Exercise-Induced Hemolysis in Sickle Cell Anemia: Shear Sensitivity and Erythrocyte Dehydration

By Orah S. Platt

We describe a steady-state patient with sickle cell anemia (SS disease) who developed sporadic hemoglobinuria, historically related to vigorous exercise. We studied him and four other patients with SS disease and demonstrated exercise-induced hemoglobinemia. To see if SS erythrocytes were abnormally fragile when exposed to shear forces that could be generated in small vessels of exercising muscles, we exposed them to physiologic shear rates in a cone-plate viscometer. We show that SS erythrocytes are more shear sensitive than normal erythrocytes. This phenomenon is directly related to the presence of dehydrated cells as demonstrated by the increasing shear sensitivity of increasingly dehydrated cells separated on Stractan density gradients. Normal shear sensitivity could be restored to dehydrated layers by restoring normal hydration. Restoration of shear stability was complete in all layers except for the most dense ISC layer. A control group of patients with SC disease exhibited no exercise-induced hemoglobinemia, no abnormal shear sensitivity of whole blood, and only rare dehydrated ISCs. These studies suggest that the exercise-induced hemolysis in SS patients is related to the lysis of dehydrated, shear-sensitive cells. This same process may also contribute to the chronic hemolysis of SS disease—a phenomenon known to correlate with the numbers of dehydrated ISCs.

OMOZYGOUS SICKLE CELL ANEMIA (SS disease) is a chronic hemolytic anemia characterized by reticulocytosis, marrow hyperplasia, indirect hyperbilirubinemia, decreased erythrocyte survival, and increased carbon monoxide excretion.1 The precise mechanism of hemolysis in this disorder is not known, but is probably a composite of general factors, including cellular sickling with spontaneous fragmentation,2,3 sickling-induced increased mechanical fragility,4 calcium accumulation with decreased cellular deformability,5 membrane injury due to increased hemoglobin binding,6,8 and decreased deformability due to cell dehydration.9 Many of these factors characterize and possibly induce the formation of the irreversibly sickled cell (ISC). It follows logically then that these ISCs have a T½ of only 2 days10 and are proportional to the degree of hemolysis in a given patient.11 Fluctuations of the degree of intravascular hemolysis in individual patients was one of the earliest clinical observations made in sickle cell anemia. Over long periods of time, patients were shown to have plasma hemoglobin levels that varied from 0 to 150 mg/dl plasma.12-16 Many had elevated levels of plasma hemoglobin during painful crises.15,16 Here we describe a steady-state patient with SS disease who developed sporadic hemoglobinuria that was historically related to vigorous exercise. He and four other patients with SS disease were shown to have exercise-induced hemoglobinemia. In this study we investigate the relationship between ISCs, erythrocyte dehydration, susceptibility to hemolysis by shear stress, and exercise-induced hemolysis in sickle cell anemia. We illustrate that SS blood is more susceptible to shear stress than SC or normal blood, and that this susceptibility is related to the presence of dehydrated ISCs.

CASE REPORT

M.B., a healthy, athletic 21-yr-old male with homozygous sickle cell anemia (SS disease) was found to have hemoglobinuria on a routine urinalysis. Previous urinalyses had been free of hemoglobin. Hemoglobin, hematocrit, and reticulocyte counts were comparable to previous steady-state measurements. He was receiving no medicines; Coombs', sugar-water, and Hinton tests were negative; and G6PD activity was normal for the degree of reticulocytosis. The patient commented that although his urine was usually yellow, it frequently turned reddish after playing basketball or practicing karate.

MATERIALS AND METHODS

Routine Hematologic Studies

Blood was drawn from this patient, apparently healthy adult volunteers, and seven other asymptomatic athletic patients with either SS disease or SC disease. Blood was collected in EDTA and processed within 2 hr of drawing.

Hemoglobin, hematocrit, red cell count, reticulocyte count, osmotic fragility, Coombs' test, and sugar-water test were performed by established procedures.17 Hemoglobin was distinguished from myoglobin in urine samples by ammonium sulfate precipitation. Irreversibly sickled cell (ISC) counts were done by enumerating the percent of cells twice as long as wide on a Wright-stained peripheral smear of well oxygenated blood. Results are expressed as the mean of

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Supported in part by Grants HL-15157 and AM-21836 from the National Heart, Lung, and Blood Institutes of the National Institutes of Health.

