Studies on Abnormal Hemoglobins

V. The Distribution of Type S (Sickle Cell) Hemoglobin and Type F (Alkali Resistant) Hemoglobin within the Red Cell Population in Sickle Cell Anemia

By Karl Singer, M.D. and Ben Fisher, M.D.

The previous communications of this series have shown that hemolysates prepared from erythrocytes of patients with sickle cell anemia contain almost constantly two varieties of hemoglobin, namely type S (sickle cell), and type F hemoglobin. The latter is an alkali resistant compound which seems to be identical with, or closely related to the physiologically occurring fetal pigment. These findings have recently been confirmed. It has also been established that the amount of the alkali resistant fraction encountered in different patients varies from 2 to 24 per cent of their total hemoglobin, without any apparent correlation to the severity of their clinical manifestations.

The question arose whether type F hemoglobin is uniformly distributed within the erythrocyte population of sickle cell anemia patients or occurs only in some of their red cells. This problem was approached by (1) transfusing erythrocytes with relatively high concentrations of F hemoglobin from patients with sickle cell anemia into normal recipients and (2) performing simultaneous determinations of the disappearance curves of the sickle cells as well as of the alkali resistant component. A close parallelism of such curves might indicate an even distribution of F hemoglobin in all of the transfused erythrocytes, whereas considerable discrepancies between the elimination rates of cells and abnormal pigment might suggest a preponderance of the latter in some portion of the erythrocyte population.

Material and Methods

For the simultaneous study of the survival time of sickle cell anemia erythrocytes and the disappearance of their alkali resistant hemoglobin under optimal experimental conditions, comparatively large amounts of erythrocytes were transfused into small children in order to keep the dilution by the recipient's own blood at a minimum.

Adults with sickle cell anemia whose hemolysates showed values of 12.3 to 16.8 per cent F hemoglobin were selected as donors. The amount of alkali resistant pigment was determined by the method described in detail in the first paper of this series. Briefly, a
hemoglobin solution, approximately 10 Gm. per cent, is prepared from oxalated or clotted blood, employing distilled water and toluene as hemolyzing agents; 0.1 ml. of this hemoglobin solution is exposed to 1.6 ml. of N/12 NaOH. After the mixture has reacted for exactly 1 minute, 3.4 ml. of a reagent which simultaneously precipitates the denatured chromogens and stops the reaction are introduced. The latter reagent is prepared by adding 1.0 ml. of 10 N HCl to 400 ml. half saturated (NH₄)₂SO₄. The final mixture is immediately filtered and the hemoglobin concentration of the filtrate determined spectrophotometrically. The percentage of undenatured hemoglobin is then calculated and is referred to as the “1 minute denaturation value.” With this method normal hemoglobin is completely destroyed within 1 minute. However, the colorless filtrate may still give spectrophotometer readings of 0.5 to 1.7 per cent of the original hemoglobin solution, due to the presence of an as yet unidentified material.

**Fig. 1**—Disappearance of transfused sickle cell anemia cells containing 16.8 per cent F hemoglobin. Recipient 1. Initial post-transfusion values (100 per cent): cells 983,000 cu.mm.; F hemoglobin, 5.9 per cent.

Approximately 400 ml. of blood were obtained by venesection and immediately replaced with normal blood. The sickle cell anemia cells were freed from plasma and re-suspended in a saline-citrate solution. Considering the anemic state of the donors, each recipient received erythrocytes equivalent to about 200 ml. of normal whole blood. Only children 12 to 17 months old, whose 1 minute alkali denaturation values were identical with those found in normal adults, were selected as recipients. The normal alkali denaturation value was considered to signify complete disappearance of the formation of fetal pigment. Since at this particular age the replacement of F hemoglobin by the adult (type N) hemoglobin is frequently still incomplete, the selection of suitable recipients—several of whom had mild nutritional anemia—was quite difficult. The re-suspended erythrocytes from the donors were transfused in two equal portions on successive days, and the values obtained 24 hours later were taken as the starting points of the disappearance curves. The method used for the determination of the life span of the transfused cells has been described in detail in previous studies. However, since larger amounts of blood were necessary for obtaining the values for post-transfusion F hemoglobin levels, about 4 ml. were always withdrawn by femoral puncture. Ten determinations of the denaturation value were performed and the results averaged. Similarly to the deduction of the recipient’s non-agglutinable cell count in the survival time experiments, the pretransfusion 1 minute
Fig. 2—Disappearance of transfused sickle cell anemia cells containing 16.8 per cent F hemoglobin. Recipient 2. Initial post-transfusion values (100 per cent): cells, 671,000/cu.mm.; F hemoglobin, 1.3 per cent.

