Metabolic Abnormalities of Erythrocytes in Severe Iron Deficiency

By Robert T. Card and Lewis R. Weintraub

Severe iron deficiency was induced in rabbits by repeated phlebotomy and maintenance on an iron-deficient diet. Erythrocytes from these animals were studied at periods of 2–10 wk following the cessation of bloodletting and were found to have a shortened survival in vivo by the 51Cr technique. These cells were found to be more susceptible than normal cells to lysis during sterile 24-hr incubation in glucose-free balanced salt solution. The severely iron deficient erythrocyte also demonstrated an increased susceptibility to sulfhydryl inhibitors in vitro. Iron-deficient erythrocytes, despite their smaller volume, were found to have an impaired ability to filter through 5-μM Millipore filters. These studies suggest the following pathogenesis for the hemolysis seen in severe iron deficiency. The abnormal plasticity may lead to excessive trapping of these cells by the spleen and reticuloendothelial cells. Within this environment these metabolically abnormal cells are exposed to hostile conditions of stasis and glucose deprivation, which may then enhance their lysis.

Iron deficiency is a systemic disorder in which the major clinical feature is decreased hemoglobin production with a microcytic, hypochromic anemia. However, there are multiple structural and functional abnormalities noted in the epithelial cells of the skin, tongue, and gastrointestinal tract that are not related to the anemia per se.1,2 In addition, there have been several reports in both man3–6 and experimental animals7–9 that have demonstrated that erythrocytes in severe iron deficiency have a shortened survival. Huser et al.7 showed in iron-deficient baboons that the life span of the red cells became shorter as the duration of the deficiency state increased. Three reports have provided evidence that the shortened survival is partially corrected when iron-deficient erythrocytes are transfused into asplenic recipients.4–6

The present study was undertaken in the rabbit in an attempt to study possible metabolic defects induced in erythrocytes by iron deficiency that
might explain the poikilocytosis and shortened survival and in turn give more insight into the tissue effects of iron deficiency.

**Materials and Methods**

**Production of Iron Deficiency in the Rabbit**

Young, male, 2-3-kg rabbits were rendered iron-deficient by diet and repeated phlebotomy. The diet consisted of pelleted rabbit diet (General Biochemicals Corp., Chagrin Falls, Ohio) containing approximately 10 µg of iron per gram of diet. Control animals were fed the same diet with 150 µg/g diet. While on the iron-deficient diet the test animals were bled by cardiac puncture three times a week. A total of 50 mg of iron was removed each week over a 3-wk period of time. Once the animals had become iron-deficient, at least 2 wk and usually a longer period of time elapsed before the erythrocytes were studied. This ensured that any stress erythrocytes produced in response to the bleeding and with known shortened survival were essentially removed from the circulation.13,14 At the time of the various studies, macrocytes were not seen on the stained smears of the erythrocytes and no population of macrocytic cells was seen on the Coulter counter (Coulter Electronics, Hialeah, Fla.) size distribution curves. The growth curves of the iron deficient animals lagged behind those of the controls during the period of phlebotomy and then paralleled the controls while on the iron-deficient diet only.

**Collection and Labeling of Erythrocytes with 51Cr**

First, 10-20 ml of blood was removed from each rabbit by cardiac puncture and mixed with 2.0 ml of acid-citrate-dextrose solution (ACD solution, Abbott Laboratories, North Chicago, Ill.). Then 40 µCi of 51Cr-labeled sodium chromate (Chromitope, E. R. Squibb & Sons, New Brunswick, N.J.), approximate specific activity 50 µCi/µg, was added and the blood was incubated for 30 min at 37°C with gentle shaking. Following incubation, 50 mg of ascorbic acid was added and the cells were washed three times in 10 volumes of normal saline.

**Erythrocyte Survival Studies**

51Cr-labeled washed erythrocytes from 10 ml of blood were suspended in a volume of normal saline equivalent to the volume of plasma removed and reinjected into an ear vein of the rabbit. Beginning 24 hr (day 1) after the infusion of the labeled erythrocytes 0.5 ml of blood was removed at frequent intervals from an ear vein over a 20-day period. The hematocrits were determined and 0.2 ml aliquots were mixed with 3 ml of distilled water in a counting tube and counted in a well-type scintillation counter (Auto Gamma Spectrometer, Model 3002, Packard Instrument Co., Downers Grove, Ill.) for at least 10,000 counts. The results were converted to cpm/ml red cells and the 51Cr half-life was estimated graphically with the counts on day 1 being 100%. The hematocrits of the rabbits varied by less than 3% during the period of these studies.