Submitted August 17, 1981; accepted January 7, 1982.

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0006-4971/82/5905-0029$01.00/0

Blood, Vol. 59, No. 5 (May), 1982
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two 500-cell counts. Mean cell volume (MCV) was calculated using hematocrit measured in a microhematocrit centrifuge and automatic red cell count (Coulter Model S).

Intracellular sodium and potassium concentrations were determined as described by Glader et al.9 using erythrocytes washed in isotonic magnesium chloride, hemolyzed in a lithium carbonate Unopette (Becton-Dickinson, Rutherford, N.J.), and measured in a flame photometer. All samples were measured in triplicate, with mean values reported.

**Exercise Studies**

To assess the effect of exercise on hemolysis, five patients with SS and three patients with SC disease had blood drawn before and after an exercise stress. Individuals with SS and SC disease who agreed to participate in this study were being seen as a routine part of their health maintenance program and not because of any intermittent illness. These particular patients were approached because of their previously demonstrated unusual physical endurance as active participants in one or more of the following organized sports: softball, basketball, soccer, or track. None of these patients were enrolled in other clinical studies at the time. Each had a urine sample and 5 ml of blood collected before and after the exercise test, which consisted of a slow and steady walk up four flights of stairs with their feet on the stair surface. This walk was tiring, but had no ill effects. None had to stop before completing the test. Plasma and urine hemoglobin was measured using the benzidine method of Crosby and Furth.19 Urine hemosiderin was determined qualitatively by microscopic analysis of Prussian blue-stained urinary sediment.

**Effect of Shear Stress on SS, SC, and Control Erythrocytes**

To measure their susceptibility to disruption by shearing forces, erythrocytes were exposed to various shear stresses and the resultant hemolysis was measured. A sample of blood to be tested was washed three times in a Heps-buffered salt (HBSS) solution, pH 7.4, with the following composition: 5 mM sodium chloride, 50 mM potassium chloride, 1 mM sodium phosphate, 1 mM magnesium chloride, 20 mM HEPES, and 100 mg/ml of bovine serum albumin and concentrated to a hematocrit of 50%–60%. Viscoity of the suspension was manipulated by adding dextran (0%–60%), with appropriate reduction of NaCl, to give a final osmolarity of 290. Plasma shear stress was applied in a cone-plate viscometer (Wells-Brookfield Co., Stoughton, Mass.) modified with the addition of a Luer port for introduction and withdrawal of the sample, a gas inlet arm, and a constant temperature water jacket. A 0.5–1.0 ml well oxygenated sample was introduced between the cone and the plate through the Luer port. The motor was started, and the cone rotated at either 0.3, 0.5, 1.5, 3.0, 6.0, 12.0, 30.0, or 60.0 rpm. The sample was exposed to the rotating cone for 2 min, then carefully withdrawn from the viscometer, and the supernatant was separated in a microhematocrit centrifuge and assayed for hemoglobin. All of the experiments were done at 37°C in an atmosphere of 100% oxygen.

**Density Gradient Fractionation of SS, SC, and Control Erythrocytes**

Discontinuous Stractan (St. Regis Paper Co., Tacoma, Wash.) gradients for separation of erythrocytes were prepared by a modification of the technique described by Crosby and Dalmark.29 Cells were washed in HBSS solution and suspended at a 5% hematocrit at room temperature in the following medium: 150 mM KCl, 5 mM NaCl, 1 mM MgCl2, 27 mM sucrose, 20 mM HBS solution, 30 mg/ml nystatin, pH 7.4, for 30 min. The nystatin was then removed by 3 washes in HBS solution warmed to 37°C, and the cells were resuspended in HBSS solution at a 20% hematocrit and allowed to equilibrate at 37°C for 30 min.

**RESULTS**

The clinical characteristics and results of exercise testing are shown in Table 1. Five patients with SS disease (including M.B., the propositus) and three patients with SC disease were studied. All started with hematologic values comparable to their previous steady-state measurements: less than 5 mg/dl plasma hemoglobin, and no urine hemoglobin. At the end of the exercise, all five patients with SS disease developed filtered through a 0.45-µm filter (Millipore Corp., Bedford, Mass.), and lyophilized. Lyophilized Stractan (200 g) was then dissolved in 200 ml distilled water and albumin was added to a final concentration of 30 mg/ml. To 9 parts Stractan solution, 1 part 0.15 M potassium phosphate buffer, pH 7.4, was added. This solution was diluted 1: 1 with buffered saline with glucose (BSG) of the following composition: NaCl 8.1 g, Na2HPO4 1.22 g, NaH2PO4 0.219 g, MgCl2·6H2O 0.406 g, glucose 2 g, and sufficient water to bring the final volume to 1000 ml. The pH was adjusted to 7.4 with HCl.

