STUDIES ON THE DESTRUCTION OF RED BLOOD CELLS

V. IRREVERSIBLY SICKLED ERYTHROCYTES: THEIR EXPERIMENTAL PRODUCTION IN VITRO

By Shu Chu Shen, M.D., Eleanor M. Fleming, A.B. and W. B. Castle, M.D.

It is well known that in fresh preparations of the blood of patients with sickle cell disease the erythrocytes can be sickled immediately by displacement of the oxygen from the hemoglobin, for example, by carbon dioxide or by nitrogen. These sickled cells can be no less rapidly restored to their normal form when the blood is reexposed to oxygen. However, even in stained smears of the peripheral blood of some of these patients a few sickled cells may be present, despite the inevitable exposure of the film of fresh blood to atmospheric oxygen. These cells, then, differ from the majority of the sickled erythrocytes artificially produced in fresh preparations in that they have somehow acquired an inability to revert to the normal discoidal form upon exposure to oxygen. Moreover, though crescentic or elliptic, these cells do not display filamentous extremities as do freshly sickled erythrocytes in wet preparations.

Although in patients with sickle cell disease the majority of the erythrocytes when exposed to a range of hypotonic concentrations of sodium chloride exhibit a so-called "increased osmotic resistance," critical study of the phenomenon indicates that in certain patients a small percentage of the erythrocytes may actually possess a slightly decreased osmotic resistance relative to the normal range; that is, they are hemolyzed in concentrations of sodium chloride, somewhat more concentrated than those which initiate the osmotic lysis of normal blood. Because it is possible to cause any type of red cell so far studied to acquire decreased resistance to osmotic lysis by sterile incubation in vitro, the question arose as to whether they irreversibly sickled erythrocytes and those erythrocytes with decreased resistance to osmotic lysis are in fact the same cells. It also appeared to be possible that both characteristics were the result of the same process, namely, stagnation of the red cells in vivo in the tissue capillaries. Accordingly, the peripheral bloods of 4 patients with sickle cell disease were studied with respect to the natural presence of irreversibly sickled erythrocytes and as to their artificial production in vitro.

Methods

The conventional characteristics of the formed elements of the peripheral blood were determined by the usual methods. The percentages of irreversibly sickled erythrocytes in samples of capillary or of defibrinated venous blood following exposure to air or to 90 per cent oxygen and 10 per cent carbon dioxide in a tonometer were determined while counting 1,000 or more red cells in blood films prepared...
and treated with Wright’s stain in the usual manner. The percentages of reticulo-
cytes were also determined in blood spread upon coverslips previously prepared
with a dried film of brilliant cresyl blue. Thereafter the dried blood films were
counterstained with Wright’s stain and the number of reticulocytes determined
by counting 1,000 or more red blood cells.

In order to observe the effect of sterile incubation at 37.5 C. for 2.4
hours in vitro
on the reversibility of the sickling phenomenon, 7 cc. samples of sterile defibrinated blood were equilibrated in 250 cc. tonometers with gas mixtures contain-
ing either 90 per cent oxygen and 10 per cent carbon dioxide or 90 per cent nitrogen
and 10 per cent carbon dioxide. Each tonometer, which was equipped with a long
glass capillary pipet inserted through a hole in a rubber stopper closing the open
end, was sterilized prior to each experiment. With the tonometer lying on its side
on a table with the stopcock open, the apparatus was carefully rotated back and
forth about its long axis usually during three periods of 3 minutes each, separated
by short intervals. During each of these periods the gas mixture, which was stored
in a cylinder equipped with a reducing valve, after bubbling through water, was
allowed to flow freely though appropriate rubber tube connections to the capillary
pipet and so into and through the tonometer. The stopcock and the inflow tubing
were then closed. During a 24-hour period in an air incubator the blood in the
tonometers was reequilibrated three or four times with the same gas mixture. At
the end of the incubation period the blood in the tonometers was again carefully
equilibrated with whichever of the two gas mixtures was required by the experi-
mental procedure, in the same fashion as in the beginning.

