytes. Oxidative inhibition of phospholipid repair is thus one possible pathophysiologic process. (3) Alternatively, acyl-Cn accumulation, if more than that predicted from the reticulocytosis, might contribute to the mechanism.

### Table 2. Arachidonic Acid Uptake into RBC Lipids

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Patient</th>
<th>C</th>
<th>Pt</th>
<th>C</th>
<th>Pt</th>
<th>C</th>
<th>Pt</th>
<th>C</th>
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<td>1</td>
<td></td>
<td>687</td>
<td>1,528</td>
<td>287</td>
<td>3,712</td>
<td>15.3</td>
<td>12.1</td>
<td>6.4</td>
<td>29.4</td>
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<td>2</td>
<td></td>
<td>2,966</td>
<td>2,778</td>
<td>3,728</td>
<td>2,565</td>
<td>12.0</td>
<td>17.1</td>
<td>15.0</td>
<td>15.8</td>
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<tr>
<td>3</td>
<td></td>
<td>4,059</td>
<td>4,120</td>
<td>3,472</td>
<td>15,074</td>
<td>18.7</td>
<td>10.4</td>
<td>16.0</td>
<td>38.0</td>
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<td>4</td>
<td></td>
<td>11,974</td>
<td>3,569</td>
<td>2,301</td>
<td>8,397</td>
<td>31.0</td>
<td>10.8</td>
<td>5.9</td>
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<td>5</td>
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<td>4,352</td>
<td>6,339</td>
<td>1,401</td>
<td>13,351</td>
<td>15.5</td>
<td>10.2</td>
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<td>6</td>
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<td>17,861</td>
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<td>4,099</td>
<td>3,288</td>
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<td>6.9</td>
<td>16.2</td>
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<td>7</td>
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<td>3,417</td>
<td>2,807</td>
<td>2,630</td>
<td>4,825</td>
<td>20.5</td>
<td>14.1</td>
<td>15.8</td>
<td>24.9</td>
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<tr>
<td>Mean</td>
<td></td>
<td>6,474</td>
<td>3,468</td>
<td>2,560</td>
<td>7,330</td>
<td>20.5</td>
<td>12.9</td>
<td>10.1</td>
<td>24.5</td>
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<td>SEM</td>
<td></td>
<td>2,137</td>
<td>1,498</td>
<td>515</td>
<td>1,923</td>
<td>2.8</td>
<td>1.0</td>
<td>1.9</td>
<td>2.9</td>
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Abbreviations: C, control; Pt, patient; P, probability.
of hemolysis by producing membrane changes as a result of accumulation of this amphiphile. Functional and morphologic abnormalities produced in vitro by incubating RBCs with 100 μmol/L palmitoylcarnitine have been noted that, if present in spur cell anemia, could contribute to hemolysis by destabilizing the membrane.

In the past, most emphasis has been placed on exchange of lipids with the plasma by RBCs as an explanation of RBC abnormalities in spur cell anemia. However, for maintenance of the molecular species composition of RBC membranes, specific acylation of lysophospholipids by fatty acids activated as acyl-CoA must proceed normally. Acyl-Cn may be an integral link in this pathway. In this report, we present evidence of impaired phospholipid metabolism in spur cell anemia.

REFERENCES
Abnormal phospholipid metabolism in spur cell anemia: decreased fatty acid incorporation into phosphatidylethanolamine and increased incorporation into acylcarnitine in spur cell anemia erythrocytes

DW Allen and N Manning