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Fragility of abnormal erythrocytes evaluated by response to shear stress

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Shear stress is a potential cause of erythrocyte fragmentation and hemolysis in flowing blood. In this study, the response of abnormal human erythrocytes to shear stress in vitro was evaluated using a concentric cylinder viscometer. Compared to normal red cells, deoxygenated erythrocytes from persons with sickle cell anemia were particularly susceptible to fragmentation and hemolysis by shear stress. Oxygenation of sickle cell blood improved the resistance of those red cells to shear stress; they remain, however, more susceptible to shear stress than normal erythrocytes. Erythrocytes from patients with iron deficiency, thalassemia minor, and erythrocyte pyruvate kinase deficiency showed fragmentation and hemolysis at threshold shear stresses intermediate between those observed for blood from patients with sickle cell anemia and normal persons. Blood samples from patients with hereditary spherocytosis were more resistant to shear stress than normal blood. These results indicate that there are important differences in the response of various red cells to shear stress.

Microangiopathic hemolytic anemias and hemolysis associated with artificial valves are characterized by morphologic evidence of erythrocyte fragmentation. Shear stress is a physical force to which erythrocytes in flowing blood are exposed. Shear stresses of 2,500 dynes per square centimeter or greater applied to normal erythrocytes in vitro result in hemolysis and formation of red cell fragments resembling morphologically those observed in patients with red cell fragmentation syndromes.[1]

Fragmented red cells may be seen in small numbers in a variety of hematologic diseases including sickle cell anemia, hemoglobin C disorders, thalassemia, and iron deficiency. Fragmentation is a possible contributory mechanism to hemolysis in these anemias. In the present study, the susceptibility to shear stress in vitro of erythrocytes from patients with sickle cell anemia, thalassemia minor, iron deficiency anemia, erythrocyte pyruvate kinase deficiency, and hereditary spherocytosis has been studied. Hemolysis and morphologic evidence of fragmentation of red cells were assessed after exposure of samples of blood to various shear stresses in a concentric cylinder viscometer.

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Methods

Concentric cylinder viscometer. A concentric cylinder rotational viscometer was specially constructed to study the effect of shear stress of blood. The bottom of the device has a cone-plate configuration with an angle of 0.29 degrees. Ordinarily, a cone-cone configuration was used at the top; in a few experiments, the upper configuration was a cone-cylinder. The instrument is described in greater detail elsewhere.[2,3] Designed to achieve very high shear rates (maximum, 2×10^5 sec.$^{-1}$), the viscometer applies uniform shear stress to all of the blood sample within the gap (including the cone-plate region at the bottom and cone-cone region at the top). Both the cup and the bob of the viscometer are made of anodized aluminum and are coated with Clay-Adams Siliclad using a procedure defined by the manufacturer. The cup rotates at high speeds and can be brought to 5,000 r.p.m. within 3 seconds after starting. The gap between the cup and bob is 165 microns. Blood is introduced into the gap through a valve at the bottom of the viscometer. The volume of blood in the gap exposed to shear stress is 2.9 ml. Measurement of the torque on the bob resulting from rotation of the cup allows determination of shear stress. Under the conditions of the experiments reported here, blood flow is laminar, not turbulent. Secondary flows are insufficient in degree to distort the linear torque-shear rate relationship. Various secondary effects such as cell-solid surface interaction, cell air-interface interaction, centrifugal effects, and cell-cell interaction have been studied previously,[4] and are not of sufficient magnitude to affect significantly the results to be reported.

Temperature is monitored through a thermocouple in the bob. All experiments are initiated at a temperature of 22° C. Cooling fins on the cup prevent excessive heating due to viscous dissipation. At the highest shear stresses studied, i.e., 4,000 dynes per square centimeter applied for two minutes, the increase in temperature never exceeded 7° C.