Fig. 3—Disappearance of transfused sickle cell anemia cells containing 16.5 per cent F hemoglobin. Initial post-transfusion values (100 per cent): cells, 830,000/cu.mm.; F hemoglobin, 4.9 per cent.

denaturation value of the recipient was always deducted from the amount of F hemoglobin found. Follow-up studies were carried out as frequently as feasible, particularly during the first two weeks after the transfusion (figs. 1 to 4). The children were only hospitalized for the performance of the transfusion.
Besides the determinations of the nonagglutinable transfused cells and their F hemoglobin contents, the values for the hematocrit and the hemoglobin, red cell and reticulocyte levels were also obtained from the same specimen.

Results

1 Transfusion Experiments

The pertinent hematologic data of the donors and recipients are recorded in table 1. Three donors were used, and the blood from one of them (11) was transfused into 2 different recipients on different occasions. The time interval between the venesections of this particular donor was about eight months.

Experiments 1 and 2. Donor 11. Recipients 1 and 2. The initial values of the transfused sickle cells in the recipients were 985 x 10^6 and 671 x 10^6 per cu.mm., respectively (figs. 1 and 2). The pretransfusion F hemoglobin level of the donor was 16.8 per cent. After the transfusions, the initial F hemoglobin concentrations of the recipients were 5.9 and 4.3 per cent, respectively, due to the dilution by their blood volumes. These figures represent the corrected 1 minute alkali denaturation values of the recipients' bloods 24 hours after completion of the transfusion.

The results of these two experiments are plotted in figures 1 and 2. The initial posttransfusion values were taken as 100 per cent, and the succeeding findings expressed in per cent of these latter values. As may be seen, there is no parallelism between the disappearance rates of the cells and of their alkali resistant pigment. In Experiment 1, almost 50 per cent of the transfused cells had disappeared by the sixth day, without any change in the level of the alkali resistant pigment. On the thirteenth day post transfusion, only 30 per cent of the cells remained in the circulation, but 70 per cent of the pigment was still detectable. On the thirty-second day, only about 10 per cent of the cells had survived, but more than 30 per cent of the initial F pigment was still demonstrable.

In the second experiment, the results were quite similar. Thus, on the eighth day, more than 50 per cent of the transfused cells had disappeared, but 70 per cent of the resistant hemoglobin was still present. On the thirty-sixth day, less than 10 per cent of the cells
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were found to remain, with the pigment corresponding to 25 per cent of the original value. On the forty-fifth day, all cells as well as all the F hemoglobin had been entirely eliminated.

These experiments seem to demonstrate that there is an unequal distribution of the alkali resistant hemoglobin within the erythrocyte population of Donor #1, and that, apparently, cells with larger concentrations of F hemoglobin survive longer. It may also

