Influence of Splenectomy on Insoluble Hemoglobin Inclusion Bodies in \( \beta \) Thalassemic Erythrocytes

By L. M. Slater, W. A. Muir and R. I. Weed

Intracellular precipitates in ghosts prepared from red cells of patients with thalassemia major were described first by Hoffman\(^1\) in 1956. Such inclusions have been recognized in intact thalassemic erythrocytes by Josephson et al.,\(^2\) Fessas,\(^3\) Nathan et al.,\(^4,5\) and others. The present work was stimulated by observations of the difficulty involved in removing hemoglobin from thalassemic ghosts encountered during the course of other studies.\(^7\) The work was undertaken to quantitate the residual hemoglobin in ghosts from thalassemic cells in comparison with other hemolytic anemias and normal cells, as well as to compare ghosts from splenectomized versus non-splenectomized thalassemic patients. The role of the spleen in fragmentation of thalassemic cells has also been examined in relation to these intracellular precipitates.

Methods

Blood

Blood samples were collected from normal adult male and female donors; fetal red cells were collected from umbilical cord blood and abnormal cells were collected from patients with \( \beta \) thalassemia minor, homozygous sickle cell and sickle-hemoglobin C disease and patients with hereditary spherocytosis. The diagnosis of \( \beta \) thalassemia major was established by demonstration of the trait in both parents, on morphologic grounds and in some cases by the associated increase of fetal hemoglobin. No attempt was made to correlate clinical status with studies of residual ghost hemoglobin. Samples to be used immediately were anticoagulated with either heparin, \( \text{Na}_2\text{EDTA} \) or sodium citrate. Those received from a distance were collected into ACD. Storage of the latter samples never exceeded 96 hours. Neither the anticoagulant nor the 96-hour period of storage in ACD was found to influence the residual hemoglobin content.

The blood was centrifuged at 750 \( \times \) g. for 10 min., plasma and buffy coat removed by aspiration and the erythrocytes washed three times with 5 volumes of 1 percent \( \text{NaCl} \). Erythrocyte ghosts were prepared by technics previously described\(^8,9\) using stepwise, successive osmotic lysis in hypotonic \( \text{NaCl} \) tris buffer, pH 7.4, with osmolarity of final wash.
solution being 30 mOsm. White ghosts were produced by three washes of all lysed cells with 30 mOsm tris buffer except those of β thalassemia major which were washed four times but remained pink even after the fourth wash. Further washes did remove more hemoglobin but it was never possible to free the thalassemic ghosts completely of residual hemoglobin. During the course of the procedure to prepare thalassemic ghosts, a small amount of pink gelatinous residue was formed. Phase microscopic examination of this residue, however, revealed only rare intact ghosts and therefore this residue was discarded in spite of the fact that it contained hemoglobin. Although the hemoglobin content of ghosts prepared from thalassemic cells in the described manner is a reflection of residual intracellular hemoglobin, it probably provides a somewhat low estimate of insoluble hemoglobin in the intact cells not only because some hemoglobin was discarded with the gelatinous residue but also some insoluble hemoglobin may have become solubilized during the four washes to which the thalassemic ghosts were exposed. Table 1 illustrates the variation in residual hemoglobin content between separate ghost preparations from the blood of one post-splenectomy thalassemic patient. Although these data reflect both biologic variation and the variability of the method, prior studies8 using the same methods have demonstrated a standard deviation of ±.009 μg./cell in the residual hemoglobin content of ghosts prepared from normal red cells suggesting greater biologic variability in the thalassemic ghosts.

Residual cellular hemoglobin was measured by the benzidine method of Bing and Baker10 as modified by Dacie11 and erythrocyte ghosts were quantitated as previously described.7

Morphologic illustrative material includes phase photomicrographs of fresh, wet specimens of splenic imprint material and of intact red cells and ghosts prepared from the blood of a thalassemic patient before and after splenectomy as well as photomicrographs of splenic sections from the same patient. The splenic material was fixed in formalin-glutaraldehyde12 and imbedded in Araldite.13 Sections were stained with toluidine blue.

