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Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

RHEOLOGY OF BLOOD CELLS IN SICKLE CELL DISEASE

By

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The abnormal blood rheology in sickle cell disease is mainly due to the abnormal rheology of sickle red blood cell. A ball microrheometer is used to measure the apparent viscosity of sickle red blood cell suspended in phosphate buffer solution (PBS) and plasma at 25% hematocrit and 37°C under deoxygenation. The apparent viscosity is measured as a function of the amount of polymer formed inside the erythrocyte. The results are similar for sickle trait (AS) and sickle cell (SS) patients and show that apparent viscosity, $\eta$, and polymer fraction, PF, are linearly related ($\eta = 3.93 \text{ PF} + 1.79$, with a coefficient of correlation $r^2$ of 0.75), evidencing that sickle deoxyhemoglobin polymerization is the dominant factor governing the overall blood rheology in sickle cell disease. In addition, the effects of pharmacological agents administered to patients such as Hydroxyurea or added directly to ex vivo blood samples such as Clotrimazole are investigated using the microrheometer. The apparent viscosity seems to be a good parameter to assess the clinical severity in sickle cell disease. However, the increase in blood viscosity under deoxygenation is small indicating that other factors also contribute to the pathophysiology of sickle cell disease. Since leukocytes have been shown to play a major role in blood microcirculation, their contribution in sickle cell disease, which has not been extensively studied,
is analyzed. Using micropipette techniques, it is shown that some sickle neutrophils display slightly higher apparent viscosity than normal neutrophils. However, patient-to-patient variability is high. Finally, an experimental procedure to visualize the nucleus and cell membrane under deformation in order to characterize leukocyte rheology is presented. It is found that a Hoechst fluorescence dye can be used for that purpose without activating the cell or modifying its rheological behavior. Pictures of a lymphocyte and its fluorescent nucleus flowing into a 4 µm pipette are shown to demonstrate the feasibility of the technique for studying the rheological behavior of leukocytes.
INTRODUCTION

1.1. Motivation

Sickle cell disease (SCD) afflicts Afro-Americans in the United-States, and black people in most South-American, African, and West-Indies countries. Sickle cell disease is a genetic disorder characterized as a disease of abnormal blood rheology leading to hemolytic anemia and vaso-occlusion. In sickle cell disease, sickler patients have red blood cells (RBC) that are not capable of traversing the capillaries and consequently of delivering oxygen to the nearby tissues, which is one of the main functions of red blood cells. Sickler patients experience numerous pathological complications and frequent vaso-occlusive painful crises. Researchers have been working on sickle cell anemia for more than 30 years now and even if they have made tremendous progress into the characterization of this disease, they still do not have a complete understanding of all its mechanisms. The change in blood rheology in SCD patients is mainly caused by the abnormal rheology of sickle red blood cell. Sickle red cells have several abnormal properties. They adhere more easily to the endothelial cells than do normal erythrocytes. They become rigid under deoxygenation because of intracellular polymerization of the sickle hemoglobin. However, little is known about the relationship between the blood rheological properties and the amount of polymer formed inside the red blood cells.

Because of the nature of the disease, the focus of blood rheology in sickle cell disease has been on studying the rheology of erythrocytes or red blood cells. However, many other factors intervene in this complex disease. From a rheological point of view, leukocytes or white blood cells (WBC) also need to be investigated. Under normal conditions, RBC outnumber WBC by about 1000:1, in SCD the hematocrit of RBC is reduced by half whereas the amount of circulating leukocytes can be multiplied by two [Boggs et al., 1973; Buchanan and Glader, 1978].
Thus, the contribution of WBC to flow resistance will be increased in SCD. Because of their larger volume as compared to other blood cells, their complex structure, their ability to change from passive to active behavior in a few seconds, leukocytes play a critical role in the microcirculation. Therefore, their role needs to be better understood, in particular in SCD.

1.2. Objectives

The present study has two major objectives. The first objective is to determine if the apparent blood viscosity given by the microrheometer can be used as a tool for assessing SCD severity and treatments. The second objective is to investigate the rheological properties of WBC from sickle cell patients using a micropipette technique.

The specific aims of the first objective are: (a) to characterize the rheology of sickle RBC suspensions under deoxygenation, (b) to correlate the apparent viscosity with the amount of polymer formed inside the RBC, and (c) to investigate the effects of pharmacological agents, such as Hydroxyurea administered to patients and Clotrimazole added to ex vivo blood samples.

Sickle trait (AS) and sickle cell (SS) red blood cells resuspended at 25 % hematocrit will be used in this study. Sickle trait blood will be used because its red blood cells present minimal membrane abnormalities and minimal cell density heterogeneity. Normal red blood cells (AA) also resuspended at 25 % hematocrit will be run as control. All the red blood cell suspensions will be resuspended both in their autologous plasma and in phosphate buffer solution (PBS) at the selected hematocrit.

The specific aims of the second objective are: (a) to compare the rheological characteristics of WBC between sickle cell and normal patients, and (b) to develop an experimental protocol for observing the cell nucleus as well as the cell outer membrane. Micropipette aspiration and recovery experiments of “normal” and “sickle” leukocytes will be performed. To interpret the results of micropipette experiments, we will model the leukocyte as a simple Newtonian liquid drop. Using this model, the ratio of apparent viscosity to cortical tension can be evaluated. Although Kan [1997] has shown that this model is not adequate for
describing the rheology of leukocytes, this model nevertheless provides a means for comparing mechanical properties of leukocytes between normal and sickle subjects. It was shown by Kan [1997] that the nucleus plays a major role in the overall rheological behavior of the cell and needs to be characterized. Experimental studies will focus on the lymphocyte because its nucleus is relatively larger and more spherical than other WBC.

1.3. Overview of the Thesis

In the present study, the effect of deoxygenation on sickle cells will be simulated in vitro. Using a spincup tonometer, blood will be rapidly deoxygenated and equilibrated at different partial pressures in oxygen. The rheological properties of blood suspensions will be characterized in terms of the apparent viscosity measured with the ball microrheometer. The advantage of this instrument over other viscometers is the small volume required for a rheological measurement. The newly designed ball microrheometer provides accurate viscosity measurements even for viscosity values as low as 1 cP (water). Viscosity measurements will be performed under physiological conditions for sickle cell patients (hematocrit of about 25%) and controlled influx factors such as partial pressure in oxygen (pO₂), saturation in oxygen, temperature, pH and osmolarity. Investigations of the properties of single sickle red cells under deoxygenation using micropipette techniques have been performed by Evans et al. [1984], Mackie [1990] and Itoh et al. [1992]. Studies of cell suspensions have also been done but were at higher hematocrit (mainly 45% and 90%) and not physiological for sickle cell patients. Of interest is to find if there is a relationship between the apparent viscosity of the blood suspension and the amount of polymer formed inside the cells.

From a clinical point of view, the existence of a correlation between the apparent viscosity of sickle RBC suspensions and polymer fraction could provide a simple means for assessing the severity of the disease. Instead of performing tedious blood analyses that include a complete blood count (CBC), hemoglobin electrophoresis (HE) and calculation of the polymer fraction as a function of the oxygen saturation, a direct measurement of the viscosity could
summarize all this information. Moreover, this new approach tending to relate polymer fraction to the viscoelastic properties could also be used to assess the effects of clinical treatments. In that regard, the effects of two drugs on the apparent viscosity of sickle blood cell suspensions will be examined: (1) Hydroxyurea, a drug that increases the amount of fetal hemoglobin resulting in a lower polymer fraction, and (2) Clotrimazole, a potassium ion blocker that reduces cell sickling by preventing cell dehydration.

