Adhesion of Normal and Sickle Erythrocytes to Endothelial Monolayer Cultures

By Richard Hoover, Robert Rubin, Gary Wise, and Robert Warren

Experiments were carried out to test the hypothesis that the differences between the surfaces of erythrocytes from normal and sickle cell patients are reflected in the degree of attachment to the capillary lining. An assay was used that measured the number of 51Cr-labeled erythrocytes (normal or sickle) attaching to a monolayer of endothelium cultured from calf aortas.

Evidence has suggested that sickle cell disease may involve an alteration in the erythrocyte membrane. This evidence includes reports that the sickling phenomenon is accompanied by a reduction in deformability of the membrane as well as recent indications that there is a reduction in exposed sialic acid in the membranes of erythrocytes from sickle cell patients. Perhaps the most convincing indication that the membrane of the erythrocyte cell is altered as a result of the sickling phenomenon is provided by the studies of Lux, John, and Karnovsky and Jensen, Bromberg, and Barefield. Both have shown that irreversibly sickled cells can be lysed and ghosts prepared under the appropriate ionic and pH conditions such that the resulting resealed ghosts retain the sickled shape. Lux et al. further showed that the protein shells of the Triton-extracted irreversibly sickled cells were deformed.

We have initiated an investigation to test the hypothesis that exposed molecular constituents of the erythrocyte membrane are altered in the red cells from sickle cell patients in such a way that the binding of the erythrocytes to the inner surface of the arteries and capillaries is increased. The causes of the painful crisis syndrome of the disease are assumed to be associated with the blockage of small vessels. Frequently, it is suggested that this blockage is due to an alteration in the deformability of the cell and its inability to pass through small bends and constrictions in the vessels. We suggest that there is an alteration of the membrane such that the red blood cells of the sickle cell patient adhere more strongly to the endothelium of the capillaries. For this reason, or this reason in combination with the enhanced rigidity of the cell, these cells fail to pass through the vessels and thus block blood flow.

MATERIALS AND METHODS

Preparation of Blood

Blood from the investigators and homozygous sickle cell patients was obtained at the University of Miami Comprehensive Sickle Cell Center. The blood was drawn into heparinized tubes and centrifuged.

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at 2000 g for 2 min at 4°C to remove the serum and buffy coat. The cells were rapidly washed 4 times in phosphate-buffered saline (0.9% NaCl, 5 mM sodium phosphate, pH 7.4). With each wash, a portion of the upper layer of cells was removed and discarded.

**Preparation of ISC**

The irreversibly sickled cells (ISC) were separated from nondeformed cells by the method of Zucker and Cameron. The percentage of ISC obtained in this manner varied from patient to patient. Frequent reticulocyte counts were also made of the ISC fraction, the supernatant fraction (non-ISC), and control red blood cells (RBC) from normal blood. At no time did the ISC fraction contain more reticulocytes (15% or less) than was present in the control blood.

**Preparation of Endothelium**

Endothelium was isolated from calf aortas by collagenase treatment according to the methods of Booyse et al. After removal, the endothelial cells were plated in RPMI-1640 medium plus penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin (0.25 µg/ml) and supplemented with 35% fetal calf serum. Cultures were not used beyond the seventh passage. During this period, the cells maintained the characteristic cobblestone appearance of endothelium.

**Adhesion Studies**

The adhesion assay was carried out essentially by the methods of Walther et al. Endothelium was plated into CoStar cluster dishes (no. 3524) at a concentration of 10³ well (2 sq cm) and allowed to reach confluency as monitored by phase microscopy (usually 2 days). The monolayers were washed twice with Hanks balanced salt solution, buffered with Hepes, pH 7.4 (HH), before addition of the red blood cells. The RBC were labeled with ¹¹⁹Cr (as sodium chromate, specific activity 200-500 Ci/g chromium, New England Nuclear, Boston, Mass.) at a concentration of 0.1 ml/ml cell suspension for 1 hr at room temperature. They were washed once in HH, and 0.5 ml (10⁷ cells/ml) was added on top the washed monolayers of endothelium. The cultures were incubated for 15 and 30 min at 37°C. Preliminary experiments, which had measured adhesion over 1 hr at 5-min intervals, indicated that the maximal number of cells had adhered by 30 min. After incubation, the unattached RBC and media were removed and the monolayers washed once in HH. Then, 0.5 ml of 1N NH₄OH was added to each well and incubated overnight at room temperature to lyse the RBC and release the radioactivity. The samples were added to scintillation vials and counted on a Beckman Model LS-333 scintillation counter. Percentage of cells attaching was calculated by comparing the incubated RBC/endothelium sample to 0.5 ml of the original inoculum of RBC.