Stock densities of 1.085, 1.090, 1.095, 1.10, and 1.12 were prepared by diluting the Stractan-BSG solution with BSG and were prepared for use in these experiments. Before using previously frozen material, it was often necessary to readjust the pH to 7.4 with NaOH and the osmolarity to 300 mosmole with NaCl. The gradients were centrifuged at 4°C in a Beckman SW40 swinging bucket rotor (Beckman Instruments, Fullerton, Calif.) at 37,000 rpm for 60 min without application of a brake. Populations of cells concentrated at the interfaces of the gradient were collected by slicing the gradient tube. Cells were freed of Stractan by washing 4 times in HBS solution.

**In Vitro Alteration of Erythrocyte Density**

Dehydrated layers of sickle cells were rehydrated using nystatin and a high potassium buffer by modification of the technique described by Cass and Dalmark.29 Cells were washed in HBS solution and suspended at a 5% hematocrit at room temperature in the following medium: 150 mM KCl, 5 mM NaCl, 1 mM MgCl2, 27 mM sucrose, 20 mM HBS solution, 30 mg/ml nystatin, pH 7.4, for 30 min. The nystatin was then removed by 3 washes in HBS solution warmed to 37°C, and the cells were resuspended in HBS solution at a 20% hematocrit and allowed to equilibrate at 37°C for 30 min.

**Table 1. Clinical Characteristics and Results of Exercise Testing in SS Disease and SC Disease**

<table>
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<tr>
<th>Patient</th>
<th>Dx</th>
<th>Hct</th>
<th>MCV</th>
<th>MCHC</th>
<th>Retcs</th>
<th>ISCs</th>
<th>Plasma mg/dl Pre</th>
<th>Hb mg/dl Pre</th>
<th>Plasma mg/dl Post</th>
<th>Hb mg/dl Post</th>
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<td>24</td>
<td>85</td>
<td>34</td>
<td>19</td>
<td>16</td>
<td>≤5</td>
<td>18</td>
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<tr>
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<td>＜5</td>
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<td>89</td>
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<tr>
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<td>＜5</td>
<td>＜5</td>
<td>＜5</td>
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<tr>
<td>W.R.†</td>
<td>SC</td>
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<td>79</td>
<td>33</td>
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<td>＜5</td>
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<td>＜5</td>
<td>＜5</td>
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<tr>
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<td>30</td>
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<td>＜5</td>
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</table>

*Propositus.

‡Shear stress hemolysis data shown in Fig. 1.

†Urine positive for hemosiderin.

‡History of dark urine following exercise.
measurable plasma hemoglobin. Free hemoglobin measurements varied ± 10%. The SC patients and the AA control did not develop plasma hemoglobin. None of those tested developed urine hemosiderin before and after exercise.

Shear Sensitivity of SS Disease and SC Disease Erythrocytes

Increasing shear stresses were applied to samples of SS, SC, and control erythrocytes in a cone-plate microviscometer. The resulting hemolysis is an expression of the cells’ sensitivity to the shear stress applied. Figure 1 summarizes the data from one representative experiment in a patient with SS disease (x, M.B.), a patient with SC disease (Δ, W.R.) and control (O). Each point represents hemolysis data based on the mean of two hemoglobin determinations from a separate aliquot exposed only to that shear stress. Control erythrocytes and SC erythrocytes showed little or no hemolysis over the entire range of stresses studied. In contrast, the SS erythrocytes exhibited increasing hemolysis over the range studied. There is a clear difference between the “shear-sensitive” SS erythrocytes and the “shear-resistant” control and SC erythrocytes.

