Decreased Erythrocyte Survival in Hemoglobin H Disease
As a Result of the Abnormal Properties of Hemoglobin H: The Benefit of Splenectomy

By Demetrios A. Rigas and Robert D. Koler

With the Technical Assistance of George Cummings, Marie L. Duerst, Donald R. Malm, Katherine Swisher and Patricia Vanbellinghen

Since the first description of hemoglobin H,1 many reports have appeared indicating a wide racial and geographical distribution of this hemoglobinopathy. We reported a shortened erythrocyte survival in a case, which was attributed to the property of hemoglobin H to denature and precipitate.2 The spleen was thought to be the major site of destruction, and splenectomy was considered. Reports of improvement of patients with hemoglobin H following splenectomy3-4 strengthened this hypothesis. We will present data indicating considerable improvement of two patients with hemoglobin H disease following splenectomy and suggesting that the abnormal structure of hemoglobin H is the cause of the reduced erythrocyte survival and that its instability is enhanced by environmental factors, particularly by certain chemicals and drugs.

METHODS

Enumeration of erythrocytes and reticulocytes,5 determination of hemoglobin6 and hematocrit7 were done according to methods in the cited references. Hemolysates were prepared by addition of equal volumes of distilled water to washed erythrocytes. Because it accelerates the rate of denaturation of hemoglobin H,8 toluene was not used and clarification of hemolysates was accomplished by centrifugation at 26,000G. Unless otherwise indicated, all hemolysates were converted to carbonmonoxyhemoglobin. Paper electrophoresis analysis was done with the Spinco Model R apparatus, using veronal buffer of pH 8.6, ionic strength 0.05.

Erythrocytes were labeled with Cr51 by incubating 4 μc. of Na2CrO4* per ml. of sterile, heparinized blood at 37 C. Labeling was stopped after 30-60 minutes by addition of 50 mg. ascorbic acid per ml. of blood,9 the plasma and buffy coat were removed, the erythrocytes washed three times with sterile isotonic saline and resuspended in saline to a volume equal to that of the original sample. Erythrocyte survival was studied by re-injecting 20 ml. of the labeled cell suspension into the same subject or into compatible non-
mal recipients. Both donor and recipient were typed for A, A', B, M, N, C, D, E, c, d, and Kell, and cross-matching was done using standard saline, Coombs, papain, albumin, and bromelin methods. A blood sample drawn 15 minutes after injection was used for calculation of blood volume. Duplicate 2 ml aliquots of all blood samples, and a weighed aliquot of the spleen, were counted with a well-type scintillation counter and a pulse height analyzer. Body surface counting was done, utilizing a scintillation detector with a flat crystal.

The mathematical analysis of the Cr erythrocyte survival data was done with the aid of the equations:

\[
A_t = A_0 (1 - mt) e^{-kt}
\]

and

\[
L = \frac{1 - e^{-m}}{k}
\]

The derivation of these equations is described elsewhere. \(A_0\) and \(A_t\) represent the Cr erythrocyte (corrected for physical decay) present at times zero and \(t\) respectively; the potential mean cell life in the absence of random destruction which we shall refer to as the finite lifespan, \(L\), is equal to \(1/m\). The constant \(k\) represents the random destruction of hemoglobin \(H\) erythrocytes. Equation (2) gives the mean lifespan, \(L\), accounting for cell death due to senescence and random destruction. The generalized least squares method was used to fit the curves of the form of equation (1) to the experimental data and to calculate 99 per cent confidence belts. The "Student's" \(t\) test was used to compare data obtained at different times or on different subjects. Separation of erythrocytes by age was done by centrifugation at 1600 G for 2 hours. Oxyhemoglobin \(H\) was separated by continuous flow paper electrophoresis using the Spinco Model CP apparatus and veronal buffer of pH 8.6, ionic strength 0.02 at 2 C. The solubility of oxygenated and deoxygenated hemoglobin \(H\) was determined in 2.24 M phosphate buffer of pH 6.8 at 25 C., and in unbuffered hemolysates concentrated by evaporation under partial vacuum avoiding freezing or boiling. Intraerythrocytic inclusions were studied by staining air-dried fresh blood smears with Wright's stain or Lepehne's reagent, by the reticulocyte staining technic, and by phase microscopy.

