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RHEOLOGICAL STUDIES IN SICKLE CELL DISEASE
by
GILDA A. BARABINO

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE
DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE:

[Signature]
L. V. McIntire, E.D. Butcher Professor and Chairman
Department of Chemical Engineering
Chairman

[Signature]
J. D. Hellums, Dean of Engineering
George Brown's School of Engineering

[Signature]
C. W. Philpott, Professor of Biology

Houston, Texas
May, 1986
RHEOLOGICAL STUDIES IN SICKLE CELL DISEASE

by

Gilda A. Barabino

ABSTRACT

The abnormal adherence of sickle erythrocytes to endothelial cells (EC) has been hypothesized to play a role in the initiation of vaso-occlusion in sickle cell anemia. Erythrocyte/endothelial cell interactions were studied under controlled flow conditions for normal (AA), homozygous sickle cell (SS), sickle trait (AS), mechanically injured normal, and "high reticulocyte control" red blood cells (RBC). Human umbilical vein endothelial cells grown to confluence on glass slides formed the base of a parallel plate flow chamber into which RBC suspensions were perfused at a constant flow rate, producing a wall shear stress of 1 dyne/cm². Adhesion was monitored using video microscopy, and the number of adherent RBC was determined at ten-minute intervals during a wash-out period.

Results indicate that SS RBC were more adherent than AA RBC. Mechanically injured (sheared) RBC were also more adherent than control normal cells, but less adherent than SS RBC. AS RBC did not differ significantly in their adhesive properties from normal RBC. Less dense (younger) RBC were more adherent to EC than dense (older) cells for normal, SS and "high reticulocyte control" RBC. The average velocity
of individual SS RBC in the region near the EC monolayer was approximately half that of AA RBC at the same bulk volumetric flow rate, as determined using image analysis techniques. The influence of several factors on the adherence of normal and sickle cells to endothelial cells was examined: 1) increasing shear rates resulted in decreased adhesion, 2) pretreating EC with a chemotactic agent had little effect on adherence properties, 3) treating RBC with pentoxifylline diminished the adherence of sickle RBC, but had no effect on AA RBC and 4) suspending RBC in a protein free suspending medium did not affect the demonstration of adherence differences between SS and AA RBC.

These findings suggest that the increased adhesion of sickle RBC is at least partially related to the increased numbers of young RBC present. Increased adherence of young cells to the EC lining vessel walls could contribute to microvascular occlusion by lengthening vascular transit times of other sickle cells. Pentoxifylline may aid in the treatment of sickle cell disease by decreasing these obstructive RBC-EC interactions that may play a role in vaso-occlusion.
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Introduction

As the major component of blood, red blood cells are a critical determinant in the rheologic behavior of blood (1, 2). The red blood cell consists of a thin viscoelastic membrane which encloses an aqueous solution of respiratory pigment called hemoglobin. It is deformable and has a bi-concave shape (8 μm diameter, 1.7 μm thick) in the unstressed state. In sickle cell disease, hemoglobin is altered due to a single nucleotide mutation and amino acid substitution of valine for glutamic acid at the sixth position of each beta chain (3). Sickle hemoglobin (HbS) polymerizes into rigid rods within the cytoplasm in the deoxygenated form causing the red cells to become stiff and less deformable. Most sickle cells regain their normal shape when reoxygenated, but a certain number suffer permanent damage and become irreversibly sickled. Sickle red blood cells play an important role in the aberrant behavior of sickle blood that characterizes the disease.

Intracellular polymerization of HbS leads to an increase in intracellular hemoglobin concentration (MCHC) and viscosity (4). The increased MCHC elicits a decrease in oxygen affinity (5) and decrease in the delay time for sickling (6). Since the rate of HbS polymerization is strongly dependent upon hemoglobin concentration, cells containing the highest concentration of HbS are the most
viscous and are at the greatest risk for sickling in oxygen-poor environments. The rapid destruction of these fragile cells is responsible for the hemolytic component of the disease. There is also a vaso-occlusive component, the occlusion of vessels by plugs of sickled erythrocytes. This obstruction to blood flow could be due to decreased deformability of the cell and its inability to pass through small vessels. If this were the case, it would be reasonable to expect that the most severely deformed sickle cells, the irreversibly sickled RBC (ISC), would play an important role in the initiation of vaso-occlusion. However, the number of circulating indefeasible ISC is not well correlated with the frequency of painful crises and other microvascular occlusive phenomena (7, 8). Recent evidence suggest that microvascular occlusion may be associated with increased adhesion of sickle cells to vascular endothelial cells. A strong correlation between erythrocyte adherence and clinical vaso-occlusive severity has been reported by Hebbel et al. (9).

Hoover et al. (10) and Hebbel et al (11, 12) demonstrated increased adhesion in static tests. The technique used by these investigators consisted of determining the fraction of cells that remain adherent to a confluent layer of cultured endothelial cells after repeated washings. Normal RBC were removed almost completely after a series of washings, whereas a small fraction of sickle RBC remained
adherent. The static conditions of these experiments lack many of the dynamic variables present in vivo such as oxygen tension, flow rate, type of flow and shear forces. All of these variables may affect the propensity of erythrocytes to adhere to the vessel walls. This assay system is also limited in that it is not possible to quantitate the strength of adhesion between the red cell membrane and the endothelial surface. Using a micromechanical technique, Mohandas et al. (13) were able to determine the percentage of red cells that can adhere and to quantitate the strength of adhesion. They found that the vast majority of sickle RBC suspended in autologous plasma were strongly adherent to endothelial cells, whereas only a small fraction of normal cells were weakly adherent. The strength of adhesion was calculated to be \( \approx 2 \times 10^{-6} \) dyne and \( \approx 3 \times 10^{-6} \) dyne for normal and sickle RBC, respectively.

Certain plasma factors have been implicated as modulators of the tendency of SS RBC to adhere to endothelial cells. Hebbel et al. (9) reported that fibrinogen enhanced the adherence of sickle RBC to endothelium. One study has shown that during sickle cell crisis, plasma fibrinogen concentration is elevated (14). Mohandas et al. (15) have suggested that both calcium and collagen-binding plasma proteins play a crucial role in increased adherence of sickle RBC to vascular endothelium. It is likely that membrane changes and plasma factors play an important role in the
adhesive phenomena observed for sickle RBC. A membrane abnormality that has been implicated as being responsible for altered adhesion of SS RBC is an aberrancy of RBC surface charge topography. Hebbel et al. (9) have reported that although SS RBC have normal total sialic acid content (sialic acid being the major contributor to the RBC's negative surface charge (16)), it is distributed abnormally and may induce a propensity for adherence.

Other investigators have attempted to examine adhesion under flow conditions. Using a similar umbilical vein flow model that consisted of umbilical veins perfused with RBC suspensions, Kucukcelebi et al. (17) and Burns et al. (18) attempted to determine whether SS RBC are more adherent to vascular endothelium under flow. Kucukcelebi et al. found that sickle RBC were about ten times more adherent than normal RBC to the umbilical vein lining. On the other hand, Burns et al. reported that SS cells do not adhere to vascular endothelium any more than normal RBC under laminar flow conditions. They did report, however, that sickle RBC were significantly more adherent than normal RBC to EC lined convoluted capillary tubes. These experiments involving perfused umbilical cords have the limitations that the flow and local wall shear rates are not well controlled. In this study, the adhesion process was examined under well controlled fluid mechanical conditions which simulate the magnitude of wall shear stress found in venules.
Impedance to blood flow is the key rheologic determinant of sickle cell disease. The intracellular gelation of sickle hemoglobin appears to be the critical factor in affecting flow through the microcirculation, although other processes probably contribute. The time required for gelation is highly dependent upon the hemoglobin concentration within the cell. Data on the rates of gelation and sickling have been formulated into a kinetic hypothesis of relations between gelation, sickling and microvascular occlusion (6, 19). The hypothesis simply states that when capillary transit time is shorter than sickling time, vaso-occlusion does not occur. Conversely, when capillary transit time is delayed so that it exceeds sickling time, the cell will become rigid within the capillary and initiates vaso-occlusion. Conditions which can retard or impede capillary transit time of individual SS erythrocytes are 1) blockage of the capillary microcirculation at bifurcation points by ISC's thereby hindering the flow of non-ISC's across a given capillary bed causing them to undergo intravascular sickling, 2) capillary edema, inflammation, and the effect of vaso-active substances which may influence the capillary flow rate and substrata so as to prolong transit time, and 3) obstructive interactions of some SS RBC with EC thereby hindering the flow of other non-adherent SS RBC. It is not certain that reversible sickling is responsible for vaso-occlusion, since data suggest that when conditions in the
capillary are those which at equilibrium would cause sickling, microvascular occlusion may or may not occur—depending on the time course of deoxygenation and the time required for sickling after deoxygenation (20, 21). Presumably, under some conditions, the red cells can traverse the capillaries before sickling occurs. Most RBC probably require some impedance to passage through the microvasculature before deoxygenation-induced reversible sickling can occur (3).

