Abnormal Erythrocyte Metabolism in Hepatic Disease

By J. Robert Smith, Neil E. Kay, Arlan J. Gottlieb, and Frank A. Oski

Erythrocyte (RBC) metabolic studies were done on 114 patients with severe hepatic disease. Heinz body formation after incubation of RBCs with acetyl phenylhydrazine was found to be significantly higher in patients than in controls. RBC-reduced glutathione levels were lower than those of controls both before and after incubation with acetyl phenylhydrazine, and patients with the highest Heinz body counts had the lowest reduced glutathione levels. RBC methylene blue-stimulated hexose monophosphate (HMP) shunt metabolism and glucose recycling through the shunt were significantly lower in patients with active hepatic disease than in controls. There was no difference in resting HMP shunt activity or in resting recycling of glucose. Despite impairment of shunt metabolism, total glucose consumption was greater in patients than in controls. The patients with the lowest stimulated HMP shunt metabolism and glucose recycling had the highest Heinz body counts, lowest reduced glutathione, and highest total glucose consumption. A continuum of abnormal shunt metabolism was seen, from a mild reduction of stimulated HMP shunt activity to a severe combined decrease in both the HMP shunt and glucose recycling. When measured, glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase, and transketolase were normal or increased. Sequential studies were done on 11 patients who had abnormal metabolic studies. Coincident with improvement of HMP shunt metabolism, the Heinz body counts became lower, reduced glutathione higher, hematocrit higher, and liver function improved. Impaired HMP shunt metabolism appears to be a common, acquired RBC abnormality in patients with severe, active liver disease.

The etiology of anemia in patients with hepatic disease is diverse. A shortened red cell life span may be observed in some patients without evidence of blood loss. In addition, a number of hematologic syndromes have been described in patients with abnormal hepatic function. In 1958 Zieve described a hemolytic syndrome in patients with active alcoholic liver disease. The mechanism underlying the hemolysis observed in this condition has never been completely clarified. Spur cells and acanthocytes are found in patients with liver disease. The role of the spleen in remodeling these morphologically abnormal red cells and presumably in the induction of hemolysis has recently drawn comment. Stomatocytosis with hemolysis has been reported in alcoholics with liver disease. Acute hemolysis has also been observed in patients with acute liver disease and glucose-6-phosphate dehydrogenase deficiency. The erythrocyte metabolic defect reported here was uncovered in the course of investigating the cause of hemolysis in patients with active hepatic disease.

Erythrocytes from patients with active liver disease were found to have an increased tendency towards Heinz body formation after incubation with acetyl...
phenylhydrazine. The increase in Heinz body formation correlated with an increased instability of erythrocyte-reduced glutathione, a decrease in stimulated hexose monophosphate shunt activity, and in glucose recycling through the hexose monophosphate shunt. Our results suggested that a predisposition towards Heinz body formation and glutathione instability exists in the erythrocytes of many patients with severe active hepatic disease as a result of an acquired metabolic abnormality of the erythrocyte hexose monophosphate shunt. These abnormalities would tend to supplement the varied stresses to erythrocyte longevity which exist in severe, active, parenchymatous liver disease.

MATERIALS AND METHODS

Patient Selection

One hundred and fourteen hospitalized patients with liver disease from three hospitals associated with the Upstate Medical Center of the State University of New York (The Syracuse Veterans Administration Hospital, University Hospital, and Crouse-Irving Memorial Hospital) were studied.

The liver disease was, in the main, acute alcoholic hepatitis and/or cirrhosis. Two patients had viral hepatitis, two had hepatic involvement with metastatic tumor, one had postnecrotic cirrhosis, and one obstructive liver disease. All patients had at least one laboratory abnormality indicating hepatic dysfunction, and some had tissue confirmation of the nature of their liver disease. Patients were studied without respect to the level of hemoglobin observed but were not included in the series if either vitamin B12 deficiency, folic acid deficiency, or erythrocyte glucose-6-phosphate dehydrogenase deficiency was present. In addition, no patient was included if there was evidence of acute blood loss, immune hemolysis, a hemoglobinopathy, or disseminated intravascular coagulation. The control group consisted of healthy volunteers and hospitalized patients without hepatic or hematologic disorders.