Vaso-occlusion in sickle cell disease is a multistep process that involves initiation, propagation, and resolution phase. The role of hemoglobin polymerization in the propagation phase is evident. Concerning the initiation phase, traditional concepts of the onset of painful crisis in sickle cell disease revolve around the sequestration and entrapment of red blood cells attendant to their diminished deformability upon deoxygenation. The reduced deformability of sickle red blood cell increases the apparent viscosity of blood flow through the microcirculation, thus leading to a “vicious cycle” of flow degradation with further increases in flow resistance and attenuation of the flux of oxygen necessary to counter the rheological insults. Two major factors have been widely identified to contribute to red blood cell entrapment during crisis: the reduced deformability of sickle red blood cell and the adhesion between the endothelium and the erythrocytes. Additionally, it is likely that other blood cellular elements, namely the leukocytes, also contribute to slow down the blood flow by obstructing the capillaries, thus serving to diminish the total throughput of the flow and convective transport of oxygen to tissue, delaying the sickle RBC transit time, and eventually causing the polymerization of sickle intracellular hemoglobin.

Therefore, the role of white blood cells in sickle cell vaso-occlusion cannot be excluded. Micropipette technique will be used to investigate if the rheological properties of white blood cells change in sickle cell disease. These properties will be derived by using a Newtonian liquid drop model for the WBC. Although this model is not always adequate for describing the
rheological behavior of the cell [Kan, 1997], it provides a means for comparing the properties between normal and sickle cell subjects.

In order to understand the rheological behavior of the cell, it is necessary to characterize the behavior of its nucleus. Therefore, experimental procedures for visualizing the nucleus under deformation will be developed. A characterization of the rheological properties of the leukocytes would allow to better understand the adhesion between leukocyte and endothelium, to study the deformation of an adherent leukocyte to the endothelial wall, to assess the resistance to flow caused by adherent leukocytes in the microcirculation and to evaluate the forces necessary to dislodge a leukocyte trapped in a capillary. This increased knowledge in white blood cell rheology will provide some valuable information on the contribution of leukocytes in vasoocclusive crisis.
2.1. Role of Red Blood Cells in Sickle Cell Disease

The deformability properties of erythrocytes are critical determinant of flow in the microcirculation. Sickle cell disease involves abnormal hemoglobin within the erythrocyte that causes the normally deformable biconcave cells to stiffen and take on this elongated, sickle shape. Intracellular polymerization of sickle hemoglobin is generally believed to be the main factor affecting sickle cell erythrocyte rheology due to its effect on red cell deformability.

2.1.1. A Mutant Form of Hemoglobin

Hemoglobin molecules have four polypeptides chains: two alpha (α) and two non-alpha chains. Each chain surrounds an iron porphyrin molecule that can reversibly bind oxygen. The non-α chains, usually β chains, combine with two α chains to form normal hemoglobin A (Hb A) [Briehl and Nikolopoulou, 1993]. A structural change takes place when hemoglobin passes from the oxygenated or relaxed state to the deoxygenated or tense state. Deoxy-hemoglobin S polymer forms when β chains in different tetramers come into contact. The same conformational change occurs in normal hemoglobin but deoxy-hemoglobin A does not polymerize. The difference between normal (Hb A) and sickle hemoglobin (Hb S) is a single amino acid substitution: an hydrophobic valine replaces the negatively charged glutamic acid in the β chain of Hb S. In normal hemoglobin, the size and the charge of the glutamic acid do not allow intermolecular contact, whereas valine allows hydrophobic contacts during the deoxygenation phase. When exposed to low concentrations in oxygen, the mutant hemoglobin forms elongated crystal polymers with a helical fiber structure, that makes the cells become more rigid. Sickle cell’s flexibility, deformability and shape are thus dramatically changed. The mechanism of sickle hemoglobin polymerization is summarized in Fig. 2.1.
2.1.2. Vicious Cycle Theory

Vascular occlusion during sickle cell crisis has been attributed to intravascular sickling of sickle cells. By increasing the blood viscosity, the intracellular polymerization of sickle hemoglobin contributes to the triggering of the vaso-occlusive event. The increase of blood viscosity causes decreased blood flow in the microvasculature. To supply the tissues with an adequate amount of oxygen, slower moving cells must release more oxygen than usual into the capillaries. This may produce oxygen saturation levels low enough to initiate intracellular polymerization of Hb S. As Hb S polymerization progresses, the cells become more rigid and impede the flow, resulting in more oxygen depletion. Thus, a vicious cycle is initiated which ultimately leads to a rigid, sickled cell obstructing the capillary. Physiologically prolonged low
concentrations in oxygen can lead to organ damages, organs such as heart [Covitz, 1994], kidneys [Haynes et al., 1994] and brain [Adams, 1994].

2.1.3. Effect of Deoxygenation on Blood Rheology in SCD

Several studies have shown the effects of deoxygenation on blood rheology in sickle cell disease using traditional viscometers [Usami et al., 1975; Murphy et al., 1975; Chien et al., 1982]. Rheological studies of individual blood cells have been carried out using micropipette techniques [Mackie et al., 1990; Itoh et al., 1992] or filtration techniques [Hiruma et al., 1995 a and b; Hasegawa et al., 1995]. Polymer fraction (defined as the ratio of the amount of hemoglobin in polymer form, to the total amount of hemoglobin within the cell) has been shown to be an important factor in sickle cell disease [Schechter and Noguchi, 1994; Hiruma et al. 1995 b].

Usami and co-workers [1975] investigated the effects of deoxygennation by controlling the suspending medium, cell concentration (45%), shearing condition, temperature, gas tension and pH. A rotary-type tonometer was used for the control of gas tension and a coaxial cylinder viscometer was used to measure the apparent viscosity. It was shown that the viscosity of suspensions of Hb S erythrocytes in plasma and in Buffer solution at 37°C increased progressively when the pO$_2$ was reduced below a critical level of approximately 60 mm Hg (equivalent to a saturation in oxygen of 80 %). Higher viscosities for red blood cell suspended in plasma than in PBS suggested the role of plasma protein in red blood cell aggregation. No significant changes of viscosity with deoxygenation were found for Hb A erythrocytes (either in plasma or in Buffer solution). Murphy et al., 1975, measured the apparent viscosity of sickle RBC suspended in plasma equilibrated with air (fully oxygenated) or nitrogen (fully deoxygenated) at 37°C in a cone and plate viscometer. They showed that the viscosity of deoxygenated SS blood was disproportionally reduced by the addition of compatible AA cells. Similar results were obtained by Schmalzer et al., [1987].
The quantitative relationship between deoxygenation and rheological behavior of sickle cell suspensions (in a buffer solution at 45% hematocrit) and concentrated Hb S solutions (concentration of Hb S: from 22 to 42 g/dl) was studied under steady shear (viscosity, $\eta$) and oscillatory shear (complex viscosity, viscous and elastic components, $\eta'$, $\eta''$) with an oscillatory cone and plate rheometer by Chien et al. [1982]. An increase in $\eta$, $\eta'$, $\eta''$ for sickle cell suspensions and Hb S solutions was found for a decrease in O$_2$ saturation below 80-85 %. They investigated the influence of Hb S rheology on the blood cell suspension rheology and showed that the viscoelastic properties of SS blood suspensions become increasingly dominated by that of the intracellular Hb S as the O$_2$ saturation decreases.