RESULTS

Figure 1 presents the genetic diagram of the family studied.

Clinical Effect of Splenectomy: Subject 10: The first Cr erythrocyte survival was started on 6/15/56. On 7/9/56, a 272 Gm. spleen was surgically removed; operative and postoperative course was uncomplicated, and the patient's strength and exercise tolerance improved. A second erythrocyte survival study was started on 6/17/57. A urinary tract infection on 6/24/57 cleared promptly during a 10-day course of sulfisoxazole. On 7/3/57, after the patient had passed an adult Ascaris worm, she was treated with magnesium sulfate and hexyresorcinol. Stool samples contained no occult blood and on follow-up have been free of ova and parasites. The erythrocyte survival curve dropped abruptly at this time. Since then the patient has had a chronic urinary tract infection requiring repeated courses of antibiotics but has been well otherwise; she is active and does all her housework.

Subject 7: The first Cr erythrocyte survival was done on 3/21/55, and on 7/23/58, a 430 Gm. spleen was surgically removed. The patient's operative and postoperative course was uncomplicated. On 7/29/58, a second Cr erythrocyte
survival study was started. The patient has been well since surgery, except for an acute hemolytic episode from 10/1/59 to 11/2/59, characterized by headache, fever to 101, dark urine and malaise. Examination on 10/25/59 revealed a hemoglobin of 8.6 Gm., 27,000 leukocytes with 57 per cent lymphocytes, many of which were atypical, a total serum bilirubin of 0.8 mg. per cent, a negative heterophile agglutination, and transitory tender, red areas along the course of both superficial saphenous and right antecubital veins. This episode was self-limited and was thought to represent an acute infection, possibly of viral etiology, with hemolysis and superficial phlebitis. Hemoglobin on 11/2/59 had returned to 11.0 Gm. and reticulocyte count, which was elevated to 10 per cent during this period, dropped to 4 per cent. The patient is now operating his own business and has noted considerable increase in strength and exercise tolerance since splenectomy.

_Erythrocyte Lifespan._ Figure 2 demonstrates the Cr\(^{51}\) erythrocyte survival before and after splenectomy for subjects 7, 10 and 12, with the normal range based on 16 subjects. The normal values calculated from these data are: k = 0.0132 ± 0.001 (Cr\(^{51}\) elution), L = 117.9 ± 0.61 days, and T\(_{1/2}\) = 28.5 ± 4.9 days (50 per cent survival). The erythrocyte survival of subject 12, who has hereditary spherocytosis trait but no hemoglobin H, falls within the normal range. In both subjects 7 and 10, the rate of Cr\(^{51}\) disappearance was much greater than normal before splenectomy and decreased significantly following splenectomy but did not become normal. The finite lifespan, L, and random loss, k, are given in table 1. Subject 7, who had no complications during the Cr\(^{51}\) survival study, had an erythrocyte finite lifespan of 41 days before and 117 days after splenectomy. The presplenectomy finite lifespan for subject 10 was 45 days. If all the data obtained following splenectomy are used, the subject showed an increase to 52 days, whereas, if the points obtained after the 21st day are rejected, her calculated postsplenectomy red cell finite lifespan was normal.
SUBJECTS 7 AND 12 AND RANGE OF NORMALS

Mean survival of normal erythrocytes

Normal range

Subject 12

Before Splenectomy

99% Confidence belt before splenectomy

After Splenectomy

99% Confidence belt after splenectomy

RICAS AND KOLEB

100

90

80

70

60

50

40

30

20

10

0

lime in days

Fig. 2.—Chromium erythrocyte survival curves of subjects 7, 10, and 12, and normal range.