Erythrocyte Metabolic Studies

Heinz body formation was studied after incubation of 2 ml of whole blood for 4 hr at 37°C with 40 ml of a 0.066 M phosphate buffer solution containing 40 mg of acetyl phenylhydrazine. Heinz bodies were developed by staining with crystal violet and the percentage of cells with five or more Heinz bodies determined. Erythrocyte-reduced glutathione was measured before and after a 2-hr incubation in the same erythrocyte-acetyl phenylhydrazine-buffer mixture used for the Heinz body test. Red cell glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase, and transketolase were measured using the methods cited. A Gilford 2000 recording spectrophotometer was used for all enzyme and glutathione measurements.

Erythrocyte hexose monophosphate shunt activity was measured using glucose labeled in the first position (14C1), and glucose recycling was measured using glucose labeled in the second position (14C2). Washed red cells were incubated for 2 hr in a Krebs-Ringer-bicarbonate buffer at pH 7.4 and 37°C with the appropriate isotopically labeled glucose. Resting hexose monophosphate shunt activity and shunt activity after stimulation with 10-7 M methylene blue were measured. The CO2 evolved was absorbed in hyamine hydroxide which was then added to Econofluor (New England Nuclear). The 14CO2 was counted in a Packard Tricarb liquid scintillation spectrometer. Total red cell glucose consumption was measured by a micromodification of the hexokinase assay. The data were analyzed by standard statistical methods.

RESULTS

Heinz Bodies

Heinz body formation after the incubation of erythrocytes with acetyl phenylhydrazine is shown in Fig. 1. Patients with hepatic disease had a signifi-
Heinz body counts were performed after incubating erythrocytes in an acetyl phenylhydrazine-buffer solution for 4 hr at 37°C. The percentages of erythrocytes with five or more Heinz bodies are shown in 146 controls and 114 patients.

Incubation studies were performed using 2 ml whole blood incubated for 2 hr at 37°C in 40 ml of solution containing 40 mg acetyl phenylhydrazine in 0.066 M phosphate buffer (pH 7.4).

*p values compare patients and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Preincubation Reduced Glutathione (mg/100 ml RBC)</th>
<th>Postincubation Reduced Glutathione (mg/100 ml RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>49</td>
<td>65.6 ± 8.1</td>
<td>49</td>
</tr>
<tr>
<td>All patients</td>
<td>114</td>
<td>57.3 ± 14.1 (&lt;0.001)</td>
<td>112</td>
</tr>
<tr>
<td>Patients &lt;35% HB†</td>
<td>45</td>
<td>62.6 ± 11.5 (NS)</td>
<td>45</td>
</tr>
<tr>
<td>Patients ≥35% HB†</td>
<td>64</td>
<td>53.7 ± 14.9 (&lt;0.001)</td>
<td>64</td>
</tr>
</tbody>
</table>

Incubation studies were performed using 2 ml whole blood incubated for 2 hr at 37°C in 40 ml of solution containing 40 mg acetyl phenylhydrazine in 0.066 M phosphate buffer (pH 7.4).

*p values compare patients and controls.

†HB are Heinz body counts after incubation with acetyl phenylhydrazine. Heinz body counts ≥35% are >2.5 SD from controls.

A significant increase in Heinz body formation when compared to normal controls (p = <0.001). The mean value for 114 patients was 47.3 ± 19.1% of erythrocytes with five or more Heinz bodies, and 16.9 ± 6.9% in 146 controls. Five patients with incubated Heinz body counts in excess of 80% had Heinz bodies demonstrable in 10%–20% of their circulating erythrocytes.

**Reduced Glutathione**

Erythrocyte-reduced glutathione was determined in 114 patients with hepatic disease, both before and after incubation with acetyl phenylhydrazine, and compared to 49 controls. Results of these studies and the significance of the data obtained are shown in Table 1. A significant difference in the level of reduced glutathione, both before and after incubation, was observed in patients as compared to controls (p < 0.001).