Shear Sensitivity of Erythrocytes Separated on Stractan Gradients

To assess the relative effect of dehydration on shear sensitivity, SS, SC, and control erythrocytes were separated on discontinuous Stractan gradients. Some characteristics of these separated layers in one representative experiment are described in Table 2. The general pattern was the same in all three samples. The reticulocytes were concentrated in the least dense (top) layer. In the more dense layers, the cells progressively gained sodium and lost potassium. Potassium loss predominated, resulting in a net decrease in total cations. This cation loss was associated with a shrinking MCV and increasing MCHC—both evidence of progressive intracellular dehydration. ISC are characteristically dense cells and they concentrated in the most dense layers. The major difference seen in these experiments was in the relative distribution of the erythrocytes in the different layers. In the control experiment the bulk of the cells were in layers 1 and 2, with 3% of the total in layer 4 and none in layer 5. In the SS experiment, more cells were found in the bottom of the gradient with 19% in layer 4 and 3% in layer 5. The SC erythrocytes were similar to the controls, but slightly more dense, with the bulk of the cells in layers 2 and 3, only 6% in layer 4, and none in layer 5.

Table 2. Characteristics of Control, SS, and SC Erythrocytes Separated on Stractan Gradients

<table>
<thead>
<tr>
<th>Layer</th>
<th>Density (g/ml)</th>
<th>Reticulocytes (%)</th>
<th>ISCs (%)</th>
<th>Percent Total</th>
<th>Na (meq/liter RBC)</th>
<th>K (meq/liter RBC)</th>
<th>Na + K (meq/liter RBC)</th>
<th>MCV (cu μ)</th>
<th>MCHC (g/dl)</th>
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<tr>
<td>Control erythrocytes</td>
<td>1.095</td>
<td>4</td>
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<td>15</td>
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<td>107</td>
<td>117</td>
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<tr>
<td></td>
<td>2.090</td>
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<td>0</td>
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<td>0</td>
<td>3</td>
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<td>50</td>
<td>72</td>
<td>81</td>
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<td>0</td>
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<td>SS erythrocytes</td>
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<td>3</td>
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<td>SC erythrocytes</td>
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<td>113</td>
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<td>72</td>
<td>78</td>
<td>36</td>
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</table>
The shear sensitivity of these different layers is shown in Fig. 2. In each experiment the pattern was the same: the more dehydrated the erythrocyte layer, the more shear sensitive it was. The relative shear resistance of layer 1 cells eliminates reticulocyte artifact as the cause of the increased sensitivity of SS erythrocytes. The increased sensitivity of the SS erythrocytes (Fig. 1) is related to the relatively large population of dehydrated fragile cells.

Reversibility of the Dehydration Effect

To see if normal shear resistance could be restored in dehydrated cells by rehydration, four layers of SS erythrocytes were rehydrated by exposure to nystatin in a high potassium buffer. In all cases, rehydration was complete as judged by normalization of the MCHC and the mean osmotic fragility. The data are presented in Fig. 3. When normal hydration was restored to control layer 3, SC layer 4, or SS layer 3, normal shear sensitivity was also restored. This implies that the largest determinant of shear sensitivity is cell dehydration with concentration of cell contents. However, as seen in Fig. 3D, when SS layer 4 is rehydrated, normal shear sensitivity is only partially restored. The major difference between SC layer 4 and SS layer 4, as seen in Table 2, is the presence of 52% ISC's in SS layer 4. After rehydration these cells maintain an abnormal shape, although they are somewhat more ovoid and less pointed.

DISCUSSION

In individuals with normal erythrocytes, exercise-induced hemolysis is associated with exposure of the cells to the tremendous mechanical forces generated during traumatic activities such as prolonged marching or jogging on pavement, karate exercising,27 or conga-drum playing.28 We have recently described an individual with hereditary xerocytosis and abnormal dehydrated erythrocytes who experienced atraumatic exercise-induced hemolysis during swimming.29 This patient's erythrocytes were more susceptible to hemolysis by shear forces than normal erythrocytes. This shear susceptibility was proportional to the degree of erythrocyte dehydration and could be reversed by

Fig. 2. (Top panel) Hemolysis of layers 1 (○), 2 (△), and 3 (□) of control erythrocytes with increasing density separated by Stractan density gradient and exposed to various shear stresses for 2 min at 37°C, 100% O₂ in a microviscometer. (Middle panel) Hemolysis of layers 1 (○), 2 (△), 3 (□), 4 (x), and 5 (△) of SS erythrocytes with increasing density separated by Stractan density gradient and exposed to various shear stresses for 2 min at 37°C, 100% O₂ in a microviscometer. (Bottom panel) Hemolysis of layers 1 (○), 2 (△), 3 (□), and 4 (x) of SC erythrocytes with increasing density separated by Stractan density gradient and exposed to various shear stresses for 2 min at 37°C, 100% O₂ in a microviscometer.