The early senescence of hemoglobin H erythrocytes has been postulated to result from precipitation of hemoglobin H in old cells which are then rapidly destroyed in the spleen. Such precipitated hemoglobin was not seen in erythrocytes prior to splenectomy, but smears of postsplenectomy venous blood contained erythrocytes with large spherical inclusions. These inclusions stained specifically for hemoglobin with Lepehne's reagent but were also seen in Wright's-stained preparations or after staining 1 minute with brilliant cresyl blue (figs. 3a,-b, -c). They were seen only in the mature erythrocytes, never in reticulocytes. They appeared to be preformed in the erythrocytes in contrast to the artifactitious inclusions which were observed in presplenectomy blood only after in vitro exposure to brilliant cresyl blue for 15–30 minutes and which represent precipitation of hemoglobin H by this dye. After 3 hours of incuba-
Table 1.—Hematological Values Before and After Splenectomy

<table>
<thead>
<tr>
<th>Subject</th>
<th>Hemoglobin, Gm. %</th>
<th>Before Splenectomy</th>
<th>After Splenectomy</th>
<th>Before Splenectomy</th>
<th>After Splenectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.5 ± 0.4 †</td>
<td>(9.7–11.3) †</td>
<td>12.2 ± 0.6</td>
<td>(10.3–13.9)</td>
<td>8.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>n = 5 ‡</td>
<td>n = 7</td>
<td>n = 21</td>
<td>n = 45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobin H, %</td>
<td>5.2 ± 0.3</td>
<td>5.9 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>RBC × 10⁶ mm³</td>
<td>(4.8–5.6)</td>
<td>(5.3–6.9)</td>
<td>(3.2–5.6)</td>
<td>(3.5–6.3)</td>
</tr>
<tr>
<td></td>
<td>n = 4</td>
<td>n = 7</td>
<td>n = 9</td>
<td>n = 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hematocrit, %</td>
<td>40 ± 1.8</td>
<td>41 ± 1.7</td>
<td>31 ± 0.6</td>
<td>34 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(35–45)</td>
<td>(34–45)</td>
<td>(27–34)</td>
<td>(28–38)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reticulocytes, %</td>
<td>7.3 ± 2.2</td>
<td>5.9 ± 1.1</td>
<td>5.6 ± 1.6</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(3.4–13.9)</td>
<td>(2.8–9.7)</td>
<td>(2.5–22.1)</td>
<td>(1.8–8.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 5</td>
<td>n = 7</td>
<td>n = 12</td>
<td>n = 41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobin H, %</td>
<td>40 ± 0.5</td>
<td>27 ± 0.2</td>
<td>35 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>total <em>%</em> of</td>
<td>(38–43)</td>
<td>(24–30)</td>
<td>(23–28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 5</td>
<td>n = 7</td>
<td>n = 12</td>
<td>n = 41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Finite life span (L) in days</td>
<td>41 ± 6</td>
<td>117 ± 12</td>
<td>45 ± 6</td>
<td>52 ± 3</td>
</tr>
<tr>
<td></td>
<td>Random destruction (k) in days</td>
<td>0.04 ± 0.007</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.005</td>
<td>0.01 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>Mean life span (L) in days</td>
<td>21 ± 6</td>
<td>30 ± 10</td>
<td>29 ± 7</td>
<td>40 ± 14</td>
</tr>
<tr>
<td></td>
<td>T₁/₂ (half life of injected Cr⁵¹)</td>
<td>11 ± 2</td>
<td>17 ± 2</td>
<td>14 ± 1.5</td>
<td>20 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>RBC containing large spherical preformed inclusions</td>
<td>0</td>
<td>4.0 ± 0.5</td>
<td>0</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>n = 13</td>
<td>(1.6–6.5)</td>
<td>(1.7–2.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RBC containing small Heinz-like preformed inclusions</td>
<td>0</td>
<td>16.2 ± 1.8</td>
<td>0</td>
<td>20.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>n = 13</td>
<td>(5.1–23.5)</td>
<td>(15.8–23.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data obtained during a two-month period following splenectomy were not used as the patient had been transfused prior to operation.
†Mean value ± its standard error.
‡Observed range.
§Number of determinations.
Fig. 3.—See legend, facing page.
per gram of hemoglobin as the water extract, which is consistent with the higher Cr\textsuperscript{51}-binding capacity of hemoglobin H\textsuperscript{15}.