**Timing of ISC Isolation and Endothelial Assay**

Blood was drawn from sickle cell patients in Miami, and the ISC fraction was isolated within 4 hr. Normal erythrocytes were also drawn from donors in Miami and treated in the same manner as those from the sickle cell patients. After isolation, the normal, the ISC, and the non-ISC red blood cells were resuspended in phosphate-buffered saline, placed in small closed containers, and mailed by express to the Harvard Medical School in Boston. This usually took 19 hr. On occasion, the samples took up to 4 days to reach Boston. On those occasions, the samples provided results that were within the range of the values obtained for blood that arrived within the 19-hr time period. Thus, we concluded that shipping delay did not affect the binding assay.

**RESULTS**

To date, 12 patients have been examined (Table 1). In all but one case, the erythrocytes from sickle cell patients showed a higher binding to the endothelial cultures than did the normal red cells. The average number of normal erythrocytes adhering to endothelium was 250,000 ± 8300 and 500,000 ± 13,100 for 15 and 30 min, respectively. No correlation could be made between the degree of binding of any fraction and the percentage of reticulocytes counted from that fraction. Attempts were also made to correlate differences in the degree of binding of the
Table 1. Adhesion of RBC to Endothelial Cultures

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Normal 15 min</th>
<th>Normal 30 min</th>
<th>ISC 15 min</th>
<th>ISC 30 min</th>
<th>Non-ISC 15 min</th>
<th>Non-ISC 30 min</th>
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<td>0.97</td>
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Mean 1.00 1.00 2.13 ± 0.44 1.73 ± 0.28 1.92 ± 0.22 1.72 ± 0.25

Binding values for all normal cells were arbitrarily set at unity; however, the percentage of cells adhering after 15 min and 30 min in the control cells averaged 5% and 10%, respectively. The differences between the controls and the ISC and non-ISC had a level of significance of $p < 0.02$; however, there was no significant difference between the adhesion of the ISC and the non-ISC.

ISC or supernatant fractions to the severity of the symptoms of the individual patient involved. Again, no correlation between the frequency or severity of the symptoms and the quantitative binding assay could be made. This was true even when one compares the extreme cases (patient 4 versus patient 7).

DISCUSSION

The results clearly indicate that the erythrocytes (ISC and non-ISC) from symptomatic sickle cell patients bind to endothelial monolayers at a frequency much greater than that of control cells.

The mechanisms for this enhanced adhesion is not clear at this time. In another study we have demonstrated that ISC from many sickle cell patients have a statistically significant reduction in the number of cationized ferritin-binding sites (presumably sialic acid) on their outer surfaces when compared to normal erythrocytes from non-sickle blood. This reduction in anionic surface sites could produce an increased adhesion of the cells to the endothelial surface, since, presumably, one of the mechanisms of maintaining cell separation in the erythrocyte is the large negative charge. Similarly, reduction in negativity has also been shown to be effective in increasing the number of polymorphonuclear leukocytes attaching to endothelium.11

It also should be pointed out that the degree of cell adhesion for the erythrocyte preparations is less than was observed for leukocytes.11 This might be expected, since neutrophils normally bind to endothelium prior to leaving the peripheral blood; for example, as a result of an inflammatory stimuli.12 In the case of the red blood cells, movement through the capillaries is such that the cells flow in single file, many times deforming the capillary and being squeezed against the endothelium. If under these conditions, a more adhesive cell enters the capillary, the chances for a stable adhesion would be increased, consequently blocking the capillary.
We propose that the mechanism of vessel blockage experienced in sickle cell disease includes an enhanced adhesion of the red blood cells to the endothelial surface of the vessels. This does not exclude other mechanisms in this blockage, such as a reduced deformability of the irreversibly sickled cell. However, our results do demonstrate that the non-ISC fraction also shows this increase in adhesion, and these cells presumably are readily deformable. Moreover, the enhanced binding of the non-ISC fraction suggests that cell shape may not be a major factor in promoting adhesion to the endothelium because cells in the non-ISC fraction have a normal morphology. Thus, it is possible that the painful crisis syndrome is caused by the cells of the non-ISC fraction as well as those of the ISC fraction.

Our results also indicate that it is incorrect to associate the severity and frequency of symptoms with the percentage of irreversibly sickled cells circulating in the patient. Symptomatic improvement following transfusion could be due to replacement of both the ISC and non-ISC fractions. One possible complicating factor is the presence of reticulocytes in the non-ISC fraction. The ISC fraction always contains a reticulocyte count comparable to that of the control sample. The non-ISC fraction frequently has reticulocyte counts that are greater than that found in the control sample. Thus, the results with the non-ISC fraction are probably not directly comparable to the ISC or control samples.

Even considering these technical problems, our results clearly indicate that the entire ISC cell population from symptomatic homozygous sickle cell patients shows an almost twofold enhancement of binding to endothelial cells in comparison to normal erythrocytes. Both studies suggest the possibility that this enhanced binding may be related to the production of some of the symptoms of sickle cell anemia. In a recent abstract, Hebbel et al. have also shown that sickle cells attach more readily to endothelium than to normal RBC.

REFERENCES

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