There is considerable evidence that red cell membrane damage acquired as a secondary consequence of the hemoglobin abnormality plays an important role in the pathophysiology of sickle cell disease. The membrane damage has been most clearly characterized by ISC formation and altered RBC topography. The process of ISC formation is not well understood, however. It probably begins soon after cell release from the marrow (22) and is thought to occur, in part, from permanent changes in the membrane structure (23). The cell loses part of its membrane by the repetitive process of sickling on deoxygenation and unsickling on reoxygenation, leading to cellular crenation, increased cell density and decreased resistance to osmotic and mechanical stress. ISCs have multiple membrane abnormalities. These abnormalities include increased rigidity and decreased deformability (24) and associated changes in protein organization, particularly the spectrin-actin cytoskeleton (25–27, Figure 1). Some
Figure 1. Erythrocyte membrane alterations in the sickling phenomenon. (From "General Pathology Principles and Dynamics," p. 121, D. W. King, C. M. Fenoglio and J. H. Lefkowitch, eds. Lea and Febiger, Philadelphia, 1983).
possible contributory mechanisms have been noted from in vitro studies: ATP depletion (28) hemoglobin binding to the membrane and RBC fragmentation (29 - 31), Ca++ accumulation (32, 33) and dehydration (4, 34 - 37). ISC membranes are also excessively permeable to cations. Increased calcium, decreased potassium and cell dehydration have been demonstrated in oxygenated sickle cells (4, 36). Cellular dehydration arising from a loss of effective ion regulation causes ISCs to have elevated mean cellular hemoglobin concentration (MCHC) (4). The high MCHC in ISC has been suggested to be an important determinant of their rheologic behavior (24, 38). Changes in the organization of bilayer phospholipids in the membrane have been demonstrated in deoxygenated sickle RBC (39 - 41). Other topographical changes have also been shown by studies of surface antigens (42, 43). A possible result of cumulative changes in the sickle RBC plasma membrane could be an altered interaction of sickle RBC with other cells.

In an attempt to assess the mechanisms whereby differences between the interaction of sickle and normal RBC with endothelial cells arise and the critical alterations in the SS RBC that produce them, different fractions of SS RBC as well as manipulated normal and sickle RBC were studied. Erythrocytes in sickle cell anemia are heterogeneous in both their rheological and hemodynamic characteristics (44). If
differences between fractions in one of these characteristics correlates with differences in behavior in endothelial adhesion, studying AA RBC which have been altered to mimic SS RBC in one or more characteristics will give some insight into the reason for altered adhesion of SS RBC.

Fractionation of SS RBC by density reveals cells of much greater density than normal cells as well as an increased number of low density (or young) cells (23, 45). Thus, density separation is a logical approach to try to identify important abnormalities. Cell density reflects the degree of dehydration of the SS RBC, and many of the abnormalities of the SS RBC, such as lack of deformability, appear to relate closely to its low water content. Furthermore, the ISC, which correlates with RBC survival in sickle cell anemia, if not with other clinical features of the disease, is a markedly dehydrated cell. Fractionation by density serves to a degree to separate out reticulocytes which concentrate in the light fractions. Comparison of SS to normal RBC is complicated by differences in RBC age and numbers of reticulocytes. "High reticulocyte controls" taken from patients with other diseases were also examined along with density-separated cohorts of normal and SS RBC to attempt to identify the influence of cell age (density) on adherence to EC.

Shear stress is a physical force to which erythrocytes in flowing blood are exposed. In vivo shear stresses range
from about 0 - 150 dyne/cm$^2$. Shear stresses can be imparted on cells \textit{in vitro} by shearing them in a concentric cylinder viscometer. Shearing or mechanically injuring normal cells results in their having decreased deformability (46), increased intracellular calcium (36) and membrane damage (47). Sickle RBC, by comparison, have been shown to be characterized by calcium accumulation with decreased cellular deformability (48), membrane injury due to increased hemoglobin binding (49, 50), and decreased deformability due to cell dehydration (24). It is reasonable to examine sheared RBC as a possible model for sickle RBC since perturbing normal RBC by shearing them mimics some of the abnormalities of SS RBC. Sickle RBC, both oxygenated and deoxygenated, have been shown to be more sensitive to mechanical trauma than normal erythrocytes (51). That is, sickle cells hemolyze at stresses which do not cause hemolysis of normal red cells. The ISC population is the most shear sensitive (52). Normal and sickle trait RBC subjected to subhemolytic shear stresses were studied.

Heterozygosity for HbS or sickle trait (HbAS) is an asymptomatic carrier state without hemolysis or clinically significant abnormalities unless the individual is exposed to extremely low oxygen tension. AS RBC change shape and lose their deformability under low oxygen tensions and can even be induced under certain incubation conditions to
become ISC's. These RBC were examined to see if there were any abnormal adherence properties of RBC in the heterozygous form of the disease.

Despite significant gains in the understanding of the molecular pathogenesis of sickle cell anemia, a specific and nontoxic therapy has not yet been found to prevent or treat the clinical manifestations of the disease. At present, medical treatment is usually supportive and symptomatic only, attempting to alleviate pain or combat infections as they occur. Attempts have been made to treat sickle cell disease by modifying the hemoglobin S molecule either directly or indirectly in a manner that will suppress the sickling process. Examples of such anti-sickling agents include urea (53), cyanate (54), procaine (55), zinc (56), and pyridoxine (57, 58). Clinical trials of these agents have proved disappointing. Piracetam is another anti-sickling agent that has been used in the treatment of sickle cell disease (59). This agent reportedly enhances the deformability of SS RBC (60) and causes decreased adherence of sickle erythrocytes to cultured vascular endothelium (61). Pentoxifylline, a methylxanthine, is a drug that has received considerable attention as an agent that causes an improvement in blood flow, presumably, due to its beneficial effect on red cell deformability (62). The effect of pentoxifylline on the adhesion of normal, sickle and sheared RBC was examined.
Interactions between cells and endothelial surfaces in blood vessels occur under dynamic conditions. Cell adhesion is affected by processes connected with the movement of cells and fluid medium with respect to the endothelium. Inhibition or promotion of adhesion may occur depending on factors such as the velocity of cells and medium. Presumably, cells that are moving slower would have a greater chance of making contact and interacting with the endothelial surface. Inward axial migration of red cells in vessels whose diameter is larger than the RBC diameter leaves a cell-poor layer near the vessel wall. Cells, therefore, are transferred from a region of high shear rate and low velocity (close to the wall) to a region of lower shear rate and high velocity (closer to the axis of flow). Altered flow characteristics of sickle blood under both oxygenated and deoxygenated conditions have been reported in microvascular studies using intravital techniques (63, 64). LaCelle et al. (65) reported that at low flow rates, some adherence of sickle cells to endothelium occurred. Klug et al. (63) reported that adherence to the vessel wall did not occur at moderate and high flow rates. The mean transit time of sickle RBC was higher than that for normal RBC during their passage through coronary circulation (66). Since sickle cells have a propensity for interactions with the endothelium lining the vessel walls, it would be interesting to note whether these cells are moving slower or
faster than normal RBC in the region near the endothelial surface due to increased interactions. Using image analysis techniques, the velocities of individual SS and AA RBC were determined.

Sickle cell anemia is a complex genetic blood disorder with altered blood rheology due to the presence of sickle hemoglobin. Two common hematologic manifestations of the disease are recurring painful crises as a result of vaso-occlusion and chronic hemolysis. The severity of expression of both these manifestations varies greatly among different individuals with the disease. Microvascular occlusion may be related to the increased adhesion of sickle red cells to vascular endothelial cells. To evaluate the phenomenon of red cell adhesion to endothelial cells, a rheological technique is used in this study that allows for the visualization of the adhesion process under well controlled flow conditions similar to what would be found in venules. Venules are probably an important site for adhesive events, as the wall shear rates (and therefore fluid drag forces) are quite low. Density separated, mechanically injured (sheared) and drug treated RBC are also examined to aid in identifying characteristics of certain RBC that make them more adherent.
Materials and Methods

Blood was drawn by venipuncture into heparinized tubes from patients with homozygous sickle cell disease, volunteers with sickle cell trait, other patients with high reticulocyte counts ("high reticulocyte controls"), and normal subjects. After centrifugation at 1000 x g for ten minutes, the plasma and buffy coat were removed. The RBC were washed three times and then suspended in tissue culture medium 199 supplemented with 20% fetal calf serum (FCS, Hyclone), 100 U/ml penicillin and 100 mcg/ml streptomycin. All RBC suspension were adjusted to 1% hematocrit by dilution. To determine hematocrits, capillary tubes filled with blood were centrifuged at 14,500 rpm for five minutes in a microhematocrit centrifuge and then read on a microhematocrit reader.

For some experiments, patient erythrocytes and/or normal control RBC were manipulated in various ways as described below:

a) density fractionated RBC: Red cells of varying cell densities were obtained by high speed centrifugation of cells suspended in plasma as described by Murphy (67). Suspensions were centrifuged at 30°C at 30,000 x g for one hour in an angle rotor. After centrifugation, the column of RBC was separated into five equal fractions. The top, middle and bottom 20%, representing the low, middle and high
density fractions, respectively, were isolated. After density separation, the cells were washed and suspended in supplemented medium as described above.

b) sheared RBC: Sheared RBC were obtained by subjecting suspensions of cells to shear stresses ranging from 1000 – 2000 dyne/cm² for two minutes in a concentric cylinder rotational viscometer. Red cell suspensions were sheared at 30% hematocrit and then adjusted to 1% after the shear.

c) Pentoxifylline treated RBC: Pentoxifylline (PTX, Hoechst-Roussel Pharmaceuticals, Inc.) was dissolved in saline at concentrations from 1.0 to 100.0 mmol/L and then added to 1% red cell suspensions at a volume ratio of 1:9, thus, diluting the drug to one tenth of its initial concentration. Control and treated suspensions were incubated for one hour at 37°C.