The development of preformed inclusions and the decrease in hemoglobin H in aged erythrocytes were demonstrated as follows: During the postsplenectomy Cr\textsuperscript{51} erythrocyte survival study in subject 7, all samples were centrifuged at 1600G and the packed column of erythrocytes was divided into three equal layers. The rate of disappearance of Cr\textsuperscript{51} from each layer is plotted in figure 5. Separation of the erythrocytes according to age with the youngest at the top and the oldest at the bottom is indicated by the disappearance of Cr\textsuperscript{51} first from the top and last from the bottom layer, and by the relative number of reticulocytes in the three layers as noted in table 2. No Cr\textsuperscript{51} was found in the top and middle layers of the 67th and 88th day samples. The percentage of erythrocytes containing preformed inclusions increased while the amount of soluble hemoglobin H decreased progressively from the top to the bottom layer (table 2 and fig. 6).

Participation of the spleen in the removal of old erythrocytes containing precipitated denatured hemoglobin H is supported by the following observations. Erythrocytes containing preformed inclusions were present in smears prepared from minced splenic pulp (figs. 4b and 4c) but were not found in splenic imprints, indicating that such erythrocytes adhere strongly to the matrix of the spleen. Accumulation of Cr\textsuperscript{51} in the spleen was also significant; of the total Cr\textsuperscript{51} remaining in subject 10 at the time of splenectomy, 31 per cent was accounted for in the spleen and blood. A third of it was present in the 272 Gm. spleen, and two-thirds in the 3700 ml. of blood, i.e., there was at least a sevenfold concentration of Cr\textsuperscript{51} in the spleen as compared with the blood which perfused it. Histologic sections of both spleens showed marked congestion of the sinusoids with erythrocytes and extensive deposits of hemosiderin. Finally, when Cr\textsuperscript{51} labeled postsplenectomy erythrocytes from subject 10 were given to two compatible normal recipients, the radioactivity of the blood samples fell precipitously, reaching 30.5 per cent in one and 45 per cent in the other within 30 minutes and less than 5 per cent in both within 20 hours. Surface body counts indicated sequestration within 30 minutes of much of the activity in the spleen followed by slight decrease in splenic activity and continued accumulation over the liver, sacrum, and lung and negligible activity over the thigh. The per cent activity was calculated from the blood volume obtained by a later study using the recipient's own erythrocytes labeled with Cr\textsuperscript{51}.

The following observations indicate that oxidation to methemoglobin is responsible for the rapid denaturation and precipitation of hemoglobin H in cells over 40–45 days old, but is prevented in younger cells, and explain the shortened erythrocyte finite lifespan prior to splenectomy. In vitro studies dis-

Fig. 3.—Photomicrographs of erythrocyte inclusions from subject 10 following splenectomy: (a & b) whole blood stained with Wright's and Lepehne's stain respectively; (e & d) brilliant cresyl blue stain of whole blood for 1 minute and 180 minutes respectively; (e & f) inclusions from residue following hemolysis and exhaustive washing with water and stained with Lepehne's and Wright's stain respectively.
Erythrocyte survival in hemoglobin H: splenectomy

Fig. 5.—Disappearance of Cr⁷¹ from layers of centrifuged erythrocytes during survival study of subject 7.

closed that oxidation of pure hemoglobin H to methemoglobin is accompanied by greatly accelerated denaturation.⁸ When the erythrocytes of subject 10 were subjected to the action of methemoglobin-forming agents such as 0.004 M amyl nitrite or 0.0058 M sodium nitrite in an isotonic and neutral pH environment for 4 to 24 hours, all the erythrocytes developed inclusions which stained immediately with brilliant cresyl blue (fig. 4d). Hemolysates of such erythrocytes were found to be devoid of hemoglobin H (fig. 7). Spectra of the hemolysates showed a strong band at 630 μm indicating methemoglobin formation. Reduced glutathione and sodium ascorbate counteracted the effect of these agents. No inclusions were detected in normal human erythrocytes subjected to the action of these substances although methemoglobin was formed. Erythrocyte enzyme systems are known to protect hemoglobin from oxidation,¹⁶,¹⁷ but they decrease with cell age and rapid accumulation of methemoglobin begins at a cell age of 40–45 days.¹⁸ In hemoglobin H disease we have found that several of these protective enzymes and the reduced glutathione decrease as the erythrocytes age.¹⁹

The abrupt drop in the erythrocyte survival curve of subject 10 and the occasional decreased hemoglobin values (8–9 Gm. per cent) without reticulocytopenia, all of which were observed after therapy with sulfisoxazole for urinary tract infections, suggested that this drug might be responsible for the hemolytic