Experimental procedure. Informed consent was obtained from blood donors and the research was carried out according to the Declaration of Helsinki. Blood samples of 100 ml. were collected in plastic bags containing acid-citrate-dextrose (ACD) (NIH formula A) or heparin from 6 normal persons, 6 patients with sickle cell anemia (hemoglobin SS), 2 patients with thalassemia minor, 2 patients with iron deficiency anemia, 2 patients with hereditary spherocytosis, and 1 patient with erythrocyte pyruvate kinase deficiency. All studies were conducted on blood samples at a hematocrit of 40 per cent. Thus, bags containing blood from anemic subjects were centrifuged lightly and plasma removed to adjust the hematocrit to 40 per cent. Studies of blood from patients with sickle cell anemia were conducted under 4 atmospheric conditions: pure nitrogen atmosphere, venous blood as drawn, room air, and 100 per cent oxygen. All studies were initiated at room temperature and begun within 1 to 2 hours after collection of the blood.

Blood was introduced into the gap and the viscometer started and brought to a desired shear stress within 5 to 10 seconds. The range of shear stresses studied varied between 250 and 4,000 dynes per square centimeter. The duration of application of shear stress was always 2 minutes. Plasma hemoglobin was determined by the benzidine method[5] and total blood hemoglobin by the cyanmethemoglobin method on blood samples prior to exposure to shear stress and on blood after application of shear stress in order to calculate the percentage of hemolysis. When hemolysis was substantial, values for paired samples varied by ±5 per cent, whereas variation was as great as ±15 per cent when hemolysis was minimal. Blood films were prepared before and after application of shear stress, stained with Wright's stain, and examined for morphologic changes.

Results

Normal blood. The effects of shear stress on normal erythrocytes were studied 21 times on samples of blood obtained from 6 healthy donors. Results were similar for blood anticoagulated with ACD and with heparin. Hemolysis was less than 1 per cent with shear stresses below 2,500 dynes per square centimeter. Hemolysis significantly increased at shear stresses above 2,500 dynes per square centimeter varying between 2 and 4 per cent at 3,000 dynes per square centimeter and between 8 and 19 per cent at 4,000 dynes per square centimeter. The range of normal values is shown in Fig. 1. No morphologic abnormalities were detected in normal blood subjected to shear stresses of 500 and 1,000 dynes per square centimeter. With a shear stress of 1,500 dynes

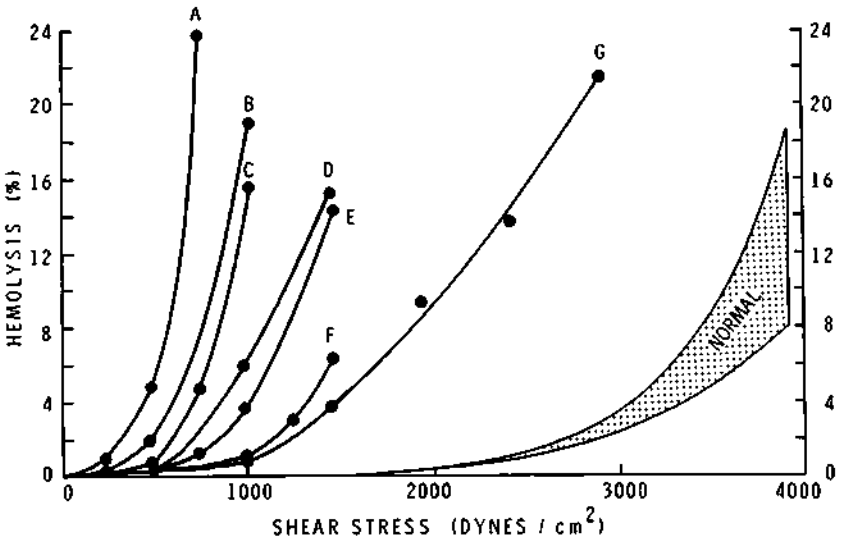

Fig. 1. The percentage of hemolysis in various samples of blood from patients with sickle cell anemia is shown in relationship to the shear stress to which the sample was exposed. Samples *A* and *D* are venous blood collected in ACD; samples *C* and *E*, venous blood collected with heparin as the anticoagulant; sample *B*, blood collected in ACD and kept under nitrogen atmosphere; sample *F*, venous blood collected in ACD and thoroughly mixed with room air; sample *G*, venous blood collected in ACD and thoroughly mixed with 100 per cent oxygen. The range of values for 21 experiments on blood from 6 normal donors is shown by the shaded area.

per square centimeter, a significant number of spherocytes and schistocytes was observed (Fig. 4, *A* and *B*). As shear stress was increased to 2,000 and 3,000 dynes per square centimeter, morphologic abnormalities substantially increased to the point that at 3,000 dynes per square centimeter, most erythrocytes were damaged (Fig. 4, *C*). Three types of morphologic abnormalities were observed: spherocytes, dense contorted poikilocytes, and small red cell fragments.