The effect of formylmethionylleucylphenylalanine (FMLP) on adhesion of RBC to EC was also examined. FMLP, a synthetic tripeptide, has been shown to increase the adhesion of leukocytes to EC. FMLP (Sigma) was dissolved in dimethyl sulfoxide (DMSO) at $10^{-3}$ M and then further diluted in the suspending medium to give a concentration of $10^{-6}$ M. This treated medium was then used in the adherence assay as described below.
Endothelial Cell Cultures

Human umbilical vein endothelial cells were harvested from umbilical cords using culture procedures adapted from those of Gimbrone et al. (68) and grown to confluence on glass slides. To remove the endothelial cells, the veins were cannulated, rinsed with 100 ml phosphate buffered saline (PBS) and then filled with 0.03% collagenase in PBS and incubated for 30 minutes at room temperature. After incubation, the enzyme solution was flushed through the cord with 100 ml PBS, and then the effluent was collected and centrifuged at 100 x g for 10 minutes. The cell pellet was resuspended in medium 199, supplemented with 20% FCS, penicillin (100 units/ml) and streptomycin (100 mcg/ml). Penicillin and streptomycin were added to retard bacteria growth. Cell suspensions were then seeded onto 75mm x 38mm glass slides (Fisher) which had been pretreated with 0.5 M NaOH for 2 - 3 hours and rinsed. Cultures were incubated at 37°C and became confluent after 3 - 4 days. Experiments were performed within four days after cultures reached confluency.

Red Cell-Endothelial Cell Adherence Assay

Adhesion was monitored using video microscopy. Endothelial cell monolayers on glass slides formed the base of a modified Richardson flow chamber (Figure 2) which was mounted in the video microscopy system on the microscope.
Figure 2. Parallel plate flow chamber. Endothelial cells are coated on the glass coverslip which forms one plate of the flow chamber. The plates are held together by a vacuum which is transmitted through holes in the rectangular silastic gasket, forming a flow channel of parallel plate geometry. The height of the flow channel (gap width) is controlled by the thickness of the silastic gasket. RBC suspensions and culture medium entered and exited the channel through two slits machined in the polycarbonate base.
stage (Figure 3). The parallel plate chamber and glass slide are held together by a vacuum maintained at the periphery of the slide, forming a channel of parallel plate geometry. The height of the flow channel (gap width) is controlled by the thickness of the rectangular silastic gasket through which the vacuum is transmitted. For these experiments, the chamber depth was machined to be either 170 μm or 104 μm. RBC suspensions and culture medium enter and exit the channel through two slits machined in the polycarbonate plate.

Once the flow chamber was assembled and the vacuum connected, RBC suspensions at 1% hematocrit were perfused into the chamber for 5 - 10 minutes at a constant flow rate (wall shear stress = 1 dyne/cm²) followed by a 20-minute rinse with cell-free suspending medium at the same flow rate. In some experiments, a ten-minute incubation period without flow preceded the rinse, during which time the RBC were allowed to settle onto the endothelial cell monolayer. Perfusion was achieved with a Harvard syringe pump. The red cell suspensions were maintained in a 37°C water bath and the chamber was maintained at 37°C by an air curtain incubator. The number of adherent RBC was determined at 0, 10 and 20 minutes of rinse for the same single microscope field. After 20 minutes of rinse, multiple microscope fields were examined. The 0-minute count was made following incubation and an one-minute period of rinsing to remove
Figure 3. Schematic of video microscopy system. Flow is controlled using the Harvard syringe pump. For chambers used in these experiments, the range of wall shear rates possible is 0 - 4000 sec\(^{-1}\) covering most of the range of physiological interest.
non-adherent settled cells. In the continuous flow experiments where the incubation period was eliminated, the rinse immediately followed the RBC perfusion and the number of adherent RBC was determined at 10 and 20 minutes for the same microscope field.

In experiments examining the effect of FMLP on adhesion, prior to the RBC perfusion, medium treated with FMLP was perfused into the flow chamber for 12 minutes followed by a 10-minute rinse with untreated medium. The rinse with untreated medium was necessary to remove any unbound FMLP, assuming FMLP would bind to EC receptor sites until a saturation point was reached. The number of adherent RBC was then determined as described above.

A video camera (RCA TC 1005) mounted to the microscope (Nikon, Diaphot-TMD) was used to record and display experiments. Experiments were recorded on video tape (Gyrry TLC 2001) for later playback and analysis and displayed on a high resolution TV monitor. Tapes were analyzed using image analysis techniques.

For parallel plate geometry where \( z \) is the flow direction and \( x \) the coordinate normal to the flat plate, with the origin at the centerline, at low Reynolds number, the velocity profile is given by

\[
V_z = \frac{3}{2} U_0 \left( 1 - \left( \frac{x}{a} \right)^2 \right).
\]
The wall shear stress, \( \tau \), can be calculated from the momentum balance for a Newtonian fluid, assuming fully developed laminar flow, i.e.

\[
\tau = \mu \frac{dV_z}{dx} \bigg|_{x=a} = \frac{3U_0 \mu}{a} = \frac{3 \mu Q}{2a b} \quad \text{dynes/cm}^2
\]

where

- \( a \) = half height of the chamber, cm
- \( b \) = channel width, cm
- \( U_0 \) = average velocity, cm/sec
- \( Q \) = volumetric flow rate = \( U_0 \cdot 2ab \), cm\(^3\)/sec
- \( \mu \) = fluid viscosity, dynes sec/cm\(^2\)

\[
\frac{dV_z}{dx} \bigg|_{x=a} = \text{wall shear rate, s}^{-1}
\]

Thus, given the volumetric flow rate, chamber dimensions, and the fluid velocity, one can determine the wall shear stress, and therefore, the hydrodynamic forces exerted on attached cells.

**Velocity Determination**

Using a microcomputer for digital image processing, velocity determinations were made from taped experiments. Individual red blood cells were traced by noting their
position in two consecutive digitized frames separated by a given delay time (Figure 4). The velocity in pixels/sec was determined from the number of pixels (picture elements into which a digitized frame is divided) between the two frames and the delay time. All velocities were converted to microns per second after calibrating on the microcomputer.

Statistical Analysis

Student's t-test was used to compare the results and to determine the statistical significance.

Viscometry

The concentric cylinder viscometer used in this work was designed and built at Rice University. It is made so that the other cylinder (cup) rotates about the stationary inner cylinder (bob). The sample is enclosed in the angular region between the concentric cylinder by a cone-and-plate region below and a cone-and-cone region above the concentric cylinder region (Figure 5). Dimensions were chosen such that a uniform shear stress is applied throughout the sample. The cup radii are about 3.5 cm. Shear stress is calculated from the torque applied to the bob. Torque is measured by the slight rotation of the inner bob against a calibrated spring; the rotation is measured with an angular displacement transducer. Shear rate is determined from the angular velocity of the rotating cup and the dimensions of
Figure 4. Velocity determination using image analysis techniques. Using videotapes of experiments, the distance travelled by a red cell after a period of one second is determined by marking the cell's position in one frame and then marking its position 30 frames later (30 video frames per second).
Figure 5. Schematic of the Rice University viscometer. Note cone and plate section at the bottom, concentric cylinder section in the middle, and cone and cone section on the top. The entire sample is exposed to the same shear stress, with no regions of stasis.
the cup and bob. A detailed description of the viscometer is given elsewhere (69). The sample volume is about 4 ml.

Digital Image Analysis

Analysis of taped experiments was performed using a digital image processor (Perceptive Systems Inc., Model 327). The system consisted of a LS1-11/23 16-bit high speed, high performance microcomputer designed for image processing and a RT11 operating system which is integrated with ITI (Image Technology Inc.) IP-512 image processing boards.
Results

When analyzing videotapes of these experiments, one can distinguish three types of movement for the red blood cells: (1) tumbling end-over-end of red cells high in the flow field, (2) sliding of red cells along the endothelial cell monolayer, and (3) stationary (adherent) red cells that have made contact and formed a "stable" bond with the endothelial cells. Red cells will adhere for a long period of time (throughout the course of the experiment) or adhere momentarily and then detach and move downstream. Thus, the adherent RBC population is dynamic in that sense that new RBC make contact and become adherent as other RBC, already adhering, detach and rejoin the flow. In general, the passage of sickle cells across the endothelial cells appeared to be slower than that of the normal cells at a flow rate corresponding to a wall shear rate of \( \sim 1 \text{ dyne/cm}^2 \). A still photograph (Figure 6) taken during the rinsing phase of a typical experiment with sickle RBC illustrates the type of data obtained.