Although the rheological behavior of sickle erythrocytes is highly dependent on oxygen tension (pO$_2$) and temperature [Coffey, 1991], very little data exist regarding the effects of individual SS cells at body temperature. A system with pO$_2$ and temperature control, using a micropipette aspiration was designed by Mackie et al. [1990] and Itoh et al. [1992]. Unlike Mackie et al., who studied a “population behavior” of cells at each oxygen tension, Itoh et al. were capable of following a single cell under microscopic observation at body temperature as a function of pO$_2$. As pO$_2$ was decreased, the membrane static rigidity (elasticity of the membrane E) and the dynamic rigidity (viscosity of the membrane $\eta$) showed no significant changes before sickling. But once sickled, morphologic alteration of the cell as well as an increase for the elasticity and the viscosity of the membrane and the hemoglobin viscosity (100 to 1000 times) were observed. The change in rheologic properties of RBC membrane was shown to be almost “all or none.” They also showed a hysteresis between deoxygenation and reoxygenation; in contrast to the observed sharp onset of the reduction in individual cell deformability upon deoxygenation, the rheological response is more gradual after reoxygenation. Mackie et al. indicated an increase of the static rigidity of the membrane E as pO$_2$ decreases. In addition, they
measured an increase of the dynamic constants: the recovery time $t_c = \eta/E$ and the unfolding time $t_u$. This last parameter is governed by the intracellular viscosity.

Another technique for quantifying the ability of erythrocytes to deform is filtration. The deformability of sickle cells was studied using a tiny mesh filtration system. Hasegawa et al. [1995] found that for air equilibrated SS/AA cell mixtures the fraction of dense cells (Mean Corpuscular Hemoglobin Concentration : MCHC>37g/dl) is a determinant factor in filterability and that dense cells contribute in a linear fashion to the loss of filtration up to 15% dense cells. It is to be noted that dense cells affect the filterability of erythrocyte suspensions about 25 times as much as that of nondense SS cells. It shows that reducing the proportions of non-dense and dense sickle cells circulating is the key to improvement in the rheological characteristics of blood in SS patients regularly transfused. Filterability of AS and $\beta^+$ Thalassemia red blood cells was expressed as a function of polymer fraction [Hiruma et al., 1995 a]. Sickle trait and sickle $\beta^+$ Thalassemia were used because these cells present minimal membrane abnormalities [Platt et al., 1985; Richieri et al., 1986] and minimal density heterogeneity [Serjeant et al., 1982]. Hiruma et al. [1995 a] showed that, when there are no membrane abnormalities, intracellular Hb S polymerization contributes in a linear fashion to the loss of filtration up to 30 % polymer fraction. At higher values of polymer fraction, this relationship is no longer linear because significant plugging of filter pore occurs under these conditions. This work confirms that sickle hemoglobin polymerization is definitely the dominant factor in sickle cell disease. Hiruma et al. [1995 b] examined whether sickle cell morphology, independent of the polymer fraction was somehow related to the cell rheology. This work suggests that polymer fraction is a better parameter for characterizing sickle cell pathophysiology than cell morphology. It was found that cell morphology did not strongly correlate to the cell filterability and that on the other hand polymer fraction did.
2.1.4. Evaluation of Polymer Fraction

Biochemical and Biophysical studies have led over the years to the development of a thermodynamic analysis of the polymerization of sickle hemoglobin [Minton, 1977; Hofrichter, 1979; Gill et al., 1980, and Sunshine et al., 1982]. Based on this thermodynamic description, Noguchi and co-workers [1983] were able to generate the polymer fraction profiles as a function of oxygen saturation and to predict the behavior of polymer in unfractionated blood.

The amount of polymerized sickle hemoglobin can also be measured by using C/H magnetic double-resonance spectroscopy [Noguchi et al., 1980 and 1981]. This study shows that Nuclear Magnetic Resonance techniques can provide quantitative information about the ratio of intracellular hemoglobin in a polymer form to the total amount of hemoglobin within the cell. These techniques appear to provide accurate quantitative results and do not require separation of the sample into a fluid phase and a solid phase.

Even if polymerization is a sine qua non parameter to provoke vaso-occlusive crisis, it may not be the original factor. Studies [Mozzarelli et al., 1987] have shown that, in steady state, the delay time of polymerization is longer than the capillary transit time (less than 1 second), meaning that in physiological conditions, polymerization should not cause occlusion in the microcirculation. However, this model assumes that no polymer is initially formed but this may not be true as the cell goes through the circulation, since Green et al, [1988] showed that in dense cells, the hemoglobin polymerization starts at arterial oxygen saturation. An understanding of the pathophysiology of sickle cell crisis requires knowledge of various factors, beside polymerization, especially red cell density heterogeneity and interactions of blood cells with the endothelial wall [Hebbel, 1991].

2.1.5. Cell Density Heterogeneity

Another important factor in sickle cell anemia is the cell density heterogeneity. Quantification of the erythrocytes density profile is well known [Rodgers et al., 1985].
Discontinuous Stractan density gradient technique was used to separate all the subpopulations of erythrocytes of close MCHC. It was found that sickle cells show a wider and broader distribution in its MCHC profile. This profile differs from normal cells by the presence of very light cells, and very dense cells, which are not found in a normal cell MCHC profile [Rodgers et al., 1985]. Hebbel et al. [1980] found a strong correlation between clinical severity and the tendency for sickle cells to adhere to vascular endothelium. Recent studies [Kaul et al., 1989a] using animal model have shown that the lightest fractions of sickle cells were most adherent to vessel wall, probably because of the increased tendency for reticulocytes (young RBC) to adhere to the endothelium [Barabino et al., 1987]. However, these models also showed that occlusions of microvessels did not occur in the absence of dense sickle cells even though the endothelial surfaces were covered with adherent light cells. The requirement for dense cells in causing vaso-occlusion was confirmed in a separate animal model by Fabry et al. [1989]. Since dense cells contain a higher intracellular hemoglobin concentration, they polymerize faster. Kaul et al. [1989 b] proposed that preferential adherence of deformable sickle cells in postcapillary venules would cause a narrowing of the vessel and thus increase the blood cell transit time in the capillary. This slow down of the blood flow will allow the most dense cell to polymerize, rigidify and obstruct the capillaries. Adherence of neutrophils to endothelial wall or plugging of the flow by the less deformable leukocytes in the capillaries would produce the similar effects in initiating the crisis.

2.2. Action of Drugs

Many approaches and treatments for sickle cell disease have been evaluated this last decade. The fundamental understanding of sickle hemoglobin polymerization, membrane ion transports, adhesion factors has lead to new therapeutic approaches. But so far, none of them has been fully effective.