Five or more Heinz bodies were found in 35% or more of the erythrocytes in 64 patients.* The erythrocyte-reduced glutathione level in this group was

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*Heinz body counts of 35% or more are greater than 2.5 standard deviations from the mean of the control group.
Table 2. Erythrocyte Glucose Consumption of Patients With Hepatic Disease

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose Consumption</th>
<th>p</th>
<th>Glucose Consumption</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated*</td>
<td></td>
<td>Stimulated*</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>No. 41</td>
<td>2.21 ± 0.35</td>
<td>&lt;0.001</td>
<td>40</td>
</tr>
<tr>
<td>Patients</td>
<td>No. 48</td>
<td>2.66 ± 0.69</td>
<td></td>
<td>49</td>
</tr>
</tbody>
</table>

*Glucose consumption was measured with and without methylene blue stimulation (10^-7 M final concentration) in red cells incubated for 2 hr in Krebs-Ringer bicarbonate buffer, pH 7.4.

53.7 ± 14.9 mg/100 ml RBC before incubation and 21.5 ± 13.6 mg/100 ml RBC after incubation with acetyl phenylhydrazine. In 45 patients with less than 35% Heinz bodies, the erythrocyte-reduced glutathione was 62.6 ± 11.5 mg/100 ml RBC prior to incubation and 37.3 ± 15.0 mg/100 ml RBC after incubation. The difference in the levels of reduced glutathione both before and after incubation was found to be highly significant when the patients with less than 35% Heinz bodies were compared to the patients with greater than 35% Heinz bodies (p < 0.001). Patients with Heinz bodies of less than 35% did not differ significantly from controls in their levels of reduced glutathione either before or after incubation. Thus, reduced glutathione levels and the stability of reduced glutathione showed good correlation with Heinz body formation.

Erythrocyte Glycolysis

When erythrocyte glucose consumption of 48 patients with hepatic disease was compared to controls (Table 2), the mean unstimulated glucose consumption was 12% higher in patients (p < 0.001). After methylene blue stimulation, erythrocytes of the patients with hepatic disease averaged 11% greater glucose consumption than the erythrocytes from controls (p < 0.005). The highest glucose consumption was seen in the erythrocytes of patients with the severest defects in stimulated hexose monophosphate shunt metabolism and stimulated glucose recycling (vide infra).

Patients were found to have slightly higher resting shunt activity as de-

Table 3. Erythrocyte Hexose Monophosphate Shunt Activity of Patients With Hepatic Disease: 14CO2 Production (µmoles/ml RBC/hr)

<table>
<thead>
<tr>
<th>No. 14C1 Glucose</th>
<th>14C1 Glucose (Stim)*</th>
<th>p</th>
<th>14C2 Glucose</th>
<th>14C2 Glucose (Stim)*</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls 41</td>
<td>0.132 ± 0.022</td>
<td>0.778 ± 0.093</td>
<td>0.007 ± 0.002</td>
<td>0.132 ± 0.026</td>
<td>0.778 ± 0.093</td>
</tr>
<tr>
<td>Per cent glucose consumption</td>
<td>6.0</td>
<td>60.5</td>
<td>6.0</td>
<td>60.5</td>
<td></td>
</tr>
<tr>
<td>Patients 49</td>
<td>0.141 ± 0.026</td>
<td>NS 0.692 ± 0.111</td>
<td>&lt;0.001</td>
<td>0.008 ± 0.004</td>
<td>NS 0.116 ± 0.046</td>
</tr>
<tr>
<td>Per cent glucose consumption</td>
<td>5.3</td>
<td>23.7</td>
<td>0.3</td>
<td>4.0</td>
<td></td>
</tr>
</tbody>
</table>

Erythrocyte hexose monophosphate shunt activity and glucose recycling were studied by incubating erythrocytes for 2 hr at 37°C in a Krebs-Ringer bicarbonate buffer (pH 7.4) with 14C1 or 14C2-labeled glucose.