RESULTS

Table 2 summarizes the residual hemoglobin of 30 normal samples, 12 umbilical cord (fetal) blood samples, 6 β thalassemia major (splenectomized),
Table 3.—Thalassemia Major µg/Ghost

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>C. A.</td>
<td>.1234</td>
<td>E. R.</td>
</tr>
<tr>
<td>A. A.</td>
<td>.0246</td>
<td>A. M.</td>
</tr>
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<td>R. B.</td>
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<td>M. P.</td>
</tr>
<tr>
<td>M. S.</td>
<td>.1070</td>
<td>C. I.</td>
</tr>
<tr>
<td>D. M.</td>
<td>.0964</td>
<td>C. K.</td>
</tr>
<tr>
<td>C. C.</td>
<td>.0440</td>
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$x = \frac{.0710}{.0191}$  \hspace{1cm} $x = \frac{.0245}{.0072}$

S. E. = ± .0191 \hspace{1cm} S. E. = ± .0072

Fig. 1.—Phase photomicrograph of ghosts prepared from β thalassemic erythrocytes. A—Pre-splenectomy. B—Post-splenectomy.

5 β thalassemia major (post-splenectomy), and 3 β thalassemia minor samples as well as 3 samples from patients with homozygous sickle disease and 3 patients with sickle-hemoglobin C disease. The residual hemoglobin in ghosts prepared from post-splenectomy thalassemic cells is greater than normal by highly significant amounts ($p < .01$) while the hemoglobin content of pre-splenectomy ghosts differ from normal to a lesser extent but still have a significantly greater amount ($p < .05$). Although the data do not contain any values on ghosts prepared from splenectomized normal individuals, the low
value obtained from the splenectomized HS patients indicates that splenectomy alone cannot account for the elevated values in the thalassemic ghosts.

Table 3 analyzes the hemoglobin content of ghosts from splenectomized versus non-splenectomized patients with \( \beta \) thalassemia major. The mean ghost hemoglobin concentration was \( .071 \pm .0191 \) S.E. \( \mu g. \) /cell in the splenectomized group compared to a mean ghost hemoglobin concentration of \( .0245 \pm .0072 \) S.E. \( \mu g. \) /cell in the non-splenectomized group. This difference is significant \((p < .05)\). Figure 1 compares the appearance of \( \beta \) thalassemia major ghosts prepared from the blood of the same individual before and after splenectomy. Figure 1A illustrates the appearance of ghosts prepared prior to splenectomy. It is apparent that there is only a rare intracellular precipitate in the pre-splenectomy ghosts and marked deformity resembling that seen in the intact cells. Figure 1B, after splenectomy, shows ghosts that are larger, more uniform and virtually every cell can be seen to contain intracellular precipitates. Occasional extracellular precipitates are visible and can be produced by vigorous shaking of such a ghost suspension, with resultant escape of the rigid intracellular precipitates from the ghosts. Although the intracellular precipitates react with benzidine, they do not stain with Prussian blue and thus, their composition is consistent with hemoglobin but not hemosiderin. Figure 2 represents the appearance of the intact erythrocytes from which the ghosts in Figure 1 were prepared. In the pre-splenectomy blood (Figure 2A), little
Table 4.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre-Splenectomy</th>
<th>Post-Splenectomy</th>
<th>Prior Transfusions</th>
<th>Prior Transfusion</th>
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<tr>
<td></td>
<td>MCV</td>
<td>MCH</td>
<td></td>
<td>MCV</td>
</tr>
<tr>
<td>C. C.</td>
<td>64.3</td>
<td>19.0</td>
<td>0</td>
<td>80.2 (immed. post-splen.)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>81.4 (4 weeks)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>70.8 (8 weeks)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>75.2 (12 weeks)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>73.6 (26 weeks)</td>
</tr>
<tr>
<td>A. A.</td>
<td>55.8</td>
<td>14.8</td>
<td>0</td>
<td>77.0</td>
</tr>
<tr>
<td>A. S.</td>
<td>71.8</td>
<td></td>
<td>q 4–6 weeks</td>
<td>90.8</td>
</tr>
<tr>
<td>M. S.</td>
<td>75.0</td>
<td></td>
<td>q 6 weeks</td>
<td>85.6</td>
</tr>
</tbody>
</table>

Fig. 3.—Phase photomicrograph of wet imprint preparation of spleen from patient with β thalassemia. Note the cell in the central channel which has condensation of hemoglobin at the ends and appears to be partially torn in half.

or no intracellular precipitation of hemoglobin is evident in the intact red cells as seen under the phase microscope but much fragmentation and many of the typical tear-drop-shaped red cells can be seen. The arrow in the center of Figure 2 A indicates a cell which appears to have been drawn nearly in two
Fig. 4.—Section of spleen from patient with β thalassemia major. Note deformed cell in passage from splenic cord (upper left) to splenic sinus (lower right).

with dense masses of hemoglobin at the ends connected by a thin outer membrane. Prolonged observation of such cells revealed no tendency for them to regain a more regular shape or for the hemoglobin to redistribute itself more uniformly. Maintenance of such irregular shapes and uneven intracellular distribution of hemoglobin must indicate cell membrane rigidity and/or intracellular gelation of hemoglobin. By contrast, Figure 2 B shows larger erythrocytes from which the ghosts in 1 B were prepared and also demonstrates the existence of multiple intracellular precipitates as well as intracellular vacuoles but less evidence of cellular distortion.