The first set of experiments were conducted using a chamber with a 170 \( \mu \text{m} \) gap width (i.e. flow channel height = 170 \( \mu \text{m} \)) and included a ten minute static incubation period (Table 1). When examined under these conditions, sickle RBC were more adherent to endothelial cells than normal RBC. From microscopic observation, it appears that all of the RBC
Figure 6. Sickle cells adhering to endothelial cell monolayer.
TABLE 1

Adhesion of Normal, Sickle, Sickle Trait and Sheared Red Cells to Endothelial Cells

Adherent RBC/mm² at Rinse Times

<table>
<thead>
<tr>
<th>RBC</th>
<th>0 Min</th>
<th>10 Min</th>
<th>20 Min</th>
<th>Multiple Fields at 20 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (n = 19)</td>
<td>24.78 ± 16.46</td>
<td>10.96 ± 8.81</td>
<td>8.11 ± 5.28</td>
<td>6.62 ± 6.10 (f=284)</td>
</tr>
<tr>
<td>SS (n = 13)</td>
<td>165.70 ± 142.62</td>
<td>72.43 ± 67.24</td>
<td>52.56 ± 44.08</td>
<td>39.32 ± 29.72 (f=211)</td>
</tr>
<tr>
<td>AS (n = 2)</td>
<td>10.41</td>
<td>10.41</td>
<td>8.33</td>
<td>9.25 ± 8.25 (f= 32)</td>
</tr>
<tr>
<td>AA Sheared (n = 10)</td>
<td>40.83 ± 11.42</td>
<td>21.25 ± 7.96</td>
<td>17.08 ± 8.66</td>
<td>22.25 ± 13.46 (f=165)</td>
</tr>
<tr>
<td>AS Sheared (n = 2)</td>
<td>29.16</td>
<td>16.66</td>
<td>16.66</td>
<td>17.16 ± 9.04 (f= 32)</td>
</tr>
</tbody>
</table>

In these experiments, there was a 10-minute incubation period before the rinse was begun. The flow chamber gap width was 170 μm. The data at 0, 10, and 20 minute rinse represent the means of single fields counted in n experiments. The data in the far right column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse. f = total fields in all n experiments. The differences between the AA controls and the SS, AA sheared, and AS sheared RBC had a level of significance of p<.01 (Student's t-test) for all times given. There was no significant difference between the adhesion of AA and AS RBC.
entering the flow chamber settle to form a monolayer over the endothelial cell surface during the ten-minute incubation period. In addition, the number of RBC calculated per chamber volume for a 1% hematocrit suspension is approximately equal to the number of RBC needed to form a monolayer. From these calculations, the percent of red cells contacting endothelial cells that remained adherent following one minute of rinsing was determined. A larger fraction of settled RBC remained adherent for SS RBC (1%) than for AA RBC (0.16%). RBC from individuals with sickle cell trait did not differ significantly from normal in their adhesion to endothelial cells. Mechanically traumatized (sheared) RBC, both normal and sickle trait, were also more adherent than normal RBC, but not as adherent as sickle RBC. The percentages of settled RBC which remained adherent following one minute of rinsing for AS, sheared AA, and sheared AS RBC were 0.07%, 0.26% and 0.19%, respectively. The differences between the AA controls and the SS, AA sheared and AS sheared RBC had a level of significance of p <.01 for all times given in the table. Results from experiments performed using RBC from a splenectomized patient with AA hemoglobin indicated that these RBC behaved like normal cells with respect to adhesion to endothelial cells (Table 2).

Increased adhesion of sickle, sheared, and "high reticulocyte control" RBC was demonstrated using a chamber with a
TABLE 2

Adhesion of Normal Red Cells and Red Cells from a Splenectomized Patient to Endothelial Cells

Adherent RBC/mm² at Rinse Times

<table>
<thead>
<tr>
<th>RBC</th>
<th>0 Min</th>
<th>10 Min</th>
<th>20 Min</th>
<th>Multiple Fields at 20 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>16.67</td>
<td>8.33</td>
<td>8.33</td>
<td>10.42 ± 6.92 (f = 16)</td>
</tr>
<tr>
<td>AA (Splenectomized)</td>
<td>12.50</td>
<td>8.33</td>
<td>4.17</td>
<td>9.08 ± 3.83 (f = 16)</td>
</tr>
</tbody>
</table>

In these experiments, there was a 10-minute incubation period before the rinse was begun. The flow chamber gap width was 170 μm. The data at 0, 10, and 20-minute rinse represent the means of single fields counted in n experiments. The data in the far right column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse. f = total fields in all n experiments. There was no significant difference between the adhesion of AA RBC from a normal subject and AA RBC from a splenectomized patient.
smaller gap width (104 μm) in experiments that included a ten-minute static incubation period (Table 3). AA (high retic) RBC were obtained from two donors, a patient responding to treatment for iron deficiency anemia and a patient with hereditary spherocytosis. The latter was studied on two occasions. The differences between the AA controls and the SS, AA sheared and AA (high retic) RBC had a level of significance of $p < .005$ for multiple fields at 20 minutes, while the differences between SS and AA (high retic) RBC had a level of significance of $p < .0005$.

Experiments on density-separated sickle, normal and "high reticulocyte control" RBC revealed that the least dense fraction (fraction with the highest percentage of reticulocytes) was more adherent than the denser fractions and the unfractionated sample. The data for a representative experiment for each RBC type is given in Table 4. AA (high retic) RBC were obtained from a patient responding to treatment for iron deficiency anemia. The "high reticulocyte control" RBC, although not as adherent as sickle RBC, were more adherent than normal, suggesting that the degree of adhesion may be partially related to the percentage of reticulocytes in the cell population. These experiments were performed using a chamber with a 104 μm gap width and included a ten-minute incubation period.

The same differences in adhesion between AA, SS, AA sheared and AA (high retic) RBC were noted when the
<table>
<thead>
<tr>
<th>RBC</th>
<th>10 Min</th>
<th>20 Min</th>
<th>Multiple Fields at 20 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (n = 3)</td>
<td>11.13 ± 5.21</td>
<td>9.71 ± 3.92</td>
<td>6.88 ± 4.03 (f = 48)</td>
</tr>
<tr>
<td>SS (n = 1)</td>
<td>141.67</td>
<td>95.83</td>
<td>86.17 ± 36.96 (f = 16)</td>
</tr>
<tr>
<td>AA (Sheared (n = 1)</td>
<td>41.67</td>
<td>41.67</td>
<td>23.38 ± 13.25 (f = 16)</td>
</tr>
<tr>
<td>AA (High Retic)* (n = 3)</td>
<td>130.54 ± 33.29</td>
<td>90.29 ± 25.75</td>
<td>48.08 ± 22.95 (f = 48)</td>
</tr>
</tbody>
</table>

In these experiments, there was a 10-minute incubation period before the rinse was begun. The flow chamber gap width was 104 μm. The data at 10 and 20-minute rinse represent the means of single fields counted in n experiments. The data in the far right column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse. f = total fields in all n experiments. The differences between the AA controls and the SS, AA sheared, and AA (high retic) RBC had level of significance of p < .0005 (Student's t-test) for multiple fields at 20 minutes while the differences between the SS and AA (high retic) RBC had a level of significance of p < .0005.

* AA (high retic) RBC were obtained from two donors, a patient responding to treatment for iron deficiency and a patient with hereditary spherocytosis. The latter was studied on two occasions.
**TABLE 4**

Effect of Cell Density on Normal and Sickle Cell Adherence to Endothelial Cells

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>AA (High Retic)</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%ISC</td>
<td>%Retic</td>
<td>Adherent RBC*</td>
</tr>
<tr>
<td>Whole</td>
<td>16.9</td>
<td>16.1</td>
<td>87.75 ± 17.88</td>
</tr>
<tr>
<td>Top</td>
<td>9.6</td>
<td>33.2</td>
<td>92.42 ± 31.58</td>
</tr>
<tr>
<td>Middle</td>
<td>22.6</td>
<td>6.7</td>
<td>52.63 ± 11.67</td>
</tr>
<tr>
<td>Bottom</td>
<td>28.6</td>
<td>8.7</td>
<td>51.29 ± 13.29</td>
</tr>
</tbody>
</table>

* Average number of adherent RBC ± standard deviation per microscope field (16 fields counted after 20-minute rinse).

In these experiments, there was a 10-minute static incubation period before the rinse was begun. The flow chamber gap width was 104 µm. The data are from a representative experiment for each RBC type. AA (high retic) RBC were obtained from an iron deficient patient responding to treatment. The differences between the SS and AA (high retic) RBC had a level of significance of p < .0005 (Student's t-test) for whole blood samples. For fractionated samples, there was no significant difference between the adhesion of middle and bottom cells, but the differences between the top and middle and the top and bottom cells had a level of significance of p < .0005 for SS and AA (high retic) RBC.
ten-minute incubation period was eliminated so that the entire experiment was conducted under flow conditions, using the chamber with the narrower gap width (104 μm). Under conditions of continuous flow, a smaller gap width minimizes the effects of inward radial migration and wall exclusion of RBC which occur in flow in large channels and result in a cell-poor layer along the channel walls. This effect diminishes as channel size decreases, thereby reducing the total flow area and increasing the potential for RBC-EC interactions. The flow rate was adjusted for the smaller gap width to maintain a wall shear stress of 1 dyne/cm². Data for these continuous flow experiments is given in Table 5. AA (high retic) RBC were obtained from the same two donors mentioned previously. The differences in adhesion between the AA controls and the SS, AA sheared and AA (high retic) RBC had a level of significance of p < .0005 for multiple fields at 20 minutes.