Sickle cell anemia. Hematocrits of the blood studied from the 6 patients with sickle cell anemia varied between 19 and 27 per cent. Substantial hemolysis occurred with exposure of sickle cell blood to shear stresses causing virtually no hemolysis of normal erythrocytes (Fig. 1). Sickle cell blood studied under venous oxygenation conditions or kept under a nitrogen atmosphere showed significant hemolysis at shear stresses of 500 to 1,000 dynes per square centimeter. Sickle cell blood oxygenated with room air or 100 per cent oxygen was more resistant to hemolysis by shear stress than poorly oxygenated sickle cell blood, but considerably more susceptible to the hemolytic action of shear stress than normal blood. Although the pH of blood collected in ACD was lower than that of blood collected with heparin, little difference was seen in the effects of shear stress related to the anticoagulant.

In sickle cell blood kept in a nitrogen atmosphere, small numbers of spherocytes and fragments were detected in blood exposed to shear stresses as low as 250 dynes per square centimeter. For blood studied under venous oxygenation conditions, morphologic changes were noted with application of

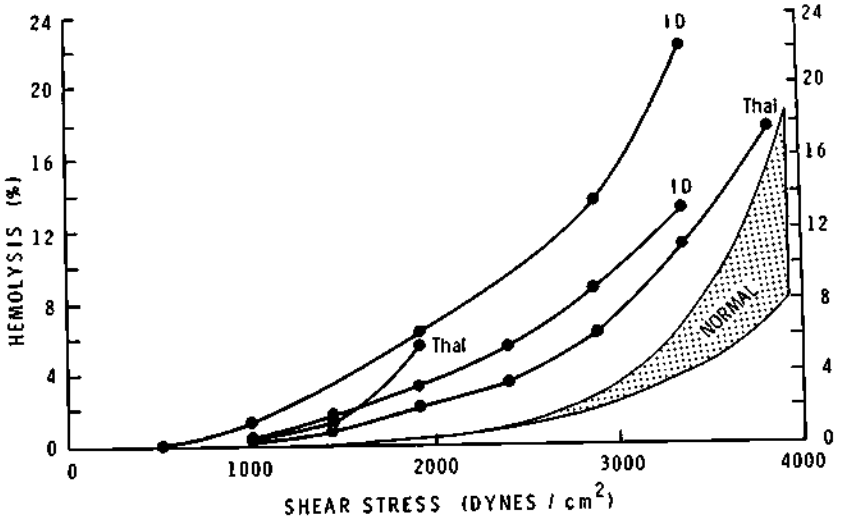

Fig. 2. The percentage of hemolysis in samples of blood of two patients with iron deficiency (ID) and two patients with beta-thalassemia minor (Thal) is shown in relationship to the shear stress to which the samples were exposed. The range of values for normal donors is shown by the shaded area.

shear stresses as low as 500 dynes per square centimeter. Spherocytes and small red cell fragments appeared and sickled cells diminished in number (Fig. 5, *A*, *B*, and *C*). These morphologic changes were more pronounced in blood subjected to shear stresses of 1,000 and 1,500 dynes per square centimeter. Very few sickled cells remained in blood exposed to shear stress of 1,500 dynes per square centimeter. In sickle cell blood, oxygenated with room air or 100 per cent oxygen, morphologic abnormalities appeared with application of shear stresses as low as 500 dynes per square centimeter, but quantitatively these abnormalities were less at 500, 1,000, and 1,500 dynes per square centimeter than observed in poorly oxygenated sickle cell blood studied at the same shear stresses.