Table 4 compares pre- and post-splenectomy mean red cell corpuscular volumes on blood from four patients and mean corpuscular hemoglobin on two. Note that the pre-splenectomy values for mean cell hemoglobin and mean cell volume are very low, consistent with the many small fragmented cells which are present. One patient received a total of only one unit of transfused blood, given prior to splenectomy and the other three patients required fewer transfusions after splenectomy. Thus, transfused blood cannot account for the observed differences. The Table illustrates that following splenectomy there was an increase in mean cell hemoglobin and mean cell volume. Although there is an increase in reticulocytosis after splenectomy (from 4–8 percent in patient C. C.), which contributes to the greater MCV, the decrease in small fragmented forms apparent in the peripheral blood suggests that in addition to the reticulocytosis, the decrease in fragmentation may contribute to the changes in volume.
Fig. 5.—Section of spleen from patient with $\beta$ thalassemia major. Note deformed cells in passage from splenic cord to splenic sinus (top to bottom of Figure).

Spleen

Figure 3 is a phase photomicrograph of a wet imprint preparation from the spleen from the patient whose cells and ghosts are illustrated in Figures 1 and 2. Figures 4 and 5 are light micrographs of sections of the same spleen. The splenic sections illustrate passage of thalassemic red cells through the basement membrane separating the splenic cords from the splenic sinus. A portion of each cell appears to be held up in passage while the distal portion has
passed through the basement membrane, acquiring a tear-drop configuration and appears to be in the process of breaking or fragmenting off from the denser portion.

Discussion

The intracellular inclusions recognized by Hoffman et al.\(^1\) in ghosts prepared from \(\beta\) thalassemia major erythrocytes were also noted by Tishkoff\(^14\) who found them to be rich in non-heme iron. In addition, however, Fessas, Loukopoulous and Thorell\(^15\) have demonstrated that these inclusions have spectral characteristics of denatured globin-hemochrome. Fessas\(^3\) suggested that these intracellular inclusions might consist of precipitated \(\alpha\) chains and more recently through the use of peptide fingerprinting, Fessas\(^16\) has demonstrated that these inclusions consist predominantly of precipitated material whose composition is consistent with \(\alpha\) chains. The basis of the occurrence of such precipitates of \(\alpha\) chains was suggested by Heywood, Karon and Weissman\(^17\) and Weatherall, Clegg, and Naughton\(^18\) who demonstrated an asymmetric rate of incorporation of C\(^{14}\)-labeled leucine into \(\alpha\) and \(\beta\) chains respectively, with the specific activity of \(\beta\) chains being much lower. Bank and Marks\(^19,20\) have demonstrated that the relatively lower rate of \(\beta\) chain synthesis represents decreased synthesis rather than an absolute increase in \(\alpha\) chain synthesis. In addition, the observations of Huehns, Dance and Shooter\(^21\) on the one hand and Tyuma, Benesch and Benesch\(^22\) on the other have called attention to the fact that isolated \(\alpha\) chains are known to have marked physical instability under conditions of low ionic strength, oxidation of the heme-iron to the ferric state or secondary to disulfide formation in the presence of free thiols. These charac-
teristics of isolated \(\alpha\) chains when considered in the light of the genetically determined basis for their relative overproduction suggest that \(\alpha\) chain excess is the basis for formation of intracellular precipitates in intact cells. The greater visible precipitation of hemoglobin in post-splenectomy ghosts compared to intact cells (Figure 2 B vs. 1 B) may be related to changes in ionic strength or redox potential which occur during the preparation of the ghosts themselves.