Fig. 3. Panel (A) Hemolysis of control layer 3 erythrocytes exposed to various shear stresses for 2 min at 37°C, 100% O₂ in a microviscometer. Before (□) and after (■) in vitro rehydration. (B) Hemolysis of SS layer 3 erythrocytes exposed to various shear stresses for 2 min at 37°C, 100% O₂ in a microviscometer. Before (□) and after (■) in vitro rehydration. (C) Hemolysis of SC layer 4 erythrocytes exposed to various shear stresses for 2 min at 37°C, 100% O₂ in a microviscometer. Before (□) and after (■) in vitro rehydration. (D) Hemolysis of SS layer 4 erythrocytes exposed to various shear stresses for 2 min at 37°C, 100% O₂ in a microviscometer. Before (□) and after (■) in vitro rehydration.
rehydration. We theorized that shear forces capable of hemolyzing dehydrated erythrocytes could be generated in vigorously exercising muscles, and that this might be the basis for atraumatic exercise-induced hemolysis in our patient with xerocytosis. Erythrocyte dehydration is not unique to xerocytosis, but is seen in several hemolytic anemias.30 In SS disease, for example, erythrocyte dehydration is a prominent feature. While reversible sickling is associated with increased cation permeability without dehydration,31,32 irreversible sickling is associated with the loss of total cation and water.33 The mechanism is not entirely clear but is associated with decreased active cation transport,34 possibly the result of increased intracellular calcium and metabolic depletion35 as in the Gardos effect.36 All of the five patients with SS disease in this study developed hemoglobinemia after a relatively aotumatic exercise stress, whereas the three patients with SC disease did not. These in vivo observations are compatible with the in vitro data illustrated in Fig. 1. Control erythrocytes and SC disease erythrocytes did not hemolyze in the viscometer over the entire range of shear stresses studied. In contrast, the SS disease cells were quite sensitive to shear forces greater than 600 dynes/sq cm. When separated on Stractan density gradients, the control, SS, and SC erythrocytes all exhibited the same density-related shear sensitivity that we previously observed in hereditary xerocytosis. The major difference in these samples was the distribution of the dehydrated cells. Layers 1 and 2 were shear resistant and layer 3 was only slightly shear sensitive. These three layers comprised 97% of normal erythrocytes, 96% of SC erythrocytes, but only 78% of SS erythrocytes. Twenty-two percent of SS erythrocytes fell into the denser shear-sensitive layers 4 and 5 and were responsible for the shear sensitivity of SS whole blood. These dense layers contained over 90% of the ISCs in the sample. The presence of ISCs influenced the degree to which normal shear sensitivity could be restored by rehydration. As seen in Fig. 3 the ISC-poor layers, 3 in the control, layers 3 and 4 in the SC, and layer 3 in the SS samples could all be restored to normal sensitivity by rehydration. In contrast, ISC-rich SS layer 4 only partially normalized. In this system then, the mechanical properties of the ISC membrane are different from normal, and this difference is related to maintaining membrane integrity in the face of shear stress. Such differences may be due to the subtle changes in protein–protein interactions as suggested by the work of Lux et al.37 and may be related to a dehydration-induced injury as suggested by Sullivan et al.38 and seen in our work with dehydrated normal cells.39 These findings are different from those of Clark et al.,39,40 who showed that restoration of normal hydration to ISCs restored normal deformability as measured by ectacytometry and by ability to be deformed upon deoxygenation. These differences illustrate that measures of mechanical stability are not interchangeable as they examine different membrane properties.

These studies show that individuals with SS disease have dense, dehydrated shear-sensitive erythrocytes and exercise-induced intravascular hemolysis. Individuals with SC disease have fewer dense cells, no in vitro shear sensitivity, and no exercise-induced hemolysis. These observations may partially explain why ISCs have such a short survival, why patients with SC disease have less hemolysis than patients with SS disease, and why patients with SS disease sporadically have hemoglobinemia, especially during painful crises when their red cells would be exposed to the shear stresses of a hyperdynamic circulation.

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Exercise-induced hemolysis in sickle cell anemia: shear sensitivity and erythrocyte dehydration

OS Platt