Fig. 4.—Photomicrographs of erythrocyte inclusions from subject 10: (a) Same as in fig. 3e & 3f but stained with brilliant cresyl blue; (b & c) erythrocytes from splenic pulp stained with brilliant cresyl blue for 1 minute and with Wright's stain respectively; (d & e) erythrocytes treated with amyl nitrite and sulfisoxazole respectively and then stained with methylene blue for 1 minute; (f) erythrocytes treated with methylene blue for 24 hours.
Table 2.—Distribution of Hemoglobin H and of Intraerythrocytic Bodies in Centrifuged Erythrocytes Following Splenectomy

<table>
<thead>
<tr>
<th>Erythrocyte Layer</th>
<th>Hemoglobin H % of Total</th>
<th>Reticulocyte %</th>
<th>Large Spherical Bodies</th>
<th>Small Heinz-like Bodies</th>
<th>Large Spherical Bodies</th>
<th>Howell-Jolly Bodies</th>
<th>Large Spherical Bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top</td>
<td>35</td>
<td>11.8</td>
<td>0.3</td>
<td>12.3</td>
<td>0.2</td>
<td>0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Middle</td>
<td>27</td>
<td>3.5</td>
<td>0.9</td>
<td>17.6</td>
<td>0.4</td>
<td>0.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Bottom</td>
<td>18</td>
<td>1.6</td>
<td>4.1</td>
<td>34.3</td>
<td>2.2</td>
<td>0.0</td>
<td>1.00</td>
</tr>
</tbody>
</table>

episodes. For this reason in vitro studies were done, adding small amounts of sulfisoxazole or various other drugs and chemicals to erythrocytes from subject 10 and from normals under isotonic and neutral pH conditions. It was thus found that 1-acetyl-2-phenylhydrazine at a concentration of 5 mg per ml., although not significantly affecting the GSH stability, caused methemoglobin formation, complete precipitation of hemoglobin H and formation of inclusions which were indistinguishable from Heinz bodies formed in normal erythrocytes by this substance and from those caused by amyl nitrite in hemoglobin H erythrocytes. These inclusions were more numerous and larger in the hemoglobin H erythrocytes than in normals. Hemoglobin H erythrocytes but not normal erythrocytes were similarly affected by 0.14 M sulfisoxazole-diethanolamine (not diethanolamine alone), 0.014 M of crystalline sulfisoxazole, 0.25

Fig. 6.—Paper electrophoregrams of hemolysates obtained from layers of centrifuged erythrocytes.
per cent methylene blue, 0.02 per cent dithionite and 0.14 M polyethylene glycol (figs. 4e, 4f, and 7). In a screening test, no such action was demonstrated by: a saturated solution of sulfanilamide, 10 mg. per ml. of chloramphenicol or tetracycline with glucosamine, 0.14 M of acetylanilide in glycerine, sodium ascorbate, meprobamate, mandelamine, acetophenetidine, sodium salicylate, and sodium acetyl salicylate. Because of its effect, sulfisoxazole has been withheld for the past eight months and tetracyclines have been used to treat flare-ups in the patient’s urinary tract infection. During this period the patient’s hemoglobin was stable at 10.5 to 11.0 Gm. per 100 ml. of blood.

The in vivo exponential loss of Cr$^{51}$ from the erythrocytes of subjects 7 and 10 was strikingly higher than normal (table 1) but, in contrast to normal erythrocytes, no elution of Cr$^{51}$ from these erythrocytes could be demonstrated in vitro.$^{15}$ This indicates that the exponential disappearance of Cr$^{51}$ from the erythrocytes of these patients is not due to elution but to random destruction of erythrocytes. That intracellular precipitation of hemoglobin H by the
Na₂CrO₄ was not responsible for this random destruction was indicated by the fact that labeled and unlabeled cells, refrigerated for 12 days and then hemolyzed, were found to contain 17.7 and 16.9 per cent hemoglobin H respectively. That the random destruction of hemoglobin H erythrocytes was probably due to an innate cell defect was suggested by the observation that anoxic conditions lead to development of intracellular inclusions.² Capillary blood of subjects 7 and 10 obtained by fingerstick prior to and after a 3 minute application of a tourniquet to the finger, diluted with the patient's deoxygenated plasma, and examined immediately under phase microscopy, showed that 30 and 90 per cent of the erythrocytes, respectively, contained such inclusions, which disappeared upon reoxygenation.