Whenever a sample of blood was to be removed for study, the tonometer was
held in a vertical position. The long glass capillary pipet, which then dipped be-
neath the surface of the blood, was operated as a bulb pipet and a small amount of
blood was sucked into it and so removed from the tonometer without contact with
room air. After withdrawal from the tonometer, the tip of the pipet was inserted
beneath the surface of a sterile pool, on the surface of a glass slide, composed of two
drops of a 40 per cent formalin solution diluted 10 times by volume with physiologic
salt solution. A drop of blood was then expelled and immediately mixed with the
formalin solution. From this mixture as well as directly from other drops of blood
expelled from the pipet without contact of its tip with coverslips, blood
smears were prepared using either plain coverslips or coverslips previously filmed
with brilliant cresyl blue. If the experiment was to continue, the remaining blood
was expelled from the pipet which was then reinserted in the tonometer with
appropriate sterile precautions. The blood remaining in the tonometer was then
equilibrated twice with the appropriate gas mixture as described above and the
apparatus was returned to the incubator. Later the blood films were treated with
Wright’s stain in the usual manner and the percentages of sickled erythrocytes and
of sickled reticulocytes were determined.

Results

As shown in table 1, sickled red cells persisted in the blood of 3 of the 4 patients,
Cases 2, 3, and 4, after exposure of capillary or defibrinated venous blood to atmos-
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phasic air or even after the further equilibration of the latter with a gas mixture consisting of 90 per cent oxygen and 10 per cent carbon dioxide. In Cases 3 and 4, the number of sickled erythrocytes differed strikingly at the time of the two observations made on each patient’s blood. As noted by others,2,4,6 these "irreversibly" sickled cells, although exhibiting the "sickle" or "oat" shaped form in fixed as well as in wet preparations, did not possess the filaments which are seen in wet preparations of blood artificially sickled by exposure to nitrogen or to carbon dioxide gas.

### Table 1.—Percentages of Irreversibly Sickled Erythrocytes in Peripheral Blood of 4 Patients with Sickleemia

<table>
<thead>
<tr>
<th>Case Number</th>
<th>Capillary Blood</th>
<th>Venous blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Delbrinated in air</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>16.8</td>
</tr>
<tr>
<td>(after splenectomy)</td>
<td>4.2</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>14.0</td>
<td>11.8</td>
</tr>
<tr>
<td>4.6</td>
<td>5.0</td>
<td>5.6</td>
</tr>
</tbody>
</table>

### Table 2.—Characteristics of the Peripheral Blood of 4 Patients with Sickleemia

<table>
<thead>
<tr>
<th>Case Number</th>
<th>R.B.C. Mls.</th>
<th>Hgb. g %</th>
<th>W.B.C. Thous.</th>
<th>Retic. %</th>
<th>Hematocrit %</th>
<th>M.C.V. μm³</th>
<th>M.C.H. C. %</th>
<th>M.C.H. H. %</th>
<th>I.E. units</th>
<th>Osmotic fragility of R.B.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.35</td>
<td>51</td>
<td>16.1</td>
<td>7.4</td>
<td>38.9</td>
<td>72.7</td>
<td>32.4</td>
<td>23.6</td>
<td>10</td>
<td>0.58 0.34 0.19 0.20 0.16</td>
</tr>
<tr>
<td>2</td>
<td>2.72</td>
<td>52</td>
<td>8.0</td>
<td>1.1</td>
<td>33.9</td>
<td>87.9</td>
<td>33.9</td>
<td>29.8</td>
<td>5</td>
<td>0.38 0.31 0.18 0.21 0.16</td>
</tr>
<tr>
<td>3</td>
<td>3.07</td>
<td>57</td>
<td>13.1</td>
<td>7.6</td>
<td>27.5</td>
<td>89.6</td>
<td>32.0</td>
<td>28.7</td>
<td>10</td>
<td>0.44 0.38 0.36 0.27 0.15</td>
</tr>
<tr>
<td>4</td>
<td>2.69</td>
<td>46</td>
<td>11.1</td>
<td>9.0</td>
<td>21.4</td>
<td>79.6</td>
<td>33.1</td>
<td>16.4</td>
<td>7</td>
<td>0.47 0.34 0.16 0.18 0.14</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.43 0.40 0.39 0.36 0.33</td>
</tr>
</tbody>
</table>

* 15.6 grams of hemoglobin are considered to be 100 per cent.