The remaining experiments described were all performed using a chamber with a 104 μm gap width under conditions of continuous flow (no incubation period) unless otherwise indicated.

**Effect of Pentoxifylline on Adhesion**

The adherence of sickle, but not normal RBC was significantly diminished by treatment with pentoxifylline. Adherence of sickle RBC treated with 0.1, 1.0 and 10.0 mmol/L of
Table 5

Adhesion of Normal, Sickle and Sheared Red Cells to Endothelial Cells
Under Conditions Of Continuous Flow

Adherent RBC at Rinse Times

<table>
<thead>
<tr>
<th>RBC</th>
<th>10 Min</th>
<th>20 Min</th>
<th>Multiple Fields at 20 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (n = 11)</td>
<td>14.77 ± 6.49</td>
<td>11.36 ± 8.16</td>
<td>11.48 ± 6.83 (f = 176)</td>
</tr>
<tr>
<td>SS (n = 11)</td>
<td>60.98 ± 22.56</td>
<td>59.09 ± 35.08</td>
<td>45.12 ± 26.76 (f = 164)</td>
</tr>
<tr>
<td>AA Sheared (n = 6)</td>
<td>22.92 ± 9.85</td>
<td>20.14 ± 6.55</td>
<td>20.14 ± 7.80 (f = 95)</td>
</tr>
<tr>
<td>AA (High Retic)* (n = 5)</td>
<td>80.00 ± 32.64</td>
<td>50.00 ± 18.82</td>
<td>43.71 ± 23.59 (f = 76)</td>
</tr>
</tbody>
</table>

In these experiments, there was no static incubation period before the rinse was begun. The flow chamber gap width was 104 μm. The data at 10 and 20-minute rinse represent the means of single fields counted in n experiments. The data in the far column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse. f = total fields in all n experiments. The differences between the AA control and the SS, AA sheared and AA (high retic) RBC had a level of significance of p <.0005 (Student's t-test) for multiple fields at 20 minutes.

*AA (high retic) RBC were obtained from two donors, a patient responding to treatment for iron deficiency anemia and a patient with hereditary spherocytosis.
pentoxifylline was reduced to about 65%, 53% and 39% of that for the untreated control respectively (Table 6). Sheared RBC were also affected by treatment with pentoxifylline. Adherence of sheared RBC treated with 0.1 and 1.0 mmol/L of pentoxifylline was reduced to about 80% and 49% of that for the untreated control respectively (Table 7). There was no significant difference between the adherence of treated and untreated normal RBC (Table 8). The differences between the untreated controls and the treated RBC had a level of significance of p < .005 for sickle RBC for each drug concentration. The differences between the untreated and treated sheared RBC were significant at 1.0 mmol/L concentration.

Effect of Shear Rate on Adhesion

To determine the dependence of normal and sickle RBC adherence to EC on wall shear rate, experiments were performed at various shear rates ranging from 25s\(^{-1}\) to 500s\(^{-1}\). The change in adherence as shear rate increases is plotted in Figure 7. For both normal and sickle RBC, adherence decreases as shear rate increases. Very little, if any, adhesion is noted at shear rates 167s\(^{-1}\) for normal RBC and at shear rates 252s\(^{-1}\) for sickle RBC. The difference between normal and sickle cell behavior with respect to adhesion appears to be more pronounced at the lower shear rates.
TABLE 6

Effect of Pentoxifylline on Adhesion of Sickle RBC
(n = 5)

<table>
<thead>
<tr>
<th>Pentoxifylline Concentration</th>
<th>10 Min</th>
<th>20 Min</th>
<th>Multiple Fields at 20 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM</td>
<td>95.00 ± 52.47</td>
<td>83.34 ± 49.23</td>
<td>60.42 ± 32.41 (f = 80)</td>
</tr>
<tr>
<td>0.1mM</td>
<td>55.00 ± 26.14</td>
<td>44.17 ± 23.36</td>
<td>39.20 ± 19.90 (f = 76)</td>
</tr>
<tr>
<td>1.0mM</td>
<td>60.42 ± 33.66</td>
<td>42.50 ± 20.48</td>
<td>31.93 ± 14.45 (f = 80)</td>
</tr>
<tr>
<td>10.0mM</td>
<td>54.17 ± 35.94</td>
<td>41.67 ± 40.99</td>
<td>23.85 ± 15.14 (f = 80)</td>
</tr>
</tbody>
</table>

The data at 10 and 20-minute rinse represent the means of single fields counted in n experiments. The data in the far column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse. f = total fields in all n experiments.
### TABLE 7

Effect of Pentoxifylline on Adhesion of Sheared RBC

(n = 1)

Adherent RBC/mm² at Rinse Times

<table>
<thead>
<tr>
<th>Pentoxifylline Concentration</th>
<th>10 Min</th>
<th>20 Min</th>
<th>Multiple Fields at 20 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM</td>
<td>20.83</td>
<td>12.50</td>
<td>11.71 ± 7.54 (f = 16)</td>
</tr>
<tr>
<td>0.1mM</td>
<td>8.33</td>
<td>8.33</td>
<td>9.88 ± 1.92 (f = 16)</td>
</tr>
<tr>
<td>1.0mM</td>
<td>8.33</td>
<td>4.17</td>
<td>5.75 ± 4.13 (f = 16)</td>
</tr>
</tbody>
</table>

The data at 10 and 20-minute rinse represent the means of single fields counted in n experiments. The data in the far column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse. f = total fields in all n experiments.
## TABLE 8

Effect of Pentoxifylline on Adhesion of Normal RBC
(n = 3)

Adherent RBC/mm²

<table>
<thead>
<tr>
<th>Pentoxifylline Concentration</th>
<th>Multiple Fields at 20 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM</td>
<td>4.25 ± 3.12 (f = 48)</td>
</tr>
<tr>
<td>0.1mM</td>
<td>4.69 ± 3.67 (f = 48)</td>
</tr>
<tr>
<td>1.0mM</td>
<td>5.21 ± 3.24 (f = 48)</td>
</tr>
<tr>
<td>10.0mM</td>
<td>5.03 ± 3.50 (f = 48)</td>
</tr>
</tbody>
</table>

The data in the far column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse. f = total fields in all n experiments.
Effect of Shear Rate on Adhesion for Normal and Sickle RBC

Figure 7. Effect of shear rate on adherence of sickle and normal RBC to endothelial cells.
Effect of FMLP on Adhesion

When endothelial cell cultures were pretreated with FMLP at a concentration of $10^{-6}$M prior to perfusion of red blood cell suspensions, the adherence of sickle RBC was unaffected, while the adherence of normal RBC was slightly increased (Table 9). The differences in adhesion of normal RBC to untreated EC and EC pretreated with FMLP had a level of significance of $p < .005$. There was no significant difference between the adherence of sickle RBC to untreated EC and EC pretreated with FMLP ($p < .01$).

Effect of Suspending Medium on Adhesion

To determine if adherence of sickle or normal RBC to EC could be demonstrated in the absence of proteins found in serum and plasma, an experiment was performed with RBC suspended in tissue culture medium and RBC suspended in phosphate buffered saline (PBS). The results are given in Table 10. The number of adherent sickle RBC remaining adherent at the end of the 20-minute rinse period for sickle RBC in medium did not differ significantly from the number of adherent RBC for SS RBC suspended in PBS. In the case of normal RBC, RBC suspended in PBS were slightly more adherent than RBC suspended in medium. There were no significant variations in RBC indices such as mean cell volume (MCV), mean cellular hemoglobin concentration (MCHC) and mean cell
<table>
<thead>
<tr>
<th>RBC</th>
<th>Pretreatment of EC with FMLP</th>
<th>Multiple Fields at 20 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (n = 2)</td>
<td>no</td>
<td>15.50 ± 5.13 (f = 32)</td>
</tr>
<tr>
<td>AA (n = 2)</td>
<td>yes</td>
<td>23.83 ± 8.92 (f = 32)</td>
</tr>
<tr>
<td>SS (n = 4)</td>
<td>no</td>
<td>65.83 ± 26.96 (f = 64)</td>
</tr>
<tr>
<td>SS (n = 3)</td>
<td>yes</td>
<td>62.50 ± 32.79 (f = 48)</td>
</tr>
</tbody>
</table>

The data in the far column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse. f = total fields in all n experiments.
TABLE 10
Effect of Suspending Medium on Normal and Sickle Cell Adherence to Endothelial Cells

<table>
<thead>
<tr>
<th>RBC</th>
<th>Suspending Medium</th>
<th>Multiple Fields at 20 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>t.c. medium</td>
<td>39.33 ± 15.38 (f = 16)</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>41.92 ± 19.13 (f = 16)</td>
</tr>
<tr>
<td>AA</td>
<td>t.c. medium</td>
<td>14.08 ± 5.83 (f = 16)</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>21.96 ± 9.54 (f = 15)</td>
</tr>
</tbody>
</table>

The data at 10 and 20-minute rinse represent the means of single fields counted in n experiments. The data in the far column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse. f = total fields in all experiments.

t.c. medium = tissue culture medium.
hemoglobin (MCH) for sickle RBC suspended in PBS as opposed to being suspended in medium (Table 11).