Hypochromic anemia. Blood from two patients with iron deficiency anemia (hematocrits, 25 and 29 per cent) and two patients with beta-thalassemia minor (hematocrits, 39 and 43 per cent) showed more hemolysis with various shear stresses than normal blood but less than sickle cell blood (Fig. 2.) Fragmentation and formation of spherocytes were more striking in blood from iron-deficient and thalassemic donors studied at shear stresses of 1,500 and 2,000 dynes per square centimeter than in normal blood studied at the same shear stresses (Fig. 6, *A*, *B*, and *C*).

Pyruvate kinase deficiency. The blood of a woman with erythrocyte pyruvate kinase deficiency was studied. Hematocrit was 30 per cent and reticulocyte count 24 per cent. Splenectomy had been performed several years previously. Her blood was more susceptible to hemolysis by shear stress than normal blood (Fig. 3) and developed more spherocytes and schistocytes than did normal blood at shear stresses of 1,500 and 2,000 dynes per square centimeter.

Hereditary spherocytosis. Blood samples of two patients with hereditary spherocytosis, after splenectomy, were studied. Neither was anemic. One had

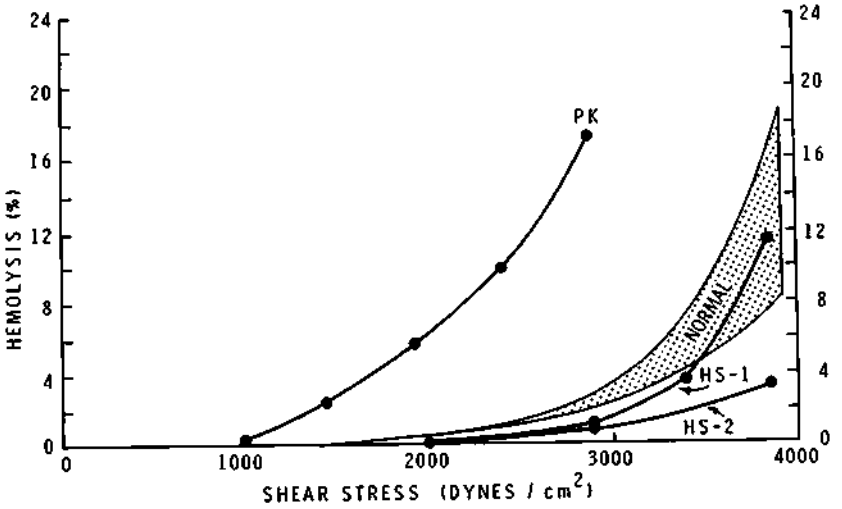

Fig. 3. The percentage of hemolysis in samples of blood of two patients with hereditary spherocytosis (HS-1 and HS-2) and a patient with pyruvate kinase deficiency (PK) is shown in relationship to the shear stress to which the sample was exposed. In fresh samples of blood, 13 per cent spherocytes were found in HS-1 and 34 per cent spherocytes in HS-2. The range of values for normal donors is shown by the shaded area.

34 per cent and the other 13 per cent circulating spherocytes. The blood of both patients was more resistant to hemolysis by shear stress than normal blood (Fig. 3). The resistance was greater in the patient with the higher percentage of spherocytes. Blood of these two patients subjected to shear stresses below 3,000 dynes per square centimeter did not differ morphologically from their blood examined prior to application of shear stress. Samples of blood studied at 3,000 and 4,000 dynes per square centimeter showed a significant increase in the percentage of spherocytes but, in contrast to normal blood studied at these shear stresses, very few red cell fragments were observed.

Discussion

The response of erythrocytes to shear stress in the concentric cylinder viscometer offers a means to estimate the resistance of red cells to a specific type of physical force. The susceptibility of red cells to physical stresses has been assessed previously by rotation of defibrinated blood in a flask containing glass beads[6,7] and by the use of the hemoresistometer.[8] With both of these techniques, multiple factors including surface effects, shear stress, and turbulence contribute to hemolysis. The principal physical force affecting red cells in the viscometer is shear stress, a force to which erythrocytes are exposed as blood flows. Surface effects are of little importance.[4] Quantitatively, the shear stress necessary to cause fragmentation and hemolysis of erythrocytes in the viscometer is an order of magnitude greater than estimates of shear rates (and shear stresses) present as blood circulates normally in the blood vessels of man.[9] However, under pathologic circumstances such as with malfunctioning valvular prostheses, shear stresses develop which are of the same magnitude as those shown to damage erythrocytes in vitro.[1]