**Incubation of Erythrocytes**

51Cr-labeled washed erythrocytes were made up to a hematocrit of 5% in Earle's balanced salt solution prepared so that it did not contain any glucose and pH was adjusted to 7.50. Streptomycin was added to this medium in the concentration of 5 µg/ml. Ten ml aliquots of the resultant mixture were incubated in 25-ml Erlenmeyer flasks under sterile conditions at 37°C in an atmosphere of 5% CO2 and 95% O2 with constant shaking. In certain of the experiments glucose was added to the medium in an initial concentration of 200 mg/100 ml. The hemolysis of the labeled cells could then be determined by measuring the release of 51Cr into the medium after a 24-hr incubation. At the end of this period of time the average pH was 7.37 (range 7.26-7.48). The 51Cr was measured in duplicate in 0.2 ml aliquots of the whole blood and supernatant after mixing the respective samples with 3 ml of distilled water in a counting tube. In pre-
liminary experiments the percentage of $^{51}$Cr or hemoglobin released from the same cells during these experiments was not statistically different. This observation would also negate the possibility that the decreased $^{51}$Cr survival of iron-deficient red cells was an artifact due to an increased rate of elution of $^{51}$Cr from these cells.

**Incubation of Erythrocytes with Sulfhydryl Inhibitors**

$^{51}$Cr-labeled iron-deficient and normal rabbit erythrocytes were incubated with the sulfhydryl inhibitors N-ethylmaleimide (NEM) and parachloromercuribenzoate (PMB) (Sigma Chemical Corp., St. Louis, Mo.). These agents were dissolved in normal saline just prior to use and added to the media in amounts such that the weight of inhibitor per milliliter of red cells was the same with the two cell types. Since the iron-deficient cells have a smaller mean cell volume, the total surface area of the iron-deficient cells is greater than that of the normal cells in an equal volume of cells. Hemolysis was measured by determining the hemoglobin concentration and $^{51}$Cr activity in the supernatant as outlined above.

**Filtration of Erythrocytes**

The ability of labeled iron-deficient erythrocytes to pass through a 5-$\mu$m Millipore filter (Millipore Corp., Bedford, Mass.), while mixed with normal erythrocytes was determined using a modification of the method of Jandi, Simmons, and Castle,11 in which 0.05 ml of iron-deficient erythrocytes, labeled with $^{51}$Cr were mixed with 0.2 ml of normal erythrocytes prepared in exactly the same manner except that “cold” sodium chromate was added to the cells in the same concentrations as had been done with the $^{51}$Cr-labeled cells. The cells were then made up to a hematocrit of 2.5% using pooled normal rabbit serum previously prepared by filtration through a 3-$\mu$m Millipore filter.

Two ml aliquots of the above mixture were filtered through a 5-$\mu$m Millipore filter, pre-wetted with a drop of serum, at constant negative pressure of $-20$ cm of water. Preliminary studies have shown that the rabbit erythrocytes could not pass through a 3-$\mu$m Millipore filter, the next smallest size. The fraction of the total hemoglobin due to either the unlabeled (normal) or labeled (iron-deficient) cells was calculated from the volume and hemoglobin concentration of each cell type added to the original mixture. The concentration of both hemoglobin (using 0.2-ml samples in 5 ml of Drabkin’s solution) and radioactivity (using 0.2 ml samples in 3 ml of distilled water and counted as before) in the original mixture and the filtrate were measured in duplicate. In each experiment the hemoglobin and radioactivity in the serum obtained from the filtrate were also measured and in all cases were less than 1% of the amount filtered. In certain experiments one part $^{51}$Cr-labeled normal cells was filtered with four parts of unlabeled iron-deficient cells.