Per cent of glucose consumption was computed by (dividing the 14CO2 production of shunt metabolism or of glucose recycling by total glucose consumption) x 100.

* Methylene blue, 10^-7 M, was used to stimulate shunt activity and glucose recycling.
terned with $^{14}$C$_1$-labeled glucose. This difference was not statistically significant (Table 3). With methylene blue in the incubation mixture, however, significantly lower stimulated shunt activity was noted in the patient group. Patients had stimulated shunt activity of $0.692 \pm 0.111 \mu M$ glucose per ml RBC per hr and controls $0.778 \pm 0.093 \mu M$ glucose per ml RBC per hr ($p < 0.001$). Six patients from this group of 49 had stimulated shunt activity which differed from controls by more than 2 SD ($<0.592 \mu M$ per ml RBC per hr). When these six patients were compared to the total group of patients with liver disease (Table 4), they had higher mean Heinz body counts, lower reduced glutathione levels before incubation and after incubation, and higher erythrocyte glucose consumption when unstimulated or when stimulated. This group also evidenced extremely low stimulated glucose recycling (mean, $0.069 \mu M$ per ml RBC per hr). It should be noted that, even when the six patients with the lowest stimulated hexose monophosphate shunt activity were excluded, the remaining 43 patients as a group still retained a statistically significant impairment in stimulated hexose monophosphate shunt activity as compared to controls (mean, $0.717 \pm 0.091 \mu M$ per ml RBC per hr, $p < 0.005$).

No significant difference was obtained between patients and controls when resting erythrocyte recycling of glucose through the HMP shunt was measured with $^{14}$C$_2$-labeled glucose. However, after methylene blue stimulation, a significant reduction in glucose recycling ($p < 0.001$) and a decreased percentage of the total glucose consumed via recycling were observed in patients when compared to controls (Table 3). Nineteen of the 49 patients studied had glucose recycling which was lower than 2 SD from the mean of the controls ($<0.100 \mu M$ per ml RBC per hr). When these 19 patients were compared to the total patient group (Table 4), they were found to have higher mean Heinz body counts, lower preincubation and postincubation reduced glutathione levels, and higher total glucose consumption both resting and stimulated.

The 19 patients with the lowest glucose recycling had lower mean stimulated hexose monophosphate shunt activity ($0.668 \mu M$ per ml RBC per hr) than the
30 patients with lesser recycling defects (0.708 µM per ml RBC per hr). There was therefore a positive correlation between the observed defect in stimulated shunt activity and stimulated glucose recycling in our patients. Stimulated glucose recycling in the remaining 30 patients did not differ significantly from controls. Heinz body formation, however, was distinctly abnormal in this latter group (40.7%), and a defect in stimulated hexose monophosphate shunt metabolism was still evident in this group when compared to controls. The stimulated shunt activity in this patient group was, as previously noted, 0.708 ± 0.089 µM per ml RBC per hr compared to controls with 0.778 ± 0.093 µM per ml RBC per hr (p < 0.005). The defect in stimulated shunt activity therefore even occurred in the erythrocytes of the patients with normal glucose recycling and would thus appear to be more pervasive than the defect in glucose recycling.

**Glutathione Peroxidase**

Erythrocyte glutathione peroxidase levels were measured in 27 patients and in 13 controls. Levels of glutathione peroxidase in the study group did not differ significantly from those found in the control group. Patients' values were 5.09 ± 3.32 IU/g Hb, and controls were 4.78 ± 2.39 IU/g Hb.

**Glucose-6-Phosphate Dehydrogenase**

Erythrocyte glucose-6-phosphate dehydrogenase activity was measured in each case, and any patient with decreased levels of this enzyme was excluded from the study.