Table 1—Hematologic Data of Donors and Recipients

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Age</th>
<th>Blood Group</th>
<th>Pre-Transfusion Values</th>
<th>Initial Post-Transfusion Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hgb Gm. %</td>
<td>RBC mill.</td>
<td>Retics./cu.mm</td>
</tr>
<tr>
<td>1. Donor</td>
<td>25 yrs.</td>
<td>O,N,Rh(D)+</td>
<td>10.2</td>
<td>3.7</td>
</tr>
<tr>
<td>Recipient</td>
<td>12 mos.</td>
<td>O,M,Rh(D)+</td>
<td>8.8</td>
<td>3.6</td>
</tr>
<tr>
<td>2. Donor</td>
<td>26 yrs.</td>
<td>O,N,Rh(D)+</td>
<td>9.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Recipient</td>
<td>14 mos.</td>
<td>O,M,Rh(D)+</td>
<td>11.1</td>
<td>4.6</td>
</tr>
<tr>
<td>3. Donor</td>
<td>18 yrs.</td>
<td>O,Rh(D)+</td>
<td>7.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Recipient</td>
<td>16 mos.</td>
<td>A,Rh(D)+</td>
<td>9.7</td>
<td>5.2</td>
</tr>
<tr>
<td>4. Donor</td>
<td>25 yrs.</td>
<td>O,Rh(D)+</td>
<td>8.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Recipient</td>
<td>17 mos.</td>
<td>A,Rh(D)+</td>
<td>11.4</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 2—Correlation between Osmotic Resistance and F Hemoglobin Distribution of Sickle Cell Anemia Erythrocytes

<table>
<thead>
<tr>
<th>Patient #</th>
<th>One Minute Denaturation Value of Hgb Solution prepared from Original Blood Sample</th>
<th>Osmotically resistant RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.8</td>
<td>13.0</td>
</tr>
<tr>
<td>2</td>
<td>5.7</td>
<td>4.4</td>
</tr>
<tr>
<td>3</td>
<td>3.9</td>
<td>3.6</td>
</tr>
<tr>
<td>4</td>
<td>4.7</td>
<td>4.1</td>
</tr>
<tr>
<td>5</td>
<td>6.6</td>
<td>7.0</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>5.3</td>
</tr>
</tbody>
</table>

be noted that the survival time curves, as well as the disappearance rates of the F hemoglobin are very similar in these 2 normal recipients.

Experiment 3. The initial transfused erythrocyte level was 830 x 10^6 per cu.mm. The blood of this donor had an alkali denaturation value of 16.5 per cent. The initial post-transfusion finding of the F hemoglobin in the recipient was 4.9 per cent. On the tenth day following the transfusion (fig. 3), almost 50 per cent of the cells were eliminated, but 75 per cent of the abnormal pigment was still demonstrable. On the twenty-second day, the number of sickle cells was about 25 per cent of the starting count, but 55 per cent of
the transfused F hemoglobin was still detectable. On the forty-third day, only 10 per cent of the "foreign" cells were found, but they contained 42 per cent of the alkali resistant pigment. Further follow-up studies, not recorded in figure 3, showed that 57 days were required for the complete elimination of this resistant pigment. In this particular instance, the F hemoglobin seemed to be concentrated to a very high degree in a relatively small number of erythrocytes.

**Experiment 4.** Again, the quite uneven distribution of the alkali resistant component is clearly noticeable (fig. 4). On the eighth day, only 30 per cent of the cells survived, but carried 65 per cent of the initial amount of the transfused pigment. On the fourteenth day, 20 per cent of the cells still contained more than 60 per cent of the F hemoglobin, and on the twentieth day, 15 per cent of the cells—with a corresponding pigment value of 30 per cent—were found. Unfortunately, the experiment had then to be interrupted because the patient left the city.

### Table 3—Demonstration of the Uneven Distribution of F Hemoglobin in Sickle Cell Anemia Erythrocytes by Means of Exposure to Mechanical Trauma