Because the hematocrit was so low, the volume of red cells actually lost during filtration compared with the total volume of cells and serum that was filtered was negligible. Therefore, the percentage of $^{51}$Cr-labeled cells filtered could be calculated by comparing the concentration of $^{51}$Cr in the filtrate with the original concentration expressed as cpm/ml. The percentage of the unlabeled cells passing through the filter was then calculated from the following formula:

$$\% \text{ Unlabeled cells filtered} = \frac{\text{Total Hb. filtered} - [\frac{\text{Total Hb before filtration}}{\text{Total unlabeled Hb before filtration}} \times \text{Total }^{51}\text{Cr after filtration}] \times 100}{\text{Total unlabeled Hb before filtration}}$$

**Statistical Analysis**

Because day-to-day variations in the result of the incubation and filtration studies were large, individual experiments were performed so that iron-deficient and control erythrocytes were handled in exactly the same manner, using identical reagents at the same time. Then the results were subjected to paired analysis using a Student’s t test.12
Table 1.—Blood Counts of Rabbits Used in the Various Experiments*

<table>
<thead>
<tr>
<th>Animals</th>
<th>n</th>
<th>Hemoglobin (g/100 ml)</th>
<th>Hematocrit (%)</th>
<th>Red Cell Count (x10^6/cumm)</th>
<th>Mean Cell Volume (μm)</th>
<th>Serum Iron (μg/100 ml)</th>
<th>Total Iron Binding Capacity (μg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22</td>
<td>12.1</td>
<td>34.7</td>
<td>5.46</td>
<td>63.6</td>
<td>166</td>
<td>330</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10.0–14.0)</td>
<td>(27.5–40.0)</td>
<td>(4.15–6.33)</td>
<td>(56.4–74.7)</td>
<td>(120–264)</td>
<td>(315–368)</td>
</tr>
<tr>
<td>Iron-deficient</td>
<td>12</td>
<td>5.9</td>
<td>20.5</td>
<td>4.31</td>
<td>47.9</td>
<td>38</td>
<td>548</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.8–9.8)</td>
<td>(12.4–32.0)</td>
<td>(2.32–5.69)</td>
<td>(40.1–56.0)</td>
<td>(11–60)</td>
<td>(471–648)</td>
</tr>
</tbody>
</table>

* Mean values and ranges.

RESULTS

Blood Counts

Two weeks following the cessation of bloodletting, the rabbits demonstrated a hypochromic microcytic anemia with a reduced serum iron and an increased total iron binding capacity (Table 1). There were large number of poikilocytic and fragmented cells present in smears of blood from these severely iron-deficient animals. Size distribution curves demonstrated heterogeneous populations of microcytes with an average mean cell volume (MCV) of 48 cu μ (Fig. 1).

Erythrocyte Survival

The 51Cr half-life was found to be 5.6, 5.7, and 5.9 days in three severely iron-deficient rabbits; these values were markedly less than the mean value of 11.2 days (range 9.2–14.8 days) in six control animals (Fig. 2).

Autohemolysis

When the erythrocytes were incubated in Earle's balanced salt solution without glucose there was a marked increase in the autohemolysis of the iron-deficient cells at 24 hr of incubation compared with normal erythrocytes (Table 2). The average value for the iron deficient cells was 46% and for the normal cells was 29%. These results when subjected to paired analysis were statistically significant with p < 0.01.

The addition of glucose to the medium markedly improved the autohemol-

Fig. 1.—Size distribution of the erythrocytes of an iron-deficient rabbit.
Based on mean values of paired experiments.

ERYTHROCYTE ABNORMALITIES IN IRON DEFICIENCY

![Graph showing survival of ⁵¹Cr-labeled iron-deficient rabbit erythrocytes. Normal range of six animals represented by the shaded area.]

Fig. 2.—Survival of ⁵¹Cr-labeled iron-deficient rabbit erythrocytes. Normal range of six animals represented by the shaded area.

ysis of both the iron-deficient and normal cells. The average value for the iron-deficient cells was 6.9% and for the normal cells was 4.5% (Table 2). This difference between the two cell types was questionably significant with $p = 0.05$.

Effect of Incubation with Sulphydryl Inhibitors

The iron-deficient cells were more susceptible to lysis when incubated with NEM for 5 hr (Fig. 3). In these studies 50% hemolysis of normal cells occurred at greater than 65 $\mu$moles NEM per ml of red cell compared to 53 $\mu$moles/ml with iron-deficient cells.

The iron-deficient erythrocytes were also more susceptible to the effects of PMB (Fig. 4); 50% hemolysis occurred at 2.7 $\mu$moles/ml iron-deficient red cells compared to 3.4 $\mu$moles/ml with normal cells.