**Glutathione Reductase**

Erythrocyte glutathione reductase was determined in 28 patients. A slight but not significant increase in FAD-saturated enzyme was noted in the group as a whole as compared to 12 normal controls. Patients' enzyme activity was 6.54 ± 2.09 IU/g Hb, with FAD 8.01 ± 2.77 IU/g Hb, and controls' activity was 5.91 ± 1.15 IU/g Hb, with FAD 7.77 ± 1.47 IU/g Hb.

**Transketolase**

Erythrocyte transketolase was measured in six patients who had abnormalities in stimulated shunt metabolism and stimulated glucose recycling. In all cases transketolase activity was not significantly different from six controls. The mean value for patients was 76.6 ± 17.3 IU/liter RBC, and controls was 88.9 ± 15.5 IU/liter RBC.

**SEQUENTIAL STUDIES**

Eleven patients who had abnormal Heinz body preparations, low reduced glutathione, impaired stimulated erythrocyte hexose monophosphate shunt activity, and low stimulated erythrocyte recycling of glucose were reexamined from 8 days to 6 wk after the initial study. Eight of the eleven patients had normal erythrocyte glucose recycling on repeat metabolic studies. Stimulated hexose monophosphate shunt activity on remeasurement remained unchanged in five patients, increased in two, and decreased in one. The eight patients whose stimulated erythrocyte glucose recycling normalized generally showed
clinical improvement, reduction of elevated enzyme levels (SGOT, alkaline phosphatase), and usually a rise in the hematocrit. The percentage of Heinz bodies decreased but did not always return to normal in those patients whose glucose recycling improved. Reduced glutathione increased, and glutathione instability was less pronounced.

DISCUSSION

Our data indicate that impaired erythrocyte hexose monophosphate shunt activity and glucose recycling through the hexose monophosphate shunt is a relatively common defect in patients with severe active hepatic disease. While erythrocyte glucose consumption was found to be significantly increased in these patients, a decreased percentage of glucose was metabolized by way of the hexose monophosphate shunt. The shunt abnormalities noted correlated with increased erythrocyte Heinz body formation in vitro, with low reduced glutathione, and with an increase in glutathione instability. In 11 patients who were studied sequentially, improvement was found in erythrocyte shunt activity as liver function improved. Concomitantly, the Heinz body count decreased or became normal, and erythrocyte reduced glutathione increased both before and after incubation with acetyl phenylhydrazine. The syndrome seemed clearly to be acquired and related to the activity and severity of the liver disease. The typical patient with this erythrocyte metabolic defect was moderately anemic, with a hemoglobin between 9 and 11 g/100 ml, and had severe active parenchymatous hepatic disease with elevations in SGOT and alkaline phosphatase.

No true incidence of the red cell abnormalities described herein can be given. The defect would appear to be rather common, since glycolytic abnormalities were found in the erythrocytes of nine of the first 20 patients studied. As more data were acquired, it became clear that patients with hepatic disease could be preselected for these erythrocyte glycolytic defects by use of the Heinz body preparation as a screening procedure.

Initially, a defect in erythrocyte glucose recycling following stimulation with methylene blue was noted, but as more patients were examined, a highly significant abnormality in stimulated hexose monophosphate shunt activity became manifest. Thus, stimulated hexose monophosphate shunt activity was found to be abnormal in the 49 patients with hepatic disease studied as compared to controls \((p < 0.001)\). While 18 of 19 patients with low stimulated glucose recycling also had low stimulated hexose monophosphate shunt activity, the remaining 30 patients whose stimulated glucose recycling was normal still showed low stimulated hexose monophosphate shunt activity \((p < 0.005)\). The frequency of abnormal erythrocyte hexose monophosphate shunt activity would therefore appear to exceed that of the defect observed in glucose recycling. The abnormalities observed in both shunt activity and glucose recycling probably represent a continuum that becomes manifest in the methylene blue-stimulated erythrocyte first in shunt activity and then later in glucose recycling. This hypothesis is further supported by the increased tendency toward Heinz body formation as well as glutathione instability noted in the 30 patients studied who did not have significant defects in stimulated glucose recycling.
Studies of patients with erythrocyte glucose phosphate isomerase deficiency provide additional evidence that abnormalities in glucose recycling alone could not be responsible for all the erythrocyte abnormalities found in our patients. Individuals with this enzyme deficiency have chronic hemolysis and very low stimulated recycling of glucose. Heinz body formation is normal, as are reduced glutathione and glutathione stability, and stimulated hexose monophosphate shunt activity is near normal.25