In Table 2 are shown the peripheral blood values including the data on quantitative osmotic fragility studies on the blood of the 4 patients. It will be noted that in the blood of Case 1 a portion of the red cells were more susceptible than are normal cells to lysis by hypotonic salt solution although no irreversibly sickled erythrocytes were seen in the blood films from this patient. Thus in Case 1, 1 per cent of the red cells were hemolyzed in 0.58 per cent NaCl instead of, as in the normal individual, in 0.43 per cent NaCl. In Cases 2, 3, and 4, on the other hand, 1 per cent of the red cells were hemolyzed in 0.38, 0.44, and 0.47 per cent NaCl respectively, and thus like the rest of the red cells of all of the patients, were less susceptible than are normal red cells to lysis by hypotonic solutions.

In an attempt to modify the capacity of the red cells for change in shape, sickling
of the erythrocytes was prevented as far as possible during sterile incubation of blood samples for 24 hours in equilibrium with a gas phase containing 90 per cent oxygen and 10 per cent carbon dioxide. As shown in table 3, experiment C, these erythrocytes almost completely lost their capacity to become sickled upon subsequent reduction of the hemoglobin by exposure to 90 per cent nitrogen and 10 per cent carbon dioxide. Likewise, except in Case 3 where the effect was only partial, the erythrocytes which were kept sickled in the nitrogen-carbon dioxide atmosphere during the incubation period largely lost their ability to reassume the dis-

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Table 3.—Percentages of Sickled Erythrocytes in Blood Samples from 4 Patients with Sicklemia following Incubation for 24 Hours in the Presence and Absence of Oxygen Respectively

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Characteristics of blood samples</th>
<th>Percentage of sickled erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Case 1</td>
</tr>
<tr>
<td>A</td>
<td>Defibrinated venous blood immediately after equilibration with 90 per cent O₂ and 10 per cent CO₂.</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Defibrinated venous blood after incubation in 90 per cent O₂ and 10 per cent CO₂ for 24 hours.</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>Same after final reequilibration in 90 per cent N₂ and 10 per cent CO₂.</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>Defibrinated venous blood immediately after equilibration with 90 per cent N₂ and 10 per cent CO₂.</td>
<td>(70)</td>
</tr>
<tr>
<td>E</td>
<td>Defibrinated venous blood after incubation in 90 per cent N₂ and 10 per cent CO₂ for 24 hours.</td>
<td>90</td>
</tr>
<tr>
<td>F</td>
<td>Same after final reequilibration in 90 per cent O₂ and 10 per cent CO₂.</td>
<td>75</td>
</tr>
</tbody>
</table>

* Figures in column are percentages of sickled reticulocytes in terms of total reticulocytes.
( ) Figures in parentheses are the results of observations using the formalin technic.

coidal form upon exposure to the oxygen-carbon dioxide gas mixture (experiment F). In table 3 are also shown the immediate effects of equilibration with the oxygen-carbon dioxide gas mixture (experiment A) and with the nitrogen-carbon dioxide gas mixture (experiment D). Also included are the results of the various experimental procedures upon the reticulated erythrocytes of Cases 3 and 4. The prolonged incubation in the oxygen-carbon dioxide gas mixture appeared effectively to prevent the subsequent sickling of the reticulocytes upon exposure to a nitrogen-carbon dioxide gas mixture (compare experiment C with experiment D).
However, incubation in the nitrogen-carbon dioxide gas mixture was not as effective in rendering reticulocytes irreversibly sickled when subsequently exposed to oxygen as it was in the case of the nonreticulated erythrocytes (compare experiment E with experiment F). The figures obtained with the formalin technic are shown in parentheses in table 3. The percentages of sickled erythrocytes in the smears prepared from the formalin solution agreed reasonably well with those made in the usual fashion. The technic could not be employed for reticulocytes, which were rendered unable to take the brilliant cresyl blue stain by previous exposure to formalin.