**Velocity Determination Using Image Analysis Techniques**

Velocities of individual sickle RBC for a given frame at a given flow rate are plotted in Figures 8 and 9 in terms of the percent of total RBC traveling at a particular velocity. At each shear rate, it is apparent that a higher proportion of normal RBC are traveling at the higher velocities. Sickle cells adhere significantly more than normal RBC and move more slowly across the endothelial cell surface. The average velocity of all of the cells in a given frame is usually twice as high for normal RBC as it is for sickle RBC at the same volumetric flow rate (Table 12).
<table>
<thead>
<tr>
<th>Sickle Red Blood Cell Indices in Various Suspending Mediums</th>
<th>Mean Cell Volume (fl)</th>
<th>Mean Cell Hemoglobin Concentration (mg/ml)</th>
<th>Mean Cell Hemoglobin (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspending Medium</td>
<td>t.c. medium</td>
<td>t.c. medium</td>
<td></td>
</tr>
<tr>
<td>plasma</td>
<td>90.4</td>
<td>361</td>
<td>32.6</td>
</tr>
<tr>
<td>PBS</td>
<td>94.8</td>
<td>349</td>
<td>33.0</td>
</tr>
<tr>
<td>t.c. medium</td>
<td>92.4</td>
<td>367</td>
<td>33.9</td>
</tr>
</tbody>
</table>

t.c. medium = tissue culture medium.
Figure 8. Velocity distribution for sickle and normal RBC for a shear rate of 126 sec\(^{-1}\).
Figure 9. Velocity distribution for sickle and normal RBC for a shear rate of 50 sec$^{-1}$. 
### TABLE 12
Average Velocities of Sickle and Normal RBC

<table>
<thead>
<tr>
<th>Shear Rate (sec(^{-1}))</th>
<th>RBC</th>
<th>Frame</th>
<th>Average Velocity * (microns/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>SS</td>
<td>1</td>
<td>7.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>11.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>23.75</td>
</tr>
<tr>
<td>126</td>
<td>SS</td>
<td>1</td>
<td>77.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>43.33</td>
</tr>
<tr>
<td>50</td>
<td>AA</td>
<td>1</td>
<td>41.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>40.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>47.50</td>
</tr>
<tr>
<td>126</td>
<td>AA</td>
<td>1</td>
<td>138.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>129.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>96.84</td>
</tr>
</tbody>
</table>

* Average of the velocities of all of the moving cells in a given frame of analysis.
Discussion

The objective of this study was to utilize rheological techniques to understand the biophysical basis of the pathophysiology of sickle cell disease. Specifically, this study examined erythrocyte/endothelial cell interactions under controlled flow conditions using video microscopy and image analysis techniques. This system has advantages over static systems in that the use of a parallel plate flow chamber with a video microscopy system allows a known uniform level of wall shear stress to be applied and provides direct microscopic visualization of the adhesion process under shear flow conditions approximating those found in vivo. In addition, rheologic data can easily be interpreted by considering the case of laminar flow between two parallel surfaces (simplified model of a blood vessel).

Sickle cells were more adherent to endothelial cells than normal RBC, both with and without a period of static incubation before the rinse period. The results from the static experiments support the work done by Hebbel et al. (11, 12) and Mohandas et al. (13). Hebbel demonstrated a marked increase in the adhesion of sickle cells to EC in static tests. He and his co-workers determined the fraction of red cells remaining adherent to endothelial cell culture plates after successive washings. Their assay system did not account for flow effects on the adhesion process or
for quantification of adhesive forces. Using a micropipette technique with single cells, Mohandas et al. (13) demonstrated enhanced adhesion of sickle cells to endothelial cells and quantitated the strength of adhesion. Our finding of increased adherence of sickle cells to EC under flow conditions without a period of static incubation is in agreement with the report by Kucukcelebi et al. (17). On the other hand, Wilkinson et al. (70) and Burns et al. (18) were unable to demonstrate significant differences between adherence of sickle and normal RBC in perfused human umbilical cords. The latter investigators did, however, report that sickle RBC were more adherent than normal cells to endothelial cell-coated capillary tubes constructed to have multiple binds. The discrepancy between the observation that SS RBC adhere more to EC under flow conditions and the find of Burns et al. that they don't, can be explained by differences in methods and in the measurement of cell adhesion. The adherence of RBC to EC is transient in that some RBC adhere only momentarily while others remain adherent for a longer period of time—the population of adherent RBC is, therefore, constantly changing. It may be that this type of adhesion is undetected in the procedure used by Burns et al. where adhesion is monitored only at the end of the experiment, after a 10 minute washout period, by means of a differential cell count. In addition, there is a progressive washout of red cells during the rinse period indicated by
Burns et al. and shown in the experiments reported in this study. Perhaps the 10 minute rinse period used in the Burns experiments is too long to detect differences in adhesion of SS and AA RBC in their particular system.

Comparison of SS to normal RBC is complicated by differences in RBC age and number of reticulocytes. Therefore, density-separated cohorts of normal and SS RBC were examined to attempt to identify the influence of cell age (density) on adherence to EC. RBC from non-SS patients with high reticulocyte counts were also studied. In all cases, less dense (young) RBC cohorts containing more reticulocytes were more adherent. Dense fractions of SS RBC, which contained most of the ISC, were less adherent. These results for fractionated RBC are in contrast with those obtained by Hebbel et al. (11) who found that adhesion increased with increased SS RBC density, namely that ISC were more adherent. This difference may be explained by the differences in the test systems. In Hebbel's static system where RBC are layered onto EC sufficient contact area is available for interactions between the two cell types despite the irregular geometry of the ISC. In a flowing systems such as the one utilized here, which involves only shearing motion, the area of ISC/EC interaction may be small due to the irregular shape of the cells and lack of any normal force to generate increased surface contact. The hydrodynamic force exerted on the ISC due to flow may actually remove it from the
endothelial surface. A recent report by Mohandas et al. (15) concurs with the findings in this study that the rigid ISC are the least adherent. Using their micropipette system, they found that the detachment of sickle RBC from EC involved peeling away large areas of red cell membrane from the endothelial surface. The force of adhesion, in this case, depends upon the area of contact and is measured by the amount of pressure required for detachment. As cells age, they appear to lose some property that plays a significant role in the adhesion process observed in these experiments. Though sialic acid, the main determinant of RBC surface charge, decreases with cell age, the surface charge density remains constant during the aging (71, 72). Thus, sialic acid loss does not readily explain the changes with cell aging. It is still possible, however, that membrane positioning of charges could play a role in the effects of RBC aging. Aging cells lose both membrane surface area and cell volume. This could help explain why surface charge density remains constant even though the main charge bearing moiety decreases.

The increased adhesion of less dense normal and "high reticulocyte control" RBC suggests that the increased adhesive properties of SS RBC may be, at least in part, a reflection of their young mean age. However, other factors may be involved. Mechanical damage of the red cell can also lead to increased RBC/EC adhesion as shown by results
obtained with sheared normal RBC. Several investigators have shown that sickle RBC are much more susceptible to shear damage than normal cells (51, 52) and that hemolysis can occur at stress levels that are obtained physiologically. Sublytic mechanical damage to sickle cells in the circulation may also predispose them to increased EC adherence. Recent evidence reported by Rodgers et al. (73) suggest that microcirculatory flow in patients with sickle cell disease is periodic. Using a laser-Doppler technique to measure blood flow, these authors found that blood flow in the forearm skin of sickle cell patients exhibited oscillations with a period of 7 to 10 seconds, that were not seen in normal subjects. Periodic increases in shear stress due to oscillatory flow may be detrimental to cells that are susceptible to shear stress, but could also facilitate the passage of red cells through small vessel by helping them to deform. Furthermore, oscillatory flow may be a mechanism by which adherent red cells may become dislodged from the endothelium.

Exposure of normal RBC to subhemolytic shear stresses is known to induce a deformability defect and lead to calcium accumulation without alterations in morphology (46, 74, 75). After sublytic shear stress exposure, both AA and AS RBC were more adherent to EC than unsheared RBC. Sickle RBC are relatively rich in calcium (76) and suffer dramatic decreases in RBC deformability. Accumulation of calcium by
normal erythrocytes leads to changes in cellular hydration and deformability very similar to those that characterize ISC's. Thus, further study of stressed AS and AS RBC may aid in determining the characteristics of the SS RBC that make them more adherent.

The influence of plasma factors on adhesion has been of interest. Hebbel and his co-workers have suggested that fibrinogen enhances SS RBC adherence (12) and Mohandas et al. have reported that both collagen-binding plasma proteins and calcium play a role in the increased adherence of SS RBC to vascular endothelium (15). Results obtained in this study indicate that adhesion differences between red cells can be demonstrated in the absence of specific human plasma factors peculiar to SS plasma, as these experiments involved washed red cells and a tissue culture suspending medium. In addition, in an experiment involving SS and AA RBC suspended in phosphate buffered saline, a significant increase in adhesion of SS RBC over that for AA RBC was demonstrated. Specific SS plasma factors may play a modulating role in the adhesion process, but were not necessary to demonstrate altered adhesion in sickle RBC in this system.