Filtration of Erythrocytes

To demonstrate that the labeling technique had no effect on the results,

Table 2.—Autohemolysis and Filtration of Normal and Iron-deficient Erythrocytes*

<table>
<thead>
<tr>
<th></th>
<th>Number of Experiments</th>
<th>Normal (%)</th>
<th>Iron-deficient (%)</th>
<th>Statistical Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autohemolysis at 24 hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without glucose</td>
<td>7</td>
<td>29</td>
<td>46</td>
<td>$p &lt; 0.01$</td>
</tr>
<tr>
<td>With glucose</td>
<td>7</td>
<td>4.5</td>
<td>6.9</td>
<td>$p = 0.05$</td>
</tr>
<tr>
<td>Filtration of erythrocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of initial cells</td>
<td>13</td>
<td>74</td>
<td>58</td>
<td>$p &lt; 0.01$</td>
</tr>
</tbody>
</table>

* Based on mean values of paired experiments.
normal cells were labeled with other normal cells and no significant differences in filtration could be found. Iron-deficient erythrocytes, despite their smaller volume, were consistently held up by the 5-μM Millipore filter to a greater extent than the normal cells (Table 2). The average number of iron-deficient cells filtered was 58%, compared with 74% of the normal cells.

Discussion

The normal erythrocyte is distorable, and this property allows it to pass through the small openings of the sinusoidal circulation of the spleen as well as the microcirculation of other organs. In contrast, the iron-deficient red cell as demonstrated by Millipore filtration experiments is less plastic. This abnormality may in part be responsible for the decreased red cell survival noted in iron deficiency. It is postulated that the rigid iron-deficient red cell would be held up in areas such as the spleen. There, the cell would be subject to conditions of stasis and glucose deprivation that would lead to their destruction. In addition, the final hemolysis of the iron deficient red cell may be hastened since the in vitro studies demonstrated an increased autohemolysis as compared to the normal cell if glucose is not added to the incubation medium.

The above sequence of events would explain the improvement noted in survival of the iron-deficient red cells when transfused into asplenic individuals. The increased sequestration of iron-deficient red cells within the spleen may also be responsible for the clinical findings of splenomegaly in patients with severe iron deficiency.

The results of the experiments with sulfhydryl inhibitors shed some light on the metabolic defect in iron-deficient cells that results in an abnormal cell
membrane. It has been postulated that the in vitro hemolytic effect of NEM and PMB is due to the action of these agents on membrane sulfhydryl groups. Although NEM and PMB attack sulfhydryl groups at similar dose levels in solutions, a significant greater concentration of NEM than PMB is necessary to produce hemolysis in normal red cells. This phenomenon is attributable to the fact that PMB is relatively impermeable to the membrane, whereas NEM readily enters the cells and combines with sulfhydryl groups of hemoglobin and glutathione thus diluting its effect on the membrane.

The increased hemolysis of iron-deficient cells with the addition of both sulfhydryl inhibitors suggests that there is a decreased number of sulfhydryl groups in the membrane. The increased susceptibility of iron-deficient cells to NEM may in part be due to a larger amount of the chemical being available at the membrane, since there is less binding by intracellular hemoglobin. However, this possibility would not be true in the case of PMB, which does not readily pass through the membrane. The surface-to-volume ratio of hypochromic red cells is greater than that of normal cells. Thus the total surface area of the iron-deficient cells per milliliter is greater than that of 1 ml of normal cells. One would then expect the total number of membrane sulfhydryl groups in 1 ml of iron-deficient cells to be greater than in 1 ml of normal cells. If this were true, then the iron-deficient cells should have been more resistant to hemolysis than normal cells with NEM and PMB since both these chemicals were added per milliliter of cells. However the iron-deficient cells were more sensitive. This suggests that the number of sulfhydryl groups in the membrane of an individual iron-deficient cell is less than one would expect from the reduction in surface area of the cell. It is conceivable that the inability of the iron-deficient red cell to maintain an adequate number of membrane sulfhydryl groups is responsible not only for decreased plasticity but also for decreased cell size secondary to premature loss of membrane.

Nonheme iron proteins are necessary coenzymes functioning as electron carriers in various dehydrogenase systems isolated from bacteria. Recently, Huennekens speculated that this may also be true in association with DPNH and TPNH-dependent methemoglobin reductase in human red cells. It is plausible that nonheme iron proteins may also be essential in other oxidation-reduction reactions that maintain the cell membrane sulfhydryl groups. If this is true, then depletion of these nonheme iron proteins in severe iron deficiency would result in a metabolic abnormality and a secondary membrane defect.

REFERENCES

Metabolic Abnormalities of Erythrocytes in Severe Iron Deficiency

ROBERT T. CARD and LEWIS R. WEINTRAUB