The following studies indicate that this reversibly precipitating material was hemoglobin H. The solubility of electrophoretically pure oxyhemoglobin H and of hemoglobin H reduced with Na₂S₂O₄ or nitrogen flow was determined in Itano's 2.24 M phosphate buffer. In contrast to hemoglobin A which is completely soluble under these conditions,¹⁴ partial precipitation of hemoglobin H occurred. The results (table 3) showed (a) that oxyhemoglobin H was less soluble than oxyhemoglobin A, and (b) hemoglobin H was less soluble in the reduced than in the oxygenated form. Precipitates thus formed were soluble in 0.1 M phosphate buffer at pH 7.0, except that formed with Na₂S₂O₄, which was partially soluble due to considerable denaturation. A deoxygenated, unbuffered hemolysate from subject 7 was concentrated until saturation and precipitate formation. Part was then clarified by filtration while in the reduced form, and part following oxygenation. It is apparent from table 3 that reoxygenation resulted in solubilization of precipitated hemoglobin H, as it increased the total hemoglobin and the percentage of hemoglobin H (determined by paper electrophoresis).

On the basis of these observations we have assumed that the random destruction of erythrocytes in hemoglobin H disease is due to this innate defect, and we have calculated the mean lifespan, $\bar{L}$, which takes into account random destruction and death due to senescence. Table 1 summarizes pre- and post-splenectomy data from subjects 7 and 10. All values indicated benefit from splenectomy, but only the increase in hemoglobin and erythrocyte survival and the decrease in the per cent of hemoglobin H are significant ($P < 0.01$).

**DISCUSSION**

The most striking changes in these patients following splenectomy were the appearance of preformed inclusions in circulating erythrocytes and the im-

### Table 3.—Solubility of Native Hemoglobin H

<table>
<thead>
<tr>
<th>Phase</th>
<th>In 2.24 M Phosphate Buffer, pH 6.8</th>
<th>In Concentrated Hemolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxyhemoglobin mg./100 ml.</td>
<td>Reduced with Na₂S₂O₄ mg./100 ml.</td>
</tr>
<tr>
<td>Supernatant</td>
<td>486</td>
<td>339</td>
</tr>
<tr>
<td>Precipitate</td>
<td>19</td>
<td>163</td>
</tr>
<tr>
<td>Total</td>
<td>505</td>
<td>502</td>
</tr>
</tbody>
</table>
ERYTHROCYTE SURVIVAL IN HEMOGLOBIN H: SPLENECTOMY

provement in red cell survival. All other effects can be related to these two changes. Preformed inclusions have also been observed in hemoglobin H patients following splenectomy by Minnich and Fessas. These inclusions differ from those observed in a type of familial hemolytic anemia, but resemble those found in three patients with leukemia.

The evidence presented here, together with our in vitro observations, indicate that there are two separate causes of hemolysis in hemoglobin H disease, both attributable to the abnormal properties of hemoglobin H. Thus the susceptibility of hemoglobin H to denaturation and precipitation, which is greatly increased by oxidation to methemoglobin, leads to formation of insoluble intraerythrocytic inclusions at a cell age of 40–45 days, and to rapid destruction of such erythrocytes by the spleen. This explains the shortened erythrocyte finite lifespan in hemoglobin H disease, its increase to normal following splenectomy, and the post-splenectomy decrease in the per cent of hemoglobin H. None of these could be explained if the inclusions but not the containing erythrocytes were removed by the spleen. In addition, the lower solubility of hemoglobin H in the deoxygenated state leads to reversible precipitation in the capillary circulation, regardless of cell age, and to random destruction of the erythrocytes. Splenectomy has little effect on this destruction as it occurs predominantly in the capillary bed. The benefit of splenectomy is primarily due to survival of cells with preformed inclusions, whose hemoglobin A presumably retains its capacity to function in oxygen transport. As the amount of hemoglobin H in the reported cases varies from 2.5 to 40.0 per cent, it is likely that the severity of the hemolytic process and the degree of improvement following splenectomy also vary. Patients with little hemoglobin H may show no shortening of the erythrocyte survival nor reversible precipitation of hemoglobin H upon deoxygenation of the erythrocytes. The various degrees of shortening of erythrocyte survival and of benefit following splenectomy that have been reported by others, and the inability of Fessas to confirm our phase microscopy observations may be explained on this basis.