<table>
<thead>
<tr>
<th>Patient</th>
<th>Duration of Exposure to Mechanical Trauma (hrs.)</th>
<th>One Minute Denaturation Value of Hgb Solution prepared from</th>
<th>Increment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Original Blood Sample, RBC, non-hemolyzed by mechanical trauma, Absolute Value</td>
<td>$%$ change from Original Value</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>6.8, 16.5</td>
<td>9.7, 142</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>15.9, 22.0</td>
<td>6.1, 38</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>9.8, 12.6</td>
<td>2.8, 29</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>6.5, 7.7</td>
<td>1.2, 18</td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>4.9, 8.0</td>
<td>3.1, 63</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>16.5, 18.2</td>
<td>1.7, 10</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>6.5, 9.0</td>
<td>2.5, 38</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>5.3, 7.6</td>
<td>2.3, 43</td>
</tr>
<tr>
<td>9</td>
<td>24</td>
<td>7.4, 8.5</td>
<td>1.1, 15</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td>5.7, 7.2</td>
<td>1.5, 26</td>
</tr>
<tr>
<td>11</td>
<td>17</td>
<td>14.4, 18.8</td>
<td>4.4, 31</td>
</tr>
<tr>
<td>12</td>
<td>24</td>
<td>6.6, 9.7</td>
<td>3.1, 47</td>
</tr>
</tbody>
</table>

Average increment in $\%$ of original: 42

**2 Attempts to Demonstrate the Uneven Distribution of F Hemoglobin in Vitro**

**Osmotic Hemolysis.** The blood from 6 patients with sickle cell anemia was hemolyzed in part, by exposing the thrice washed red cells to a hypotonic (0.2 per cent) sodium chloride solution, at 37 C., for 1 hour. These cell suspensions were frequently agitated. After this period, the nonhemolyzed cells were collected by centrifugation and used to prepare a 10 Gm. per cent hemoglobin solution. The denaturation values (F pigment) from these osmotically most resistant cells were determined and correlated with the values obtained from the whole blood samples. As may be seen from table 2, it was not possible to separate the cells containing a higher concentration of F hemoglobin by means of this procedure, since the percentage of F hemoglobin in the hemoglobin solution prepared from the osmotically resistant erythrocytes, was always approximately the same as found in the original blood specimens.

**Mechanical Hemolysis.** It has been shown that sickle cell anemia erythrocytes, when converted to the sickled shape by reduction of their S hemoglobin, exhibit an increased mechanical fragility. A similar procedure was, therefore, employed in an attempt to demonstrate in vitro the uneven distribution of F hemoglobin within the erythrocyte population in sickle cell anemia.
Oxidated blood in the amount of 4.0 ml. was placed into a small Erlenmeyer flask containing 25 glass beads, and then exposed to a stream of CO₂ until all the hemoglobin was reduced. The CO₂ was introduced by means of an inlet needle into the air-tight stoppered flask, and an outlet needle was also provided. After complete reduction of the hemoglobin was achieved—which could be seen from the change in the color of the solution—the needles were withdrawn from the rubber stopper. The flask was then placed in the holder of the standard mechanical fragility rotator and the cells were exposed to the mechanical trauma for periods ranging from 16 to 30 hours. The blood specimens of 12 patients with well established sickle cell anemia were used in these experiments. As may be seen from table 3, the hemoglobin solutions prepared from the cells most resistant to mechanical trauma, consistently showed an increase of the F hemoglobin concentrations in comparison to the values of the original samples. However, no direct correlations could be established between the total amount of F hemoglobin present, the increment of the alkali resistant pigment, and the time of exposure of the cells to the mechanical trauma. Nevertheless, these experiments suggest—similar to the transfusion experiments—that the distribution of the alkali resistant component is not a uniform one.