Blood obtained from patients with sickle cell anemia was more susceptible

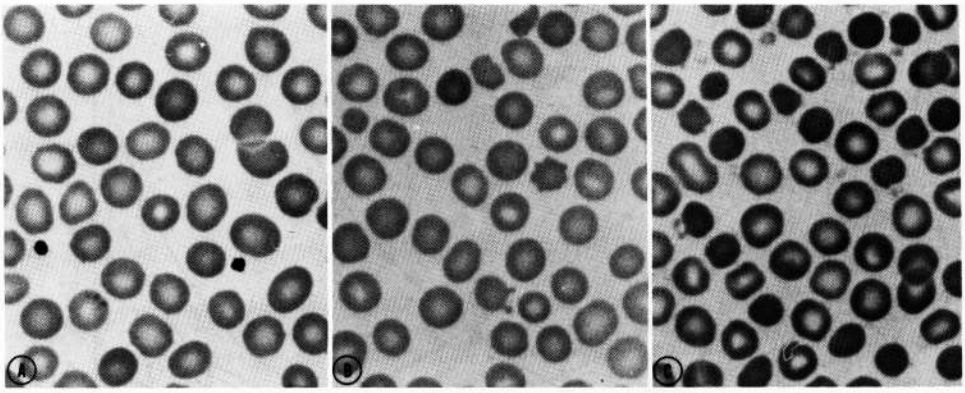

Fig. 4. Peripheral blood films, normal donor, Wright's stain, original magnification, ×640. *A*, blood prior to application of shear stress; *B*, blood exposed to shear stress of 1,500 dynes per square centimeter; *C*, blood exposed to shear stress of 2,000 dynes per square centimeter.

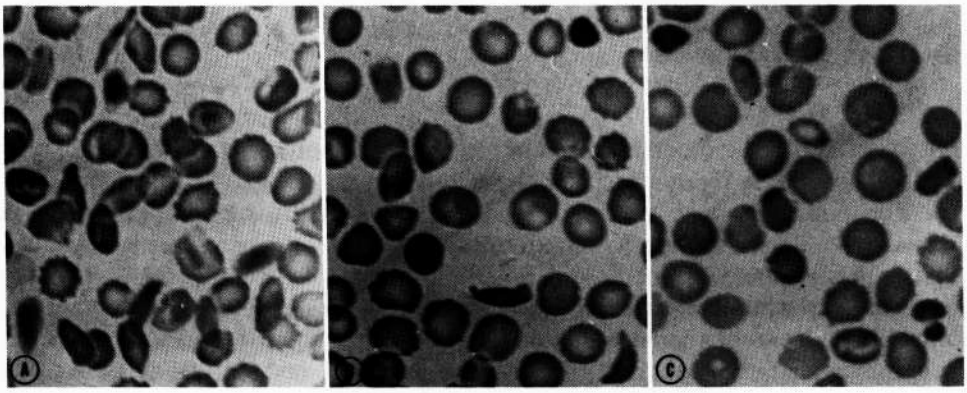

Fig. 5. Peripheral blood film donor with sickle cell anemia, Wright's stain, original magnification, ×640. *A*, blood prior to application of shear stress; *B*, blood exposed to shear stress of 500 dynes per square centimeter; *C*, blood exposed to shear stress of 1,000 dynes per square centimeter.