A chemical modulator that has been shown to greatly increase polymorphonuclear neutrophil leukocyte (PMNL)-endothelial cell interactions is the synthetic peptide N-formylmethionylleucine phenylalanine (FMLP) (77). Adherence of untreated PMNL can be greatly increased by the pretreatment
of endothelial cells with FMLP—indicating an active role of the endothelial cell (which has FMLP receptors) in this interaction. In sickle cell disease, bacterial infections frequently trigger the onset of painful crises (78). To model the effect of bacterial infection on RBC/endothelial cell adhesion, the peptide FMLP, a bacterial toxin analog was used to simulate the bacterial release of toxins during infection. Endothelial cells were pretreated with FMLP prior to the perfusion of untreated SS and AA RBC into the flow chamber. FMLP did not have a significant effect on the adhesion of SS RBC and only slightly increased the adhesion of AA RBC. These findings do not suggest a role for infection in modulating sickle cell-endothelial cell adherence.

Although pentoxifylline (PTX) had no significant effect on the adhesion of normal RBC to endothelial cells, it did cause a significant decrease in the adherence of sickle RBC which was concentration dependent. Treatment with PTX also resulted in decreased adherence of sheared RBC which have several characteristics in common with sickle RBC as previously discussed. The mechanism by which pentoxifylline effects the adhesion of SS RBC to EC is not yet known. Since the endothelial cells were not exposed to PTX prior to interaction with the RBC in the flow chamber, it is not likely that the adherence of SS RBC observed in this study is due to a change in the endothelial cells. However, further experiments will be necessary to explore the role of
endothelial effects of PTX in this system. Some altered properties of sickle cells that make them more adhesive may be affected by pentoxifylline. The least dense (reticulocyte-rich) fraction of sickle cells was found to be more adherent than older cells in this study. As cells age, they undergo several changes, such as increased hemoglobin concentration (79), decreased sialic acid and lipid content (71, 80), decreased deformability (79), and decreased ATPase activity (81). The aging RBC may also lose some property that plays a role in the adhesion observed in these experiments. It is possible that PTX may interact with reticulocytes and alter some property that makes them more adhesive. Several investigators have reported that PTX increases the deformability of red blood cells, but many of these studies were performed using whole blood filtration techniques and did not account for the presence of white blood cells and their effect on filtration measurements. Evidence has been reported recently which indicates that in whole blood filtration, the white blood cell is the main determinant of filterability (82 - 84). One leukocyte was found to be as effective as 700 erythrocytes in obstructing flow through 5 μm pores in a Nuclepore filter. Thus, what was reported as a drug effect on red cell deformability, may in fact, be an effect of PTX on white blood cells. In a recent report on filtration studies involving leukocytes, it was reported that leukocyte filterability is improved by PTX (85). PTX
is known to increase the ATP content of erythrocytes (86) and to modulate ATPase activity (87). It is thought that PTX inhibits c-AMP phosphodiesterase, leading to relaxation of smooth muscle (thereby relaxing vessel walls) and improvement in blood flow (88, 89). PTX has also been reported to reduce plasma fibrinogen (90). Fibrinogen enhances sickle RBC adherence to endothelium in vitro (9) and its levels are reportedly increased during sickle vasocclusive crises (12, 78). Sickle cell crises are also characterized by moderate leukocytosis which may contribute to rheologic difficulties in the microvasculature. PTX may also be beneficial in improving the deformability of leukocytes which could facilitate microcirculatory perfusion (85).

Results from this study indicate that the adhesion of sickle erythrocytes to endothelial cells is significantly decreased in the presence of PTX. The concentrations of PTX used, however, are greater than plasma concentrations achieved in clinical use of the drug. It is difficult to extrapolate the results from one hour incubations of RBC with relatively high PTX concentrations to prolonged exposure of RBC in vivo to much lower PTX levels. However, the PTX concentrations used here to reduce SS RBC adherence are similar to those used by other investigators to reduce white blood cell deformability (85). The effect of PTX on white blood cell deformability, plasma fibrinogen levels and RBC-
endothelial cell interactions suggest that PTX may be an important rheologically-active drug that could be of use in the treatment of sickle cell disease.

Image analysis techniques were used to analyze taped flow experiments to gain insight into red cell-endothelial cell interactions by determining the velocities of individual sickle and normal RBC. The velocity of cells plays an important role in the promotion or inhibition of cell adhesion. Because of the inward radial migration and wall exclusion of RBC which occurs in flow in channels whose height is many times that of the red cell, the bulk of the RBC in a suspension will be flowing at the same velocity in the core of the channel. Other RBC that may be traveling in the cell-poor region near the wall may actually be moving at different rates which in some cases may be slow enough to allow adhesive interactions between the RBC and the EC lining the wall to take place. From gross observation of recorded experiments, it appeared that sickle RBC traveling near the endothelial cell surface were moving slower than normal RBC near the EC surface at the same volumetric flow rate. Velocity measurements made using image analysis techniques confirmed this observation. Some red cells (particularly in the the case of sickle RBC) appeared to be sliding slowly across the endothelial surface as if temporary contacts between the cell surfaces were being made, but were not strong enough for the red cell to adhere. Differences
in the adhesive properties of SS and AA RBC could partially account for the slower velocities of sickle RBC near the endothelial cell monolayer. If sickle cells have an increased propensity for adherence, it is likely that sickle cells that do come in close contact with endothelial surface are more likely to form sufficiently stable bonds to remain in contact with the endothelial surface. Normal red cells, on the other hand, may not be "sticky" enough to form adhesive bonds even when close enough to the endothelial surface to make contact.

Sickle cells have been shown to differ from normal red cells in their adherence properties (9–13) and flow characteristics (63–66). That sickle cells travel more slowly across the endothelial surface as these studies suggest, supports the idea that sickle cells may have an increased propensity for adherence to endothelial cells. Image analysis may prove to be an useful technique that can be combined with video microscopy to study differences between sickle and normal red blood cells with respect to adhesion.

The attachment of red cells to endothelial cells could involve adhesion between cells and an absorbed film of serum, serum fractions, cell microexudates or other substances which is, at first, weak but becomes reinforced as time passes. A measure for the adhesive strength involved is the resistance of cells to separation from the substrate by shear forces. Thus, the adhesion of blood cells to the
vascular endothelium can be evaluated as resistance to the shearing force of the marginal blood stream. Grinnell (91) defined four steps leading to cellular adhesion: (1) adsorption, (2) contact, (3) attachment, and (4) spreading. Adsorption consists of the coating of the substrate with proteins which are either derived from serum added to the medium, or produced by the cells. Fibronectin has been of particular interest, since it is derived from both sources (92, 93). From the analysis of micropipette experiments examining adhesion between RBC and EC, Mohandas et al. have proposed three types of adhesive contacts, 1) laying on the surface, 2) point attachment and 3) spreading on the surface. The first type of contact is not very strong and adherent cells are easily removed. The second type involves breaking the point of attachment to remove the adherent cell, and the third type, which is probably the strongest, involves an extensive area of contact which must be broken in order to remove an adherent cell. Results from this study indicate a point attachment of RBC to EC under flow conditions, as demonstrated by increasing and decreasing the flow rate abruptly. Adherent cells adhere at a point and undergo a tear drop deformation under stress caused by abrupt changes in the flow.

The forces involved in the interaction between a red blood cell and the endothelial cell of the vessel wall to which it is adhered are (1) hydrodynamic drag, (2) normal
forces (pressure), (3) electrostatic forces of repulsion, (4) Van der Waals forces of attraction and (5) adhesive forces between the cell and substrate. The first force is brought about by local fluid mechanics, mainly through the fluid shear stress acting over the exposed cell area. The fluid shear stress force also acts at the wall over the area involved in the membrane-membrane interaction. Intercellular electrostatic forces are repulsive and non-specific, since both cell surfaces have a net negative charge. Attractive interactions between two cells in contact are of the Van der Waals type. It is possible that some plasma protein(s) may serve as a bridging molecule in the adhesive interaction between red cells and endothelial cells. Several types of attractive or adhesive forces could be involved in the interaction including covalent bonding, electrostatic attraction and hydrogen bonding. The adhesive force depends on the area of contact and the strength of interactions between cellular receptors. The magnitude of this force would be very sensitive to the biochemical state and morphology of the RBC and the endothelial cells. If an RBC flattens out or spreads on the vessel wall surface, it will reduce the hydrodynamic drag force and increase the area of contact, leading to a more stable adhesion.