**Discussion**

The transfusion studies as well as the in vitro experiments with prolonged exposure of the sickled erythrocytes to mechanical injury, reported in this paper, have demonstrated that the alkali resistant pigment is unevenly distributed throughout the red cell population of patients with sickle cell anemia. As may be seen from figure 1, one portion of the erythrocyte population does not seem to contain F hemoglobin at all, since there was a significant decrease in the number of the transfused cells without a concomitant diminution of the F pigment level. The size of this particular portion probably varies from patient to patient. F hemoglobin, on the other hand, appears to be highly concentrated in a relatively small number of the red cells. In all 4 transfusion experiments, 15 per cent or less of the donor's cells still contained as much as 30 to 40 per cent of the transfused alkali resistant compound. The gradual decline in the F hemoglobin values during the observation period may be explained either by assuming the disappearance of aged cells carrying mostly F pigment, or, more likely, by the disintegration of "intermediate" cells which contain both, type S and type F hemoglobin. Thus, the erythrocyte population in sickle cell anemia possibly consists of three fractions: (1) red cells containing S hemoglobin, and no or minimal amounts of F; (2) "intermediate" cells with S and F pigment; and (3) a small portion of erythrocytes which apparently carry mostly F and relatively little or no S pigment. Since the sickling phenomenon depends on the presence of a sufficient concentration of S hemoglobin within the erythrocyte, the last mentioned group of red cells may not be capable of sickling. Because of the observed great variations in the F hemoglobin concentration (from 2 to 24 per cent) among different patients with sickle cell anemia, the problem as to whether 100 per cent sickling can be achieved with the bloods from all such patients, requires re-investigation with particular consideration of those individuals whose hemolysates contain relatively large amounts of F pigment. Such studies on a more extensive scale are now in progress in our laboratory.

That the red cell population in sickle cell anemia is not a homogenous one has been postulated prior to the present experiments. This was inferred from the fact that in survival time determinations of sickle cell anemia erythrocytes, quite regularly certain portions of the transfused red cells are more readily
hemolyzed than others. Usually, there is a rapid initial elimination of cells, varying in extent from patient to patient; later on, the speed of hemolysis decreases and some of the remaining erythrocytes disintegrate at a much slower, but still abnormal, rate. It seems that this last mentioned portion is characterized by an increased concentration of F hemoglobin. In this respect it is of interest that Kaplan and Zuelzer noticed similar survival time patterns with erythrocytes from patients with frank Mediterranean anemia (hereditary leptocytosis). These investigators found that 25 to 50 per cent of the transfused leptocytes disappeared within 20 to 30 days, but that subsequently the slope of the survival time curve indicated a more normal rate of elimination. Since it has been established that large amounts of F hemoglobin (from 30 to 70 per cent) are present in the erythrocytes of fully developed hereditary leptocytosis, simultaneous determinations of the disappearance rates of cells and F hemoglobin in this disorder are definitely indicated.

It is very likely that, even under normal conditions, certain intra-erythrocytic constituents may show an unequal distribution within the red cell population. The phenomenon of some nonagglutinable erythrocytes, which is constantly demonstrable with the use of extremely avid anti A or B sera, suggests that some of the red cells do not contain the A or B agglutininogen at all, or only minimal traces of it. With less avid sera, the rise in the nonagglutinable cell count probably signifies that such nonagglutinated erythrocytes contain less A or B than do the easily agglutinable ones.

The unequal distribution of F hemoglobin in sickle cell anemia erythrocytes also explains the absence of any correlation between the amount of F hemoglobin found and the severity of the clinical syndrome in an individual patient. The degree of the anemia will depend on the size of that portion of the erythrocyte population which is most rapidly eliminated, and on the ability of the marrow to replace this particular portion. In figure 5, the survival time of the erythrocytes from 2 patients with sickle cell anemia are shown; the blood of one of them contains 16.8 per cent, and that of the other 6.0 per cent F hemoglobin. Nevertheless, life span studies with the blood which had the lower F hemoglobin value demonstrated that 50 per cent of the cells were eliminated within three days, whereas six days were required for a 50 per cent removal of the transfused cells of the blood which showed the greater amount of F hemoglobin. The significance of the various red cell portions has also previously been emphasized in the analysis of the mechanisms responsible for the so-called “aplastic crisis” in sickle cell anemia. In this complication, the marrow temporarily ceases to produce red cells under the influence of certain infections. The rate of decrease in the hemoglobin and erythrocyte levels in the peripheral blood will then depend on the proportion of the most rapidly eliminated red cell fraction, in relation to the whole erythrocyte population. Only when a large part of the population has a particularly short survival time will a “crisis,” i.e., a rapid fall of the hemoglobin and red cell levels, develop in a few days.