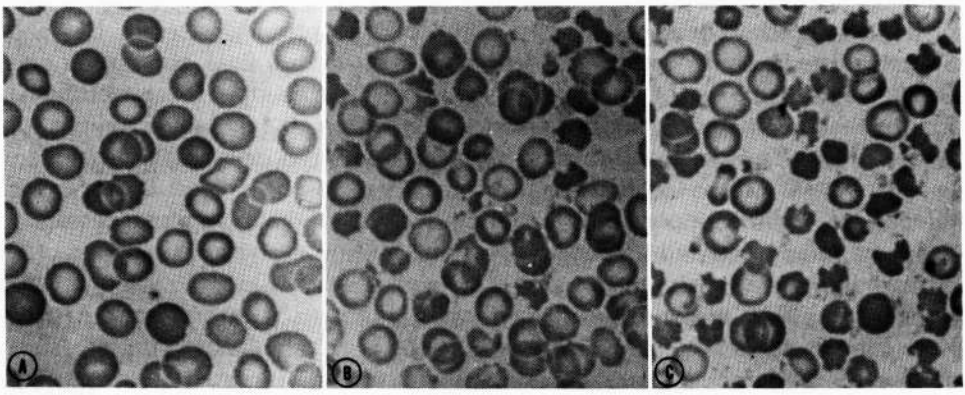

Fig. 6. Peripheral blood films, donor with iron-deficiency anemia. Wright's stain, original magnification, ×640. *A*, blood prior to application of shear stress; *B*, blood exposed to shear stress of 1,500 dynes per square centimeter; *C*, blood exposed to shear stress of 2,000 dynes per square centimeter.

to hemolysis and red cell fragmentation by shear stress in our experiments than all other types of blood studied. Hemolysis and fragmentation were greater in poorly oxygenated than in well oxygenated sickle cell blood, indicating that the presence of hemoglobin in the sickled form is contributory. The amount of hemolysis is much greater at low shear stresses than would be predicted from the number of sickled erythrocytes observed morphologically in blood prior to the application of the shear stress. This suggests that not only sickled cells but morphologically nonsickled cells must be hemolyzed at these low shear stresses. That these nonsickled cells are abnormal finds confirmation in the observation that red cells judged nonsickled by conventional microscopy often have a high percentage of hemoglobin in the sickled state when viewed by high resolution electron microscopy.[10]

Well oxygenated blood from patients with sickle cell anemia is distinctly more likely to hemolyze with shear stress than normal blood. This observation may relate to the presence of irreversibly sickled cells which amount to 11 to 44 per cent of the venous blood in patients with sickle cell anemia.[11]

The two most striking morphologic findings in sickle cell blood subjected to shear stress are the decline in the percentage of sickled cells and the appearance of significant numbers of spherocytes. The fall in number of sickled cells likely is due to their greater susceptibility to damage and destruction by shear stress than their nonsickled cohorts in the blood. Spherocytes probably form as a result of specific damage to the red cell membrane of sickled and nonsickled cells by shear stress resulting in loss of a small amount of membrane and assumption of the spherical form.

Blood samples of patients with iron-deficiency anemia and thalassemia minor are more susceptible to fragmentation and hemolysis with application of shear stress than samples of normal blood. Mild-to-moderate red cell fragmentation is observed in these disorders clinically. Jensen and Lessin[12] have suggested that small fragments develop from cells when the inner surfaces of the membrane of two parts of the cell become fused. The apposed area seals with extrusion of a small fragment. In hypochromic cells, this mechanism may be more likely than with normal erythrocytes because of the diminished content of cytoplasm and the greater deformability of hypochromic cells.

Pyruvate kinase-deficient erythrocytes may be more susceptible to shear stress because these cells have diminished ATP and suffer ion transport abnormalities which lead to cellular dehydration.[13] With the decline in ATP concentration, erythrocytes accumulate membrane calcium and become more rigid.[14] Reticulocytes in pyruvate kinase deficiency have diminished ATP and are uniquely susceptible to hemolysis in vitro.[15,16] The patient whom we studied had a reticulocyte percentage of 24.

Spherocytes are distinctly less deformable than normal erythrocytes[14] and yet are more resistant to hemolysis and fragmentation by shear stress than normal red cells. The relative resistance of the spherocytes may relate to the fact that the maximum dimension of the cell is less than with other shapes and the force exerted on a particular particle in a shear field increases with increasing particle size.

In studies with spherocytes at levels of shear stress resulting in significant hemolysis, we observed very few red cell fragments morphologically. Apparently, the cell either hemolyzes or maintains its integrity completely. With a minimum

surface-to-volume ratio, the spherocyte seems incapable of losing additional membrane and yet maintaining integrity.

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