Nathan and Gunn\(^4\) and Bank and Marks\(^{19,20}\) have discussed these mechanisms and proposed that precipitation of excess \(\alpha\) chains predisposes \(\beta\) thalassemic red cells to premature removal from the circulation. What then is the relation of the relative overproduction of \(\alpha\) chains to the red cell fragmentation noted by Whipple and Bradford\(^{21}\) and emphasized as an important feature of thalassemia major by Marmont and Bianchi\(^{24}\) and what role does the spleen play in this process? As Nathan and Gunn\(^4\) have suggested, the precipitated \(\alpha\) chains presumably give rise to Heinz bodies which may be removed by the pitting function\(^{29}\) of the spleen. Studies of the spleen by Koyama et al.,\(^{26}\) Rifkind,\(^{27}\) Weed and Weiss\(^{28}\) and Wennberg and Weiss\(^{29}\) have shown that rigid Heinz body-containing portions of cells are held back upon passage from the splenic cord to splenic sinus. The less rigid portion of the cell may fragment off into the sinus and return to the circulation.\(^{26,28}\) The studies by Wennberg and Weiss\(^{29}\) of spleen from a patient with hemoglobin H disease illustrate remarkable red cell fragmentation and pitting of Heinz bodies occurring within the spleen. These same phenomena can be recognized in thalassemic spleen (Figures 3, 4, and 5). In addition to actual Heinz body formation in intact cells, the persistent cellular deformities (Figure 2) indicate intracellular gelation of hemoglobin which undoubtedly contributes to cellular rigidity and difficulty of splenic passage.

Thus, many of the small, fragmented and distorted cells in non-splenectomized thalassemia major blood may arise from fragmentation pitting of intracellular precipitates secondary to splenic passage. Support for this suggestion is provided by the studies of Wennberg and Weiss\(^{29}\) as well as in the present studies, by the splenic morphology itself. Additional evidence includes the change in the appearance of post-splenectomy cells to less fragmented, larger cells containing inclusions and vacuoles\(^{30}\) but also by the increase in MCV and MCH of post-splenectomy cells first mentioned by Bradford and Dye.\(^{31}\) Finally, the present demonstration of a distinct increase in residual ghost hemoglobin after splenectomy suggests that the gelled or precipitated \(\alpha\) chains are pitted out of the cells by the spleen. An alternative explanation for the low residual ghost hemoglobin and the sparsity of intact cells containing vacuoles and inclusions prior to splenectomy is that spleen completely removes such cells but that they continue to circulate post-splenectomy. While sequestration of entire cells containing Heinz bodies and vacuoles undoubtedly provides a partial explanation, it does not account for the marked decrease in the number of markedly distorted and fragmented small cells which also occur post-splenectomy. If the pathologically small and distorted cells seen prior to sple-
Splenectomy were solely a product of faulty erythropoiesis, they presumably should have been predisposed to splenic removal and the apparent numbers of fragmented cells should have increased upon splenic removal, tending to balance the effect of the larger inclusion-containing cells on the MCV and MCH. Since fragmentation pitting of intracellular inclusions has been demonstrated morphologically and must result in return to the circulation of cells which have a lower MCV and MCH, the consequences of splenic passage provide a single explanation for the change in appearance of thalassemic red cells after splenectomy as well as change in their MCV and MCH and residual hemoglobin of ghosts.

Fragmentation of a rigid cell may occur anywhere within the microcirculation where deformability is required for passage, although the spleen is ideally suited anatomically to produce this phenomenon. Certainly within the bone marrow, the narrow passage from hematopoietic cords through basement membrane into sinuses leading to the central vein may play a role in enucleation of red cells as shown by Weiss. This same narrowed passage may provide the initial barrier leading to loss of rigid intracellular precipitates from thalassemic red cells as they enter the circulation. In this sense, “intra-marrow hemolysis” may represent loss of some hemoglobin from many cells as well as total loss of some cells.

Nathan and Gunn have also emphasized the increased membrane cation permeability properties of thalassemic cells. Since the changes are more marked pre-splenectomy, it is possible that the alterations may arise secondary to the distortion and damage which follows fragmentation-pitting of the insoluble hemoglobin precipitates. The increased ion permeability in turn may also hasten ultimate hemolysis of the remaining portion of the cell by predisposing them to colloid osmotic lysis.

Figure 5 summarizes a proposed pathogenetic mechanism for the hemolytic phenomena of thalassemia major. It represents a synthesis of the present observations and proposals of other workers. As Nathan and Gunn and Bank and Marks have suggested, secondary to the genetically determined decrease in β chain synthesis, the asymmetric relative overproduction of α chains with their physical instability may lead to development of the intracellular precipitates which are most evident in post-splenectomy cells. These insoluble and rigid precipitates may lead to fragmentation-pitting occurring in any area of restricted passage within the microcirculation but apparent to a marked degree within the spleen. Fragmentation of thalassemic erythrocytes may give rise to small, rigid and distorted cells which undoubtedly are predisposed either to further fragmentation or subsequent sequestration and lysis.

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