**DISCUSSION**

The fact that the sickling of the erythrocytes strikingly increases the viscosity of the blood provides an obvious explanation of the characteristic pathologic lesions of sickle cell disease, namely, the congestion of the capillaries and the multiple thromboses and infarcts including frequently the total atrophy of the spleen. However, many sickled red cells apparently traverse these areas of lowered oxygen tension and thus with proper precautions against exposure to air are demonstrable in wet preparations of venous blood. The readiness with which these cells revert to the discoidal form distinguishes them from the "irreversibly" sickled erythrocytes which may also be present in such wet preparations and which form the subject of this communication. Only the irreversibly sickled forms, however, are observed in the usual stained blood films. The hypothesis examined experimentally here is that the persistently sickled form has been assumed because of prolonged or repeated intermittent exposure to anoxia and consequent erythro-stasis in the capillaries of various organs. Janet Watson infers the effectiveness of stagnation in vivo from the finding of Diggs and Bibb of irreversibly sickled forms in the pleural or ascitic fluids of patients whose peripheral blood showed none of these forms. Clearly, from the experiments reported here, the effect of sterile incubation in vitro upon erythrocytes maintained in the sickled form under anoxic conditions is to cause loss of ability to revert to the discoidal form upon re-exposure to oxygen. Moreover, these incubated sickled erythrocytes resemble the irreversibly sickled forms seen in fixed blood smears with respect to their lack of the hairlike processes frequently extending from the ends of the crescents that are characteristic of the sickled forms artificially produced in wet preparations by exposure to nitrogen-carbon dioxide gas mixtures.

Although irreversibly sickled reticulocytes are rarely seen in the peripheral blood of patients with sickle cell disease, these cells are capable of sickling in vitro if their hemoglobin is sufficiently reduced. The present experiments indicate that after incubation in the nitrogen-carbon dioxide gas mixture a larger proportion of the reticulated than of the nonreticulated erythrocytes, especially in the blood of Case 4, were able to revert to the discoidal form upon re-exposure to oxygen. This finding confirms the suggestion already made by others that the acquisition of a permanently sickled form requires that the red cell receive a sufficiently long or repeated exposure to whatever bodily processes are concerned in order to allow for maturation of the reticulocyte to the more adult form. However,
although the blood of Case 1 showed an increased osmotic fragility of a small proportion of the red cells, a finding which conceivably could be due to erythro-stasis in vivo, no irreversibly sickled red cells were present. The fact that irreversibly sickled reticulocytes are so rarely seen in the peripheral blood is strong evidence against the possibility that they are young cells recently delivered by the bone marrow.

It may be argued that exposure to atmospheric oxygen in the preparation of the blood films would invalidate the experiments in which formalin fixation was not used. However, reversal of sickling due to exposure to atmospheric oxygen would be expected only in the case of films made from blood removed from the tonometer after equilibration with the nitrogen-carbon dioxide gas mixture, as, for example, in experiments C, D, and E of table 3. The differences between the numbers of sickled red cells in the ordinary smears and in the formalin fixed smears do not significantly alter the conclusions drawn from these experiments.

Conclusions

1. The peripheral blood of patients with sicklemia when examined in wet preparations without contact with air may contain two distinct types of sickled erythrocytes.

   The first type, which exhibits filamentous processes extending from the ends of the crescentic forms, resembles those produced by exposure of the blood to nitrogen or to carbon dioxide gas in vitro. Exposure to oxygen causes these red cells to revert to the discoidal form.

   The second type appears as 'sickle' or 'oat' shaped forms without filaments, in fixed as well as in wet preparations of the blood, and does not revert to the discoidal form upon exposure to oxygen. Such red cells rarely exhibit the vital staining properties of reticulocytes. In the blood of one patient their presence did not correlate with the presence of red cells of relatively increased osmotic fragility.

2. When samples of the blood of 4 patients with sicklemia were incubated for 24 hours at 37.5 C. in the absence of oxygen, the nonreticulated red cells largely lost their ability to resume the discoidal form when exposed again to oxygen. To a considerable extent, however, reticulocytes retained their ability to revert to the discoidal form. Similar incubation in the presence of oxygen caused loss of the ability of both adult and reticulated red cells to sickle when subsequently deprived of oxygen.

3. These studies confirm the hypothesis that sufficient intermittent or continuous stagnation of the red cells in various organs in vivo with consequent sickling may result in the production of irreversibly sickled forms.

4. The fact that reticulocytes do not as readily acquire the property of becoming irreversibly sickled after incubation in vitro as do nonreticulated red cells may explain the fact that irreversibly sickled reticulocytes are rarely seen in stained blood films.

References

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