Whether collisions of red cells with endothelial cells result in adherence depends on a delicate balance between the fluid mechanical drag force, which tends to dislodge the
RBC, and the adhesive force generated at the area of contact with the endothelium. Local blood flow rate controls the fluid mechanical drag force, with the important parameter being the velocity gradient of a wall (wall shear rate). In addition to the drag force, the electrostatic repulsive force can also tend to counteract adhesion. The adhesive force must be sufficiently large to overcome the electrostatic repulsive force, the shear stress force from the fluid acting over the exposed cell area ($F_s$), the wall shear stress acting on the area of contact ($F_w$) and the form drag ($F_d$) which should be very small, since an extremely low Reynolds number is involved. Using a component force balance on a red blood cell adhering to the endothelial cell monolayer, an approximation of the adhesive force can be obtained:

$$F_a - F_s - F_w - F_d = 0$$

Approximate values for $F_s$, $F_w$ and $F_d$ were obtained as follows,

$$F_s = (\text{fluid shear stress, } \tau) \times (\text{area of exposed RBC})$$

where area of exposed RBC is assumed to be a circle of radius $4\mu$

$$F_w = (\text{fluid shear stress, } \tau) \times (\text{area of membrane interaction})$$

where area of membrane interaction is assumed to be a very small circle of radius $2\mu$ for "point" attachment
\[ F_d = 2\pi \mu R v \text{ (Stoke's Law)} \]

where

\[ \mu = \text{viscosity} \]
\[ R = \text{radius} \]
\[ v = \text{velocity} \]

assuming one can treat the adherent RBC like a sphere as a gross approximation.

The electrostatic repulsive force is not included in this component force balance since it acts normal to the cell surface and is not considered to contribute to this balance which involves forces that act parallel to the cell surface. For a shear stress of .35 dyne/cm² and a gap width of 104\mu, \( F_a \) is calculated to be approximately 1.743 \( \times 10^{-7} \) dyne/cell. An unknown amount of the adhesive force as determined by these calculations is probably due to van der Waals forces of attraction which are very difficult to determine. Values of \( 10^{-6} \) to \( 10^{-7} \) dyne have been obtained for the force of cellular adhesion from theoretical calculations by Brooks et al. (94) and Weiss et al. (95).

From experiments examining the effect of shear rate on adhesion, it was determined that red cells virtually did not adhere at shear rates greater than about 250s⁻¹. This corresponds to a fluid shear stress of approximately 8.7 \( \times 10^{-7} \) dyne/cell acting on the exposed cell area (the predominant force acting to counteract adhesion). A simple
interpretation of the action of the hydrodynamic shearing force on cell adhesion is as follows. If at the moment when a cell adheres the force is less than that which corresponds to the strength of the adhesive bonds, the cell becomes attached to the cell surface. When the adhering cells are subjected to a force equal to approximately $8.7 \times 10^{-7}$ dyne, only an insignificant fraction of cells can adhere, and it may be concluded that the adhesion force does not attain this threshold. In this case, the adhesive force was calculated to be $\sim 2 \times 10^{-7}$ dyne which is below the threshold where practically no adhesion is observed. The fluid shear stress corresponding to shear rates near $100 \text{s}^{-1}$ is not large enough to prevent the formation of adhesive bonds and thus, more adhesion is measured at these rates. In addition, one would expect less adhesion at high shear rates where flow is faster than that demonstrated at low shear rates where flow is slow, since interaction time will be much less at high flow rates. Shear rate is an important variable that plays a role in determining the flow of sickle blood in the microcirculation. At low shear rates, significant problems may develop in the microcirculation due to obstructed flow caused by sickle cells becoming stiff and/or by SS cells interacting with endothelial cells.

Previous experimental evidence has suggested at least two phenomena which may predispose to vaso-occlusion in sickle cell disease: (1) intracellular gel formation, which
leads to shape change and decreased deformability of SS RBC (96) and (2) increased adhesion of SS RBC to EC (9 - 12). In this study, the adhesion process was examined under controlled fluid mechanical conditions in order to gain insight into events that may lead to vaso-occlusion, but do not directly involve the deformability of the SS RBC. Cells with maximum intracellular gel formation and shape change are the densest cells (44, 97), while these studies indicate that the most adhesive cells are the least dense RBC. Although the least dense cells are hemodynamically the most competent in deoxygenated blood (44), their tendency to adhere could sufficiently retard the flow in the small vessel to extend transit times for other cells beyond the critical delay time required for gel formation. The delay time for SS hemoglobin gelation is strongly dependent on the intracellular hemoglobin concentration to a very high power. The intracellular fraction of polymer will be highest in cells with high hemoglobin concentrations. Polymer fraction has been correlated with vaso-occlusive severity (98). The least dense sickle cells have a mean corpuscular hemoglobin concentration (MCHC) of 30 to 35 g/dl, while the most dense sickle cells have an MCHC of 40 to 45 g/dl (44). The pre-gelation delay time for the light cells may be as long as 40 seconds, but cells with an MCHC over 36 g/dl show gelation in less than 0.5 second (99). Since the average capillary transit time is roughly one second, cells with pre-gelation
times in this range are threatened with rheologic change in the microcirculation. Thus, the dense cells with high MCHC values may have a profound effect on the microcirculatory flow if their capillary transit times are increased. The adhesion of light (young) cells may lengthen transit times for other cells in the microvasculature leading to gel formation in dense cells and resulting in vaso-occlusion. Recently, increased adhesion of reticulocytes in SS disease to fibronectin-coated petri dishes has been demonstrated in a static system (100). The abnormal adherence of young cells may be of little or no consequence in other diseases which are characterized by increased numbers of reticulocytes, but do not involve vascular occlusive events. Increasing capillary transit times would not result in changes in the rheological properties of RBC for these diseases. Increased adherence to endothelial cells, the only factor besides delay time thought to be correlated with disease severity, may be an important feature of young RBC in sickle cell disease.

The exact relationship between rheology, sickling kinetics and SS RBC endothelial adherence remains unknown. Although the process of sickling is of central importance, it is unclear as to whether distortion of the sickle cell leads to vaso-occlusion or whether damage done to the cell by sickling lead to abnormal red cell-endothelial cell interactions. Occlusion of the microvasculature may be
initiated by cells that have been distorted due to gelation of deoxy-hemoglobin S, and, perhaps mediated in part by the abnormal tendency of these cells to adhere to vascular endothelium. It is probable that a combination of intrinsic erythrocyte abnormalities, plasma alterations, abnormal erythrocyte—vessel wall interactions, and contributions from the structure and function of the microcirculation all combine to cause vascular occlusion, and to explain the patient-to-patient variability in the disease.
References


APPENDIX

Richardson Flow Chamber

A diagrammatic illustration of the body of the flow chamber used in these experiments is given in figure 10A, where: a and b are inlet and outlet ports for the RBC suspensions and tissue culture medium; c and d are slits for entry and exit of suspensions and medium (arrow indicates the direction of flow); e is a slotted edge in which to place the rectangular silastic gasket; f is a connector for a vacuum line; g is a recessed channel that transmits vacuum and thus allows mounting of the glass slide over the gasket—both of which are watertight—onto the body of the chamber by the vacuum. Use of vacuum to hold the chamber parts together provides a uniform pressure on the rectangular gasket which results in a uniform thickness of the flow chamber. A lateral view of the assembled flow chamber is shown in figure 10B where h is the silastic gasket and i is the glass slide.

The dimensions of the flow channel formed by two parallel walls a distance 2a apart have been calculated to be:

\[ 2a, \text{ channel height} = 0.01693 \text{ cm} \]

\[ b, \text{ channel width} = 1.39 \text{ cm} \]

\[ l, \text{ channel length} = 4.33 \text{ cm} \]
Figure 10. Diagrammatic illustration of the body of the flow chamber and a lateral view of the assembled chamber.
The distance $2a$ was calculated as illustrated in the following diagram:

```
+------------------+
| chamber body     |
+------------------+
    /                |
   /                 |
  /                  |
+-----------------+/                   |
| silastic gasket  |
+-----------------+/                   |
   \                |
    \               |
  \                |
+-----------------+/                 |
| flow channel    |
+-----------------+/                 |
    \            |
     \          |
     \          |
+--------------+/        2a h       |
| glass slide  |
+--------------+/        \          |
   \          |
    \        |
+-------+/   g        |
```

$2a = h - x - g = 0.00667 \text{ in} = 0.01693 \text{ cm} = 170 \mu\text{m}$.

Similarly, the distance $2a$ was calculated to be $0.0104 \text{ cm} = 104 \mu\text{m}$ for the flow chamber with a narrower gap width.

Shear Stress Calculations

Using the equation for the wall shear stress,

$$\tau = 3 \frac{\mu Q}{2 a^2 b}$$

$\tau$ can be calculated for a given flow rate as follows:

$$\tau = (3/2) \left(\frac{\mu Q}{a^2 b}\right)$$

$$= (3/2) \left(0.00689 \frac{Q}{(7.1741 \times 10^{-5}) (1.39)}\right)$$

$$= 103.64 \frac{Q}{\text{dyne/cm}^2}$$
where \( Q \) is the flow rate in \( \text{cm}^3/\text{sec} \) and \( \mu \) is the viscosity of the medium which is assumed to be the same as the viscosity of water at the same temperature conditions (37°C), i.e. \( \mu = 0.689 \text{cp} \) or \( \mu = 0.00689 \text{ g/cm sec} \). Similarly, for the chamber with a gap width of 104 \( \mu \text{m} \), the wall shear stress can be calculated for a given flow rate from,

\[
\tau = \left(\frac{3}{2}\right) \left(\frac{0.00689Q}{(2.704 \times 10^{-5})}\right) (1.40)
\]

\[
= 273.01 \text{ Q dyne/cm}^2
\]