The most promising of these approaches has been the treatment by Hydroxyurea. Treatment by Hydroxyurea demonstrated decreased number of crisis, reduced number of blood transfusions and of acute chest syndrome [Charache et al., 1995]. The principal therapeutic effect
of Hydroxyurea in sickle cell patients is believed to be the increase in the fraction of “F cells”,
from about 30% to about 50% [Charache et al., 1995]. “F cells” contain about 20% fetal
hemoglobin (Hb F) and 80% Hb S. Although the biochemical mechanism by which Hydroxyurea
stimulates Hb F synthesis is not known yet, the biophysical mechanism of how Hb F has an
inhibitory effect on polymerization in F cells is well understood. The effect of Hb F is simply to
dilute the Hb S and consequently retard the kinetics of polymerization [Bridges et al., 1996].
Hydroxyurea increases the fraction of cells with sufficiently long delay times to escape the
microcirculation before polymerization begins. Hydroxyurea may have other attributes in
addition to its ability to induce fetal hemoglobin. For example, lower WBC counts in patients
treated with Hydroxyurea may also contribute to clinical beneficial effects observed under
Hydroxyurea treatment [Charache et al., 1995]. Orringer et al. [1994] showed that patients with
sickle cell disease who receive chronic Hydroxyurea therapy show a disappearance from their
blood of two red cell populations: the densest, most-dehydrated, irreversibly sickled cell-enriched
fraction and the least dense, most hydrated cells. The disappearance of these two red cell
populations seems to coincide closely with improvement in patient status. Rodgers et al. [1993]
have shown that the addition of recombinant human erythropoietin to intermittent therapy with
Hydroxyurea permits enhanced induction of Hb F. Also butyrate compounds have shown
stimulation of fetal hemoglobin synthesis [Dover, 1997].

Based on the understanding of red cell transport physiology, Brugnara et al. [1996], have
introduced another antisickling agent: Clotrimazole. Since cell dehydration contributes to the
formation of dense cells and the extent of sickle polymerization, Brugnara at al. proposed to use
an ion channel blocker to prevent water loss from the cell during sickling. It was shown
previously [Stuart, 1992] that under sickling, the concentration of intracellular free Ca\(^{2+}\) was
increased which activates the potassium ion channels also called Gardos channels. By inhibiting
these channels, K\(^+\) loss accompanied with water could be prevented, thus reducing cell
dehydration and the proportion of dense cells. In their study, Brugnara et al. demonstrated that
the Gardos channel plays a role in cell dehydration. Stuart et al. [1994] also showed Clotrimazole (CLT) improves sickle cell deformability.

The effects of Hydroxyurea and Clotrimazole will be investigated with the microrheometer.

2.3. Rheology of Normal Leukocytes

Leukocytes, because of the relative size and rigidity, play a significant role on the overall circulation flow. The most common circulating white cells in humans are called neutrophils or PMN (for polymorphonuclear neutrophils). The neutrophil has been the object of wide studies. This cell contains a relatively small segmented nucleus occupying about 21% of its volume, and a cytoplasm which is rich in granular bodies. Both granules and nucleus are barely discernible with an optical light microscope. The neutrophil assumes a spherical shape in its passive state. The membrane surface is highly ruffled which gives an excess surface area estimated at 110-120% of the undeformed state. This property allows the cell to deform into nonspherical shapes without subjecting the surface membrane to large stresses.

Although there are strong experimental evidences [Evans and Yeung, 1989, and Tran-Son-Tay et al., 1991] that neutrophils exhibit behavior similar to liquid drops, it is known that they cannot be modeled as simple Newtonian drops. For example, Needham and Hochmuth [1990] observe a non-Newtonian shear thinning behavior when cells are flowing into a pipette at increasing rates of deformation. In addition, cells held in pipettes for short period of time recover more quickly compared to cells held for longer time periods [Tran-Son-Tay et al., 1991]. Neutrophils initially enter into a pipette with a velocity much larger than the steady state velocity, indicating a smaller apparent viscosity at this stage. Neutrophils that are only slightly deformed in a pipette before ejection also give smaller apparent viscosity values [Hochmuth, 1993] than cells that are significantly deformed. Thus, since the apparent viscosity changes with the rate and extent of deformation, the behavior of neutrophils cannot be Newtonian. This result indicates a “fading elastic memory”, indicative of a Maxwell-type liquid. However, the Maxwell liquid is
not adequate for describing the rheological properties of neutrophils either. It is necessary to continuously adjust the material properties (viscous and elastic coefficients) of the model as the deformation proceeds in order to fit the experimental data [Dong et al., 1988].

Kan [1997] using a three-layer model was able to explain the inconsistencies observed in the micropipette experiments. This model was proposed by Hochmuth [1993] for describing the rheological behavior of the neutrophil. In this model, the outer layer is the cortical region surrounding the cytoplasm, the second layer is the cytoplasm, and the third layer represents the nucleus. Kan [1997] found that in order to understand the rheological behavior of WBC, it is essential to have information on the deformation of the cell nucleus. However, the behavior of the nucleus has not been characterized yet.

2.4. Role of Leukocyte in Sickle Cell Disease

Reports on the role of leukocytes in sickle cell disease is very limited. However, there is an increasing evidence that WBC may be involved in the initiation of vaso-occlusive crisis in sickle cell disease. Clinical studies showed that elevated total WBC counts are common in sickle cell disease patients [Boggs et al., 1973] and that WBC counts greater than 15,000 cells/ml are associated with an increased risk of early death [Platt et al., 1994]. The analysis by Mansour [1986] of data assembled from over 1000 patients enrolled in the Cooperative Study of Sickle Cell Disease clearly demonstrates that the hematological parameter that correlates the best with severity of crisis is the total leukocyte count, instead of the erythrocyte-related parameters such as mean corpuscular hemoglobin concentration, mean cell volume, and total hemoglobin. Lower WBC counts in SC disease patients [Wong et al., 1996] may contribute partially to reduced clinical manifestations reported for these subjects.

There are some instances where leukocyte plugging does not cause a complete obstruction of the capillaries and that plasma as well as platelets and erythrocytes can squeeze their way past the jammed leukocyte [Branemark and Lindstrom, 1963]. Due to the flow of plasma past a blocking leukocyte, erythrocytes may enter into the capillary where they
accumulate, densely packed, behind the leukocyte. When the leukocyte moves on, the erythrocytes will follow closely behind, forming a leukocyte-erythrocytes train. The velocity of such a train, or of a single leukocyte, in a narrow capillary is lower than that of freely moving erythrocytes. Thus, due to their lower deformability, the leukocytes lengthen the transit time of the red cells through the microcirculation. If the transit time is longer than the delay time necessary for polymerization, then intracellular polymerization occurs while the cell is squeezing through small vessels, and blockage may occur.

Recent studies [Lipowsky and Chien, 1989; Boghossian et al., 1991; Kasschau et al. 1996; Hofstra et al., 1996] have examined the role of neutrophils in the pathophysiology of vaso-occlusive sickle crisis.

Lipowsky and Chien [1989] investigated the mechanics of leukocytes-endothelium adhesion in light of its potential for obstructing the microvascular lumen and contributing to the onset of a vaso-occlusive crisis. Studying flow in skin capillaries, they found that the red cell velocity goes through a maximum during a period following an induced flow cessation. They showed that the time to reach peak velocity (T peak) is lengthened in crisis compared to crisis free conditions. They correlated the WBC count with T peak. This correlation evidenced that prolonged T peak corresponds to the washout of WBC adhered to the endothelium. This study indicates that enhanced sickle WBC adhesion affects microvascular perfusion and causes luminal obstruction.

Kauschau et al. [1996] evidenced an increased adherence of sickle neutrophils to fibronectin in static adhesion assays as compared to normal neutrophils. They showed that sickle neutrophils adhesion is enhanced significantly by autologous plasma, indicating that sickle plasma contains factors promoting the adhesion of sickle WBC. They emphasized on the role of plasma factors (particularly interleukin 6) in increasing the number of circulating neutrophils and activating them, thus mediating sickle neutrophil adhesion to fibronectin.
Another investigation [Boghossian et al., 1991] reported that sickle WBC are less adherent to glass coverslips perfused with whole blood in a dynamic adhesion assays than control WBC. This study underlines the defective role of neutrophils to adhere to the vessels in dynamic conditions and migrate through the tissues, and suggests that these defects in neutrophils adhesion and migration may contribute to the susceptibility to infection seen in sickle cell disease.