The finding that the least destructible red cell portion contains a high concentration of F hemoglobin and relatively little or no S hemoglobin could, of course, also be interpreted that it is not the presence of F, but the absence of S hemoglobin which is responsible for the longer life span of these particular
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erythrocytes. Although this interpretation may be correct, it should be emphasized that this red cell fraction still shows an abnormally short survival time. This fact makes one speculate whether the pathologic alterations in the sickle cell anemia erythrocyte may not only affect the pigment, but may also involve the red cell stroma.

In the light of this discussion, the conclusion that the absolute amount of S hemoglobin found in a patient with sickle cell anemia, represents a yardstick of the severity of the disease seems to be untenable. The interpretation of certain physico-chemical data should be coordinated with the complex pathophysiologic mechanisms operating in the disease process.

**Fig. 5—**Survival time of transfused sickle cell anemia erythrocytes containing (1) large and (2) small amounts of F hemoglobin.

The genetic determinants governing the variable amounts of S hemoglobin (and the reciprocal amount of F hemoglobin) in patients with sickle cell anemia are not yet fully understood. To date, we have examined 87 different individuals with unquestionable clinical and hematologic sickle cell anemia. In many instances, the patient's bloods were also studied electrophoretically and found to contain no other hemoglobins than types S and F. All individuals whose hemoglobin solutions contained the third variety of abnormal pigment encountered in sickle cell disease (presently called either type III or type C by some investigators) were not included in this group.* Of these 87 patients, 3 (3.4 per cent) had no F hemoglobin at all when tested with the alkali denaturation method; in the others, the resistant hemoglobin varied from 2 to 24 per cent. No especially

* The patients belonging to this latter category will be discussed in a separate report.
severe clinical manifestations were observed in the patients who had no F hemoglobin.

No satisfactory explanation can be offered for the finding that F hemoglobin is particularly concentrated in the longest surviving fraction of the red cell population in sickle cell anemia. We do not know whether some parts of the marrow produce these cells exclusively, or whether they are produced throughout the whole marrow. Experiments were performed in which sinusoidal blood was aspirated from various marrow sites, but no significant differences of the marrow fluid in resistance to alkali denaturation could be demonstrated in aspirates from the sternum, spinous process or iliac crest. We also do not know whether the appearance of qualitatively different erythrocytes is confined to the hereditary hemolytic syndrome of sickle cell anemia, or occurs also in other hemolytic disorders with a genetic intra-erythrocytic defect. The variability in cell composition of various fractions may not be limited to the hemoglobin, but may affect other erythrocyte constituents as well. To elucidate these problems will be the task of future studies.

SUMMARY

1. By transfusing sickle cell anemia erythrocytes with a relatively high concentration of F hemoglobin into normal recipients, it was demonstrated that the disappearance rates of the transfused cells and of their alkali resistant pigment consistently showed great discrepancies. These observations suggest an unequal distribution of the F pigment within the erythrocyte population. A nonuniform distribution of F hemoglobin could also be detected in vitro by exposing sickle cell anemia bloods to mechanical trauma for a longer period of time. The cells most resistant to trauma contained a higher percentage of F hemoglobin than the original blood specimen.

2. The red cell population of patients with sickle cell anemia seems to be composed of three main fractions: (1) cells containing S hemoglobin and no or little F hemoglobin, (2) cells containing both pigments and (3) cells containing F pigment with no or little S hemoglobin.

3. The erythrocytes carrying mostly S hemoglobin have the shortest life span, whereas the red cells containing mostly F hemoglobin have the longest survival time.

4. The significance of these findings in regard to clinical and genetic aspects of sickle cell anemia is discussed. No direct correlation is demonstrable in an individual patient between the absolute amounts of either type S or type F hemoglobin and the severity of the anemia. The latter depends on the variable size of the portion of red cells containing mostly S hemoglobin, and also on the ability of the marrow to replace this particular fraction.

REFERENCES


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