Splenectomy, although beneficial, does not constitute a cure of this disease as the genetic and biochemical defect is still present and the patients remain subject to hemolytic episodes due to infection, methemoglobin-forming drugs, etc. The degree of compensation for continued hemolysis varies and is higher in subject 7 than in 10 (P < 0.01), as judged by hemoglobin and hematocrit levels.

The observations made on the survival of postsplenectomy erythrocytes from subject 10 in the two normal recipients may be explained on the basis of the two hemolytic mechanisms. Thus, the accumulation of Cr in the spleen within the first 30 minutes followed by a decline thereafter is explainable in terms of the rapid sequestration and destruction in this organ of erythrocytes containing preformed inclusions. The elimination of the remaining erythrocytes and accumulation of Cr in the reticuloendothelial system is explainable by the random destruction of these erythrocytes due to reversible precipitation of hemoglobin H upon deoxygenation in the capillary bed. The very short survival of these cells in compatible normal recipients indicates that the normal spleen and reticuloendothelial system destroy them more actively than the
patient’s, perhaps because of overloading due to chronic erythrocyte destruction in the patient’s organs.

The most probable genetic basis for the appearance of the disease state demonstrated by subjects 7 and 10 is the double heterozygous occurrence of the gene for hemoglobin H and the gene for hereditary leptocytosis, although Bingle et al. found no evidence of hereditary leptocytosis in their families. Although a slight reduction of the erythrocyte survival by the Cr technic has been reported recently in six of nine cases of hereditary leptocytosis trait of the type showing elevated hemoglobin A, no information is available in regard to the type showing no such elevation. Subject 12, who falls in the latter category but has no hemoglobin H, has a normal erythrocyte survival. This observation argues against any direct effect of the hereditary leptocytosis trait in causing the hemolytic process although its presence is probably necessary for the phenotypic expression of the hemoglobin H gene.

Sickle cell anemia was the first, and now hemoglobin H disease becomes the second hemoglobinopathy in which the molecular properties of the abnormal hemoglobin account for the pathophysiology of the disease. The aging of hemoglobin H, i.e., its denaturation and precipitation at a certain age, which determines the lifespan of the erythrocyte, may not be a unique occurrence in cellular biochemistry and physiology. Recent observations suggest that hemoglobin A ages in vivo and acquires a higher affinity for oxygen although its oxygen capacity remains unchanged. Dische has proposed a theory of cataract formation involving precipitation of lens proteins by oxidation of sulfhydryl groups when the protective action of reduced glutathione is impaired. These observations may well form the basis of a concept that aging proteins play an important part in determining the lifespan and/or the function of cells and tissues not only in disease, as is the case with hemoglobin H, but also in health.

**Summary**

Two of three siblings with hemoglobin H disease were splenectomized. Both were benefited as judged by improved feeling of well-being, exercise tolerance and improvement in hemoglobin level and in erythrocyte mean lifespan. However, they were still susceptible to hemolytic crises, and their erythrocyte mean lifespan was below the normal range. Erythrocyte survival studies by the Cr method indicated a finite lifespan of 40–45 days before splenectomy, which increased to normal following splenectomy. In addition, there was random destruction of the erythrocytes, which was slightly reduced following splenectomy. The erythrocyte survival of a member of this family who had the hereditary leptocytosis trait, but no hemoglobin H, was normal.

Evidence is presented indicating that hemoglobin H denatures and precipitates irreversibly at a cell age of 40–45 days, forming intraerythrocytic inclusions which lead to shortening of the erythrocyte finite lifespan due to rapid removal of such erythrocytes by the spleen. In addition, hemoglobin H has a lower solubility in the deoxygenated state and precipitates reversibly, regardless of cell age, in the capillary bed, causing random erythrocyte destruction.
Splenectomy is beneficial because of survival of erythrocytes with inclusions whose hemoglobin A presumably retains its function in oxygen transport. Methemoglobin-forming chemicals such as amyl nitrite and drugs such as sulfisoxazole were found to denature hemoglobin H and produce in vitro inclusions in every erythrocyte of these patients but not of normal subjects. It is suggested that the shortening of the erythrocyte survival and the degree of post-splenectomy improvement is greater the higher the amount of hemoglobin H present in the erythrocytes.