Hofstra et al. [1996] reported that patients with SCD appear to have chronically activated neutrophils. They hypothesized that neutrophils become activated in patients with SCD, in part, through specific interactions with sickle RBC. They also indicated that sickle plasma contains factors that promote adhesion between sickle RBC and neutrophils.
Materials and METHODS

3.1. Instrumental Setup for Studying the Rheology of Cell Suspensions

3.1.1. The Microrheometer

Apparent viscosity measurements are performed in a modified magneto-acoustic ball microrheometer of Tran-Son-Tay et al. [1988]. The main advantages of this technique are the requirement of very small sample volume (20 µl) and the capability of studying opaque solutions. Because of the small sample size, the temperature of the sample fluid can be rapidly and accurately controlled with the help of a water bath. The new microrheometer is now capable of measuring the viscosity of water in the very short chamber. Ball displacements as small as 3 µm can be detected. These features are essential for studying the rheology of sickle cell blood.

A small stainless steel ball (Model 440-C, New England Miniature Ball CO., Connecticut) with diameters of 1.3 mm and density of 7.67 g/ml is inserted in a cylindrical glass capillary tube (internal diameter of 1.6 mm and about 10 mm long) as shown in Fig. 3.1. The tube is held vertically within a Lexan™ water jacket with an O-rings at each end to provide a tight-seal.

The motion of the falling ball is tracked by ultrasound location method. An ultrasonic pulser/receiver (Model 5052 PR, Panametrics, Massachusetts) generates and receives waves through a pressure transducer (15 MHz, Custom size ∅2 mm, Valpey-Fisher, Massachusetts) that are reflected from the top of the microrheometer chamber or the ball (when inserted). The receiver amplifies and filters the voltage signal, produced by the reflected wave. The time interval between the emitted sound wave and the returned echo is converted into a Voltage through a Time-to-Voltage Converter (Model TVC 501, Tektronix, Florida). This voltage can be displayed on the vertical axis of a 150 MHz. Tektronix digital oscilloscope (Model TDS 420,
Tektronix, Florida). Thus, the oscilloscope waveform display gives a graphic representation of the position of the ball since it is proportional to the measured time interval. The speed of the falling ball is then determined from the slope given by the oscilloscope graphic representation.

In a falling ball experiment, the ball is pulled to the top of the chamber with the DC field of the electromagnet (the AC field is used in the oscillatory mode only). The current is then cut and the ball falls. The fluid viscosity is determined from the measured terminal velocity of the ball and is given by:

\[
\eta = \frac{2g R_b^2}{9K V_t} (\rho_b - \rho)
\]

where \(\eta\) is the steady state viscosity, \(R_b\) the radius of the ball, \(\rho_b\) the density of the ball, \(\rho\) the density of the fluid, \(V_t\) the terminal velocity, and \(K\) the wall correction factor determined for a given ratio \(R_b/R_t\) (\(R_t\) is the tube radius). For a ratio of \(R_b/R_t=0.8\), \(K\) is determined to be 73.6 [Haberman and Sayre, 1958].

![Figure 3.1: Schematic of the Magneto-Acoustic Ball Microrheometer.](image)
3.1.2. Blood Preparation

The patients were selected from a population of individuals attending the Cancer Center of Shands Hospital, University of Florida. AS and SS individuals were chosen for this study. A total of twenty individuals were studied: two normal, seven sickle trait, nine sickle cell and two SC donors. A two milliliters sample was systematically drawn and sent to Shands Hospital, where an Hemoglobin Electrophoresis (HE) and a Complete Blood Count (CBC) were done within six hours after the blood was drawn. The CBC was based on the Coulter method count (Coulter Counter MAXM). The hemoglobin electrophoresis was performed on acid agar and cellulose acetate using established Methods [Huisman et al., 1977]. Proportions of the different types of hemoglobin were determined from the Hb Electrophoresis, and the mean cell hemoglobin concentration (MCHC) from the CBC.

For these studies, blood from healthy donors, from donors with homozygous sickle cell disease and from sickle trait donors is collected by venipuncture into vacutainers using sodium heparin as an anticoagulant. Blood is stored at 5°C and all measurements are made within 36 hours from withdrawal. To obtain the red blood cells, whole blood is centrifuged (Model Marathon 6K, Fisher Scientific) at 2000 g for 25 minutes after which the plasma and buffy coat are removed by aspiration and carefully stored. The red cells are then washed three times in isotonic Phosphate Buffered Saline solution (PBS) (Dulbecco’s Phosphate Buffered Saline, D-PBS, 10 X). The pH of the PBS was previously adjusted to 7.4 (with 0.1 M HCl or 0.1 M NaOH) and its osmolarity was found to be 303 mosmol. The pH is measured with a digital pH meter (Model 220, Corning, New York). Experiments are done at 25 % hematocrit to standardize the data. Hematocrit is measured by centrifugation of a 20 µl sample at 13450 g for 3 minutes (Hematocrit tube, Cat-Number 1-000-7500-C, Fisher Scientific). The suspensions are then equilibrated at ambient air for thirty minutes, to ensure that all the samples start at a total oxygenation level (saturation in oxygen of about 98.8 %).
For the experiments with Clotrimazole (CLT), a final CLT concentration of 5µmol/L (based on the CLT plasma concentration reported by Brugnara [1996]) was investigated. The Clotrimazole was dissolved in a dimethyl sulfoxide (DMSO) solution (0.05% v/v). This low level of DMSO is not known to be damaging to the cells. SS RBC were resuspended at 50% hematocrit in the autologous plasma. Viscosity measurements were performed at 37°C, for two oxygen levels: fully oxygenated and totally deoxygenated. For each patient, a control is also run without CLT.

3.1.3. Blood Deoxygenation

A spin-cup tonometer (Model IL 237, Instrumentation Laboratory, Massachusetts, NIH, Maryland) connected to three flowmeter units (Model Accucal™, Gilmont, Illinois) respectively for oxygen, nitrogen and carbon dioxide is used to deoxygenate the blood. The blood suspension is loaded into the spin-cup tonometer containing a saturated mixture of O₂, N₂, CO₂ gases. The gas mixture determines the partial pressure in oxygen (pO₂) level and its value is verified with an oxygen needle electrode (pH/Needle Electrode, Model 757, Diamond General Corporation, Michigan). To prevent the cells from drying out, the gas mixture is saturated by bubbling it twice through water to ensure 100% saturation humidity. The CO₂ concentration in the gas mixture is kept at 5.6% in order to maintain the pH of the sample around 7.4. After allowing 5 to 10 minutes for the cells to equilibrate at a given pO₂, a 0.5 ml sample is withdrawn and used for the apparent viscosity measurement. At the same time, a portion of the blood sample is placed in a co-oximeter (Model AVOXimeter 1000, Avox Systems, Texas) to measure the oxygen saturation (SO₂), the total hemoglobin concentration, and the oxygen content.

3.1.4. Determination of Polymer Fraction

The main determinant of Hb S polymer formation is oxygen saturation. The two other important variables, among sickle cell populations, are intracellular hemoglobin concentration and composition. Experimental or theoretical methods can be used to determine the extent to
which the distribution in intracellular hemoglobin concentrations found in sickle erythrocytes affects the amount of intracellular polymerization of hemoglobin S.

Noguchi et al. [1983] and Noguchi [1984] developed a numerical analysis to calculate the polymer fraction, as a function of oxygen saturation. They analyzed the effects of hemoglobin and water non ideality and of erythrocytes heterogeneity on the polymerization of hemoglobin S in cell. The skeleton of the thermodynamic model was developed by Minton, [1974]. The addition of nonideal behavior of hemoglobin was derived by Minton, [1977]. The nonideal behavior of water in the solvent to the hemoglobin non-ideality in the thermodynamic description for Hb S polymerization was added by Gill et al. [1980]. Noguchi et al., [1983] and Noguchi, [1984] showed that the addition of water non ideality greatly improved the fit of the estimated prediction of hemoglobin S polymerization to the carbon 13 nuclear magnetic resonance (C NMR) measurements. The polymer fraction, $f_p$, is defined as the following ratio:

$$f_p = \frac{n_p}{n_T}$$

where $n_p$ is number of moles of hemoglobin in polymer form, and $n_T$ the total number of moles of hemoglobin. By assuming that the intracellular hemoglobin and composition of sickle erythrocytes are known and uniform, the polymer fraction can be derived as follows:

$$f_p = \frac{C_p (C_T - C_\sigma)}{[C_T (C_p - C_\sigma)]}$$

where $C_T = MCHC$, $C_p = $ concentration of hemoglobin in the polymer phase (69 g/dl), $C_\sigma = $ solubility of hemoglobin in the mixture expressed as a function of the oxygen saturation.

When the density profile is known, a more accurate polymer fraction can be expressed as:

$$f_p = \frac{C_p}{[C_\sigma (C_p - C_\sigma)]} \times \sum F_{ci} (C_i - C_\sigma)$$

where $F_{ci}$ is the fraction of cells with intracellular hemoglobin concentration $C_i$. However, in the present study, the density profiles are not measured, so the first formulation for the polymer fraction calculation will be used.
The whole theory has been summarized by Sunshine et al. [1982] and Noguchi et al. [1983]. An hemoglobin electrophoresis and a Complete Blood Count provide the proportions of hemoglobin (Hb F, Hb A, Hb S) and the MCHC respectively. The solubility, $C_\sigma$, is calculated based on these parameters as explained in the theory. A code developed by C.T. Noguchi outputs the predicted polymer fraction, $f_p$, as a function of the saturation in oxygen.

3.2. Instrumental Setup for Studying the Rheology of Individual Cells

3.2.1. The Micropipette Technique

The experimental setup consists of a micropipette, a chamber mounted on an inverted interference contrast microscope (Axiovert 100, Zeiss, Germany) with a 100x oil immersion objective, a water reservoir to control the hydrostatic pressure at the tip of the micropipette and a video system to videotape the experiments. A schematic of the experimental setup is shown in Fig. 3.2.

**Micropipette**

The micropipettes are formed from a 1 mm-outer diameter, 0.5 mm-inner diameter glass capillary tube (A-M Systems, Inc., Everett, WA). This glass capillary is mounted on a pipette puller (Model PB-7, Narishige, Tokyo, Japan), heated and quickly pulled to form a capillary with an inner diameter of only a few microns (from 4 to 6 µm). To form a flat tip, we use a microforge (Model MF 83, Narishige, Tokyo, Japan). The microforge consists of a horizontal dissecting microscope, a heated platinum wire and soda glass bead, and a horizontally mounted micromanipulator. The flat pipette tip is made by immersing the newly formed glass capillary into the molten glass bead, which has a lower melting point than the glass capillary. The melted glass from the bead flows into the capillary as shown in Fig. 3.3.a. Then, the bead is allowed to cool. After a few seconds, the bead is withdrawn and the pipette is broken by quick fracture to leave a flat tip as illustrated in Fig. 3.3.b. The pipettes have the tendency to fracture at the point
where the glass had stopped flowing into the pipette resulting in flat-tipped pipettes. The tube and tip are backfilled with a saline solution to avoid plugging. To prevent the cells from sticking to the glass capillary, the walls of the micropipette are coated with plasma proteins.

Figure 3.2.: Micropipette Setup
Figure 3.3.: Fabrication of Flat-tipped Micropipette

**Water reservoirs or manometer**

A system of two reservoirs is used: one adjustable reservoir and a reference reservoir as illustrated in Fig 3.4. The net zero of the manometer system is adjusted by zeroing the height between the two reservoirs (by opening the valve 1 between the two reservoirs), and adjusting the height of the two reservoirs relative to the micropipette stage. To adjust the reference reservoir at the same level as the pipette tip, the micropipette is connected to the reference reservoir, and the reference reservoir is positioned using a micrometer (Model 4104M, Daedal) so that there is no movement of a cell or tiny particle towards or away from the tip (in this case, the valve 2 is closed, and valve 3 opened). Once calibrated, the micropipette is disconnected from the reference reservoir by shutting the valve 3, and connected to the adjustable reservoir by opening the valve 2. Raising or lowering the adjustable reservoir with the micrometer produces a hydrostatic pressure that is greater or less than the pressure in the chamber at the tip of the pipette. By plugging a differential pressure transducer (Model DP 45-14, Validyne, Northridge, CA) between
the two reservoirs, it is possible to have an accurate reading of the hydrostatic pressure at the tip of the pipette.

Figure 3.4.: Micropipette Setup: Pressure Control and Image Acquisition
The output signal from the transducer is decoded via a carrier demodulator (Model CD 280-2, Validyne, Northridge, CA). The pressure transducer allows to detect pressure as small as 10 dyn/cm² which corresponds to a column pressure of 1 µm of water. Higher pressures are generated by creating a partial vacuum above the water in the adjustable reservoir. This is achieved by sealing the top of the adjustable reservoir and connecting a syringe or exerting mouth suction. To detect higher pressure, a higher pressure transducer is used (Model DP 15-30-N-1-S-4-A, Validyne, Northridge, CA). The pressure range is 80,000 Dyn/cm² with an accuracy of 400 Dyn/cm².

**Chamber**

The chamber is made of a 3 mm-thick Plexiglas piece, with an opening closed on the top and the bottom by two coverslips. The microscope stage has been designed to allow insertion of the pipette into the chamber. The pipette is positioned with a three dimensional macromanipulator (Model MMN-1, Narishige, Tokyo, Japan). While the fine motion is controlled by an hydraulic Joystick (Model MMW-22D, Narishige, Tokyo, Japan).

3.2.2. Leukocyte Isolation Procedure

Venous Blood is drawn from healthy adult donors into K₃ EDTA Vacutainers to get rid of Ca²⁺ that tends to activate neutrophils [Schmid-Schonbein et al., 1981]. The blood is diluted with equal volume of Hanks’Balanced Salts Solution (Ca²⁺ and Mg²⁺ free modified HBSS, Sigma Chemical Co.) and carefully layered over Ficoll-Hypaque gradient (Sigma Histopaque-1077 and 1119) as shown in Fig. 3.5.a.

The cells are then centrifuged at 900g for 25 min. at 25°C. After centrifugation those distinct cell layers can be observed: Layer 1 (plasma-Histopaque 1.077 interface) consists of mononuclear cells and platelets, Layer 2 (Histopaque 1.077-Histopaque 1.119) consists of granulocytes as shown in Fig. 3.5.b. Plasma, layer 1 and layer 2 are saved for the experiments.
Cells from layer 1 and layer 2 are resuspended in 10 X volume HBSS and centrifuged again at 400 g for 10 min. The supernatant is then removed, and the cells are washed again with HBSS. Finally, the cells are resuspended in a small volume of 50% plasma - 50% HBSS in order to give the cells an environment as physiological as possible while minimizing the cells adhesion and activation in contact to glass surfaces in the chamber and the pipette. Viability tests are performed with Trypan Blue Dye. Dead cells are stained in blue. Approximately, 90% of the cells are alive after this separation procedure. Cells of interest are then transferred into the micropipette chamber.

![Figure 3.5. Protocol for Leukocyte Separation](image)

Figure 3.5.: Protocol for Leukocyte Separation

3.2.3. Data Analysis

Two types of experiments will be run: aspiration and recovery as shown in Fig. 3.6. In the aspiration experiments, the entry flow inside the pipette is analyzed. The aspiration pressure is fixed, and the length of the cell projection $L_p$ is measured as a function of time. In the recovery experiments, the cells are deformed from their resting spherical geometry, then released and their
recovery to spherical shape is monitored. The experimental data from aspiration and recovery tests are analyzed assuming that the leukocyte behaves as a Newtonian liquid drop. In this model, the cell interior is modeled as a uniform liquid with an apparent viscosity $\mu$. The cell membrane is modeled as a shell under a pre-stressed isotropic surface tension designated as cortical tension $T_0$. For the moment, there is no rheological model available to fully describe the hydrodynamics of the leukocyte. Since the Newtonian liquid drop model has been shown to be valid for large deformation, long holding time and low rate of deformation [Evans and Yeung, 1989; Needham and Hochmuth, 1990; Tran-Son-Tay et al., 1991], it will be used as a tool for comparing the rheological properties between the neutrophils of normal and sickle patients for experiments performed in those conditions. Comparison between sickle and normal neutrophils will be established between experiments performed in the same conditions (similar aspiration pressure, holding time, pipette radius).
3.2.3.1. WBC aspiration experiments

From the WBC aspiration experiments, the cell cortical tension and viscosity are assessed. The cortical tension $T_o$ can be derived from the Laplace’s Law.

$$T_o = \frac{P_c \cdot R_p}{2(1 - \frac{R_p}{R_{out}})}$$

where $R_p$ is the radius of the pipette, $R_{out}$ the radius of the outside portion and $P_c$, the threshold pressure needed to create an hemispherical projection inside the pipette. The threshold pressure $P_c$ is determined by increasing the aspiration pressure by small increments until the cell projection inside the pipette would just exceed a pipette radius but without further flow. The cell cortical tension is responsible for the spherical shape and produces the driving force that leads to the recovery of the cell after deformation.

The cell apparent viscosity is calculated from a linearized version of the theoretical results of Yeung and Evans [1989] derived by Needham and Hochmuth [1990]:

$$\mu = \frac{(\Delta P - P_{cr}).R_p}{\left(\frac{dL_p}{dt}\right).m.(1 - \frac{R_p}{R_{out}})}$$

for $0.5 \leq R_p/R_{out} \leq 1$

where $\mu$ is the apparent cytoplasmic viscosity, $(dL_p/dt)$ is the rate of entry into the micropipette, the constant $m$ is proportional to $\eta'$, where $\eta'$ is the ratio of the surface dissipation in the cortex $\eta_m$ to the dissipation in the cytoplasm $\mu R_p$. Evans and Yeung indicate that $\eta' \cong 0.01$ which corresponds to value of $m=6$. 

Figure 3.6.: Aspiration and Recovery Experiments
3.2.3.2. Recovery experiments

For the recovery experiments, the approach of Tran-Son-Tay et al. [1991] will be used. In this analysis, the leukocyte is modeled as a Newtonian liquid drop, and the length (D) and width (W) of the cell as it recovers can be approximated by:

\[
\frac{D}{D_o} = \frac{D_i}{D_o} + A \tilde{t} + B(\tilde{t})^2 + C(\tilde{t})^3
\]

\[
\frac{W}{D_o} = \frac{W_i}{D_o} + D \tilde{t} + E(\tilde{t})^2 + F(\tilde{t})^3
\]

where \( \tilde{t} = 2 t T_o / D_o \), \( T_o \) is the cortical tension, \( D_i \) and \( W_i \) are the initial dimension of the deformed cell, \( D_o \) is the diameter of the spherical undeformed cell. The coefficients \( A, B, C, D, E, \) and \( F \) are known and are given by Tran-Son-Tay et al. [1991]. It should be noted that these parametric solutions are valid only for \( D_i/D_o \leq 3.5 \) and \( \tilde{t} \leq 3.5 \).
RESULTS/DISCUSSION

4.1. Rheology of Red Blood Cells in Sickle Cell Disease

4.1.1. Blood Analysis

Sickle cell anemia is one of the hemoglobinopathies, characterized by a type of hemoglobin with altered oxygen affinity, that causes anemia and other clinical consequences. Sickle cell subjects exhibit a clear disorder in their hemoglobin electrophoresis results, and is characterized by a high Hb S concentration. For the SS patients used in this study, the concentration of Hb S is found to be 84 ±13 %, (Table 4.1). Sickle cell individuals are usually anemic, which is confirmed by the CBC results (see hematocrit, Hct, and total hemoglobin concentration, Hb T, in Tables 4.1 and 4.2). SS donors clearly have a lower hematocrit: 28.7±7 %, than normal donors: 43±3.2 %, whereas sickle trait donors have only a slightly lower hematocrit: 38.8±4 %. As a consequence, the total hemoglobin concentration for sickle cell individuals is only: 9.5±2.1 g/dL, as opposed to 14.8 ± 0.9 g/dL for normal donors and 12.9 ± 1.1 g/dL for sickle trait donors. Individuals SS 3 and SS 4 are treated with Hydroxyurea which increases the amount of fetal hemoglobin (Hb F). Donors SS 2 also shows 20 % Hb F. Fetal hemoglobin is found in the reticulocytes and is transformed into Hb A during cell maturation.

Donors SS 5, SS 6, and SS 7 are individuals with sickle β+-thalassemia. The β+-thalassemia gene results in a decreased synthesis of β-chains. β+-thalassemia is generally associated with 18-30% Hb A, resulting in a mild disease condition [Serjeant, G.R, 1982]. Fetal and A₂ hemoglobins are usually found in larger proportions in those patients.

Table 4.1.: Hemoglobin Electrophoresis Data
Hb A₂, Hb A are normal types of hemoglobin, Hb S is sickle hemoglobin, Hb F is fetal hemoglobin, Hb C is an abnormal type of hemoglobin, MVC designs the Mean Cell Volume, Hct stands for the hematocrit, and the unit fL corresponds to µm³.

<table>
<thead>
<tr>
<th>Hb</th>
<th>HbA/C (%)</th>
<th>Hb S (%)</th>
<th>Hb F (%)</th>
<th>Hb A (%)</th>
<th>Remark</th>
<th>MVC (fL)</th>
<th>Hct (%)</th>
<th>Sex/Race</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS 1</td>
<td>1.0</td>
<td>34.9</td>
<td>0.3</td>
<td>63.8</td>
<td></td>
<td>84</td>
<td>39.4</td>
<td>F/B</td>
</tr>
<tr>
<td>AS 2</td>
<td>2.8</td>
<td>41.6</td>
<td>0.2</td>
<td>55.4</td>
<td></td>
<td>89</td>
<td>36.2</td>
<td>F/B</td>
</tr>
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**Table 4.2.: Complete Blood Count Data**

MCH and MCHC design respectively the Mean Cell Hemoglobin and the Mean Cell Hemoglobin Concentration.
Patients SS 8 and SS 9 are sickle βo Thalassemia. The βo-thalassemia gene is associated with an absence of normal β chains and hence of HB A, resulting in a condition which is electrophoretically, hematologically, and clinically similar to homozygous sickle cell (SS) disease.

Two patients (SC 1 and SC 2) have SC disease. SC patients have only hemoglobin S (more than 50 %) and hemoglobin C which is also an abnormal hemoglobin [Burn et al., 1982]. SC patients have a higher hemoglobin level and hematocrit than SS donors. Compared with subjects with homozygous SS disease, persons with hemoglobin SC are known to have a lower incidence of bacterial infection and fewer end-organ failures [Wong et al., 1996]. Wong et al. [1996] reported that Hb SC subjects have higher hemoglobin levels than Hb SS patients, lower total leukocyte, granulocyte, monocyte and lymphocyte counts.

Normal donors show values of normal hemoglobin close to 100 % as expected. On the other hand, sickle trait individuals have both Hb A and Hb S. The present sickle trait population

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<td>40.7</td>
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has subjects with an average of 38.6 ± 3.4 % of sickle hemoglobin and 59.4 ± 3.6 % of normal hemoglobin.

4.1.2. Effect of Polymer Fraction

4.1.2.1. Polymer fraction profiles

Polymer fraction profiles were generated from the Hb Electrophoresis data, by Dr. Noguchi and co-workers (NIDDK, NIH, Bethesda, Maryland), using a code that expresses the polymer fraction in terms of the oxygen saturation. This program takes as inputs the representative proportions (in %) of normal hemoglobin, fetal hemoglobin, sickle hemoglobin, and finally the Mean Corpuscular hemoglobin Concentration (MCHC in g/dL).

The upper and lower bounds of the polymer fraction profiles for SS and AS individuals are represented in Fig. 4.1, profiles for one SC patient and one SS patient treated with Hydroxyurea are also plotted for comparison. The complete set of polymer fraction profiles for the donors studied is given in Appendix A.
Figure 4.1.: Polymer Fraction Profiles. Upper and lower bounds of the polymer fraction profiles are represented for SS and AS patients. Profiles for one SC patient and one patient treated under Hydroxyurea (HU) are also presented.

For SS patients, the range of polymer fraction formed goes from 47 to 63 % for a deoxygenation at 0 % saturation in oxygen. Polymerization of SS hemoglobin starts for a saturation in oxygen below 80 %. Polymer fraction curves for donors treated on Hydroxyurea are beneath the profiles of SS donors. Although the polymerization process for SS cells starts at a comparable value in SO₂, the amount of polymer inside the erythrocytes of SS patients treated with Hydroxyurea is considerably less at 0 % SO₂ than of those without treatment. The maximum polymer fraction formed for the represented HU patient is 38 % as opposed to 55 % for
non treated patients. The presence of Hb F in sickle erythrocytes results in a lower concentration of Hb S and therefore decreases the chance for polymerization. The same polymer profiles are obtained for the patient with a significant amount of fetal hemoglobin (patient SS 2) and patients under HU treatment. The SC profiles falls between the AS and SS profiles. The effect of Hb C on polymerization is comparable to that of Hb A, meaning that Hb C and Hb A molecules interact similarly with Hb S molecules. The SC polymer profiles are higher than AS because SC patients have more sickle hemoglobin than AS patients. As expected, polymer fraction values for AS donors are much lower than for SS individuals. The range of polymer fraction formed inside AS cells is between 27 % and 35 % at total deoxygenation. For these cells, polymer starts forming for a saturation in oxygen below 50-60 %. For AA patients, there is no polymerization of the hemoglobin under deoxygenation.

4.1.2.2. Effect of dehydration

Fig. 4.2. shows the apparent viscosity of AS and SS RBC suspended in PBS as a function of the polymer fraction. This figure underlines the effect of dehydration. In the experiments performed by Morio [1996] and in the early experiments of the present study, the fully oxygenated blood sample is gradually deoxygenated to six different levels of partial pressure in oxygen (pO₂). These experiments are thereafter referred as 1st set of experiments. Thus, in order to measure the viscosity at the six selected pO₂ levels, the blood sample must stay in the tonometer chamber for about two hours (at each pO₂ levels, the sample is incubated for 20 min. with the gas mixture). However, deoxygenation of the blood sample at body temperature (37°C) using nitrogen gas for extended time induces dehydration. This effect can be identified by measuring the hematocrit or the total hemoglobin concentration before and after deoxygenation. Dehydration is particularly observed in the high polymer fraction data corresponding to the lowest pO₂ levels. These data are from samples that have been incubated for 2 hours in the
spincup tonometer. A review of the data indicates that the hemoglobin concentrations of these blood samples have increased and therefore that dehydration has occurred.

In the experiments performed later in this study, referred hereafter as 2nd set of experiments, a new blood sample is deoxygenated for 10 minutes as opposed to 20 minutes for each of the pO₂ levels selected to avoid dehydration. This is checked by measuring the hemoglobin concentration before and after deoxygenation. The data do not show the high viscosity values at the low pO₂ levels as seen in the 1st set of experiments. It is also found that the viscosity values obtained during reoxygenation are higher than those obtained during deoxygenation. The observed hystheresis is mainly due to the drying of the blood which increases the hematocrit and consequently causes an increase of the apparent viscosity. The data without dehydration show that if the experiments are performed at a constant hematocrit of 25 %, the blood apparent viscosity varies linearly with polymer fraction: \( \eta = 3.2969 \text{PF} + 1.7907 \) with \( r^2 = 0.75 \), where PF designs the polymer fraction and \( \eta \) the apparent viscosity of RBC suspensions.

For comparison, the apparent viscosity of normal RBC suspensions in PBS at 25% hematocrit is equal to 1.73±0.06 cP. and does not depend on the level of deoxygenation.

4.1.2.3. Effect of plasma

The protocol designed for the 2nd set of experiments to avoid dehydration is used in all the experiments to follow. Thus, intracellular hemoglobin polymerization should be the only factor responsible for the increase in viscosity. Fig. 4.3 regroups the data at 25% hematocrit for AS and SS RBC suspended in PBS and autologous plasma. The viscosity data in plasma are slightly higher and more scattered than those in PBS. The best correlation between apparent viscosity and polymer fraction is obtained with the PBS data: \( \eta = 3.9269 \text{PF} + 1.7907 \) \( r^2 = 0.75 \). Plasma data give a trend similar to PBS data \( \eta = 4.176 \text{PF} + 2.8268, r^2 = 0.53 \), but are translated by a value of about 1 cP. The coefficient of correlation in plasma is lower than in PBS because of the
larger data scattering in plasma. For healthy subjects, the apparent viscosity for cell suspension is independent of the level of SO$_2$ or pO$_2$, the apparent viscosity is 1.73±0.06 cP in PBS and 2.10±0.07 cP in plasma. No polymer is formed in these healthy control cells.

Figure 4.2.: Apparent Viscosity vs. Polymer Fraction: Effect of Dehydration. AS and SS data at 25 % hematocrit are shown (Note: points at high polymer fraction are artifacts due to sample drying).
Figure 4.3.: Apparent Viscosity vs. Polymer Fraction: Effect of Plasma. AS and SS data in PBS and plasma are shown.

4.1.2.4. Effect of hemoglobin type

The apparent viscosity seems lower for AS patients than SS patients, but the difference is not statistically significant (Fig. 4.4). The linear regression for SS RBC suspended in PBS is: \( \eta = 4.078 \cdot PF + 1.8256