It is not as yet clear whether the erythrocyte metabolic defects noted in our patients result from enzyme inhibition, a deficiency state within the cell, or are imposed as a result of increased oxidative stress by environmental factors. Our attempts to induce the metabolic lesions in compatible normal erythrocytes after incubation with plasma from patients with low stimulated hexose monophosphate metabolism and glucose recycling have been inconclusive.

The erythrocytes of patients with liver disease and abnormal shunt activity behave metabolically as NADPH-depleted cells mimicking the erythrocytes in partial G-6PD deficiency. The serial metabolic events beginning with impaired shunt metabolism, then decreased erythrocyte NADPH, unstable reduced glutathione, mixed disulfide formation, and hemoglobin denaturation with Heinz body formation, may ultimately lead to damage to the erythrocyte membrane, splenic sequestration, and destruction of these conditioned erythrocytes. The hostile metabolic environment of the spleen, particularly of an enlarged spleen, an area of low oxygen tension with an acidic pH and about one-third the glucose of the peripheral blood, would likely further depress hexose monophosphate shunt activity and glucose consumption in the erythrocyte.22'23 The findings of earlier investigators of shortened red cell survival and splenic sequestration4 in patients with hepatic disease is consistent with the metabolic defect that we have demonstrated as well as the various metabolic and erythrocyte membrane defects previously observed.7'10 Clearly these phenomena are not mutually exclusive. Spur cells, for example, may also be associated with impaired shunt metabolism and Heinz body formation in addition to the lipid abnormalities of the erythrocyte membrane.

Similarly, several metabolic deficiency states present in alcoholics might intensify the metabolic lesion observed in the erythrocyte. These include thiamine,24'25 riboflavin,26'27 magnesium,28'29 and possibly selenium deficiency.30

Hemolysis has been well documented in patients with severe liver disease.3'4 Erythrocyte survival studies were not done as part of our current study. Some of the patients studied had marked reticulocytosis associated with a falling hematocrit and no evidence of blood loss. Other patients were anemic with hemoglobins between 8 and 11 g/100 ml; they had a reticulocytosis of 3'0-5'0, erythroid hyperplasia of the marrow, and showed no rise in hemoglobin despite a continued reticulocytosis. These data suggest that hemolysis was indeed occurring in these patients. All patients with abnormalities of stimulated erythrocyte hexose monophosphate shunt metabolism were anemic, although no correlation could be shown between the hematocrit or hemoglobin and degree of abnormality in shunt metabolism. Thus, while there is no direct evidence that the metabolic abnormalities demonstrated in the erythrocyte are responsible for the shortened red cell life span in patients with severe active liver disease, the
association between increased Heinz body formation and hemolysis has been well demonstrated.\textsuperscript{31,32} Heinz body formation would at least supplement the multifactorial stresses to survival encountered by the erythrocytes of the patient with active hepatic disease.

Hemolysis following administration of oxidant drugs to patients with inherited abnormalities of the hexose monophosphate shunt has been well described.\textsuperscript{33} Acquired abnormalities of the shunt may also be associated with drug-induced hemolysis. Yawata and co-workers, who demonstrated marked impairment of stimulated glucose recycling in erythrocytes of uremic patients, found acceleration of hemolysis when primaquine was administered to patients with abnormal glucose recycling.\textsuperscript{34} On the basis of the metabolic studies described in this report, oxidant drugs may potentially induce acute hemolytic reactions in patients with severe active parenchymatous hepatic disease.

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**REFERENCES**

19. Yunis J: Biochemical Methods in Red
20. Oski FA: Unpublished method
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JR Smith, NE Kay, AJ Gottlieb and FA Oski