The apparent aging of the hemoglobin H molecule which leads to shortened erythrocyte finite lifespan is discussed, and a concept of aging proteins determining the lifespan and/or the function of cells is advanced.

**SUMMARIO IN INTERLINGUA**

Duo inter tres fraternos con morbo de hemoglobina H esseva subjicite a splenectomia. Ambes beneficiava a judicar per le meliorate sensation de benessier subjective, lor tolerantia pro exercitio, e le melioration del nivello de hemoglobina e del longevitate medie del erythrocytos. Tamen, le duo continuava experienciar crises hemolytic, e le longevitate medie de lor erythrocytos remaneva infra le limites del norma. Studios del longevitate erythrocytic, effectuate per medio del methodo a Cr³⁺ indicava un longevitate finite de 40 a 45 dies ante le splenectomia. Iste valor montava a magnitudes normal post le splenectomy. In plus, il occurreva un destruction aleatori del erythrocytos le qual esseva levemente attenuate post le splenectomy. Le longevitate del erythrocytos de un altere membro del mesme familìa qui habeva le character de leptocytosis hereditari (sed nulle hemoglobina H) esseva normal.

Es presentate datos a provar que hemoglobina H deveni disnaturate e se precipita irreversiblemente a un etate cellular de 40 a 45 dies, con le formation de inclusiones intraerythrocytic que resulta in le reduction del longevitate finite del erythrocytos in consequentia del rapide elimination de tal erythrocytos per le splen. In plus, hemoglobina H ha un plus basse solubilitate in le stato disoxygenate e se precipita irreversiblemente, sin reguardo al etate del cellula, intra le vasculatura capillar, con le effecto de un destruction aleatori de erythrocytos. Splenectomy es benefic a causa del resultante superviventia de erythrocytos con inclusiones in que le hemoglobina retene presumitemente su function in le transporto de oxygeno. Esseva trovate que substantias chimic que forma methemoglobina, i.e. per exemplo nitrito amyllic e drogas del typo de sulfisoxazol, effectua le disnaturation de hemoglobina H e le production de inclusiones in vitro in omne le erythrocytos del patientes in question sed non de subjectos normal. Es formulate le these que le reduction del longevitate erythrocytic e le grado del melioration post-splenectomy es plus marcate in tanto que le quantitate del hemoglobina H presente in le erythrocytos es plus grande.

Es discutite le apparente invetulation del molecula de hemoglobina H que resulta in le reduce longevitate finite del erythrocytos. Es formulate un concepto de invetulation de proteinas que determina le longevitate e/o le functiones del cellulas.
ACKNOWLEDGMENT

The authors wish to thank Dr. Bernard Pirofsky for typing and cross-matching the blood of subject 10 and of the normal recipients, Dr. Nelson R. Niles for performing the histologic examination of the spleens, and Mr. John H. Brooke and Donald Pauling Jenkins for their help with the photomicrography and phase microscopy.

REFERENCES

15. Rigas, D. A., and Koler, R. D.: Chromate binding by hemoglobin H erythrocytes: The relative capacity and affinity of hemoglobins A and H for chromate ions. (Submitted for publication.)
ERYTHROCYTE SURVIVAL IN HEMOGLOBIN H: SPLENECTOMY


Demetrios A. Rigas, Ph.D., Associate Professor of Experimental Medicine, University of Oregon Medical School, Portland, Oregon.

Robert D. Koler, M.D., Associate Professor of Medicine, University of Oregon Medical School, Portland, Oregon.
Decreased Erythrocyte Survival in Hemoglobin H Disease As a Result of the Abnormal Properties of Hemoglobin H: The Benefit of Splenectomy

DEMETRIOS A. RIGAS, ROBERT D. KOLER, George Cummings, Marie L. Duerst, Donald R. Malm, Katherine Swisher and Patricia Vanbellinghen

Updated information and services can be found at:
http://www.bloodjournal.org/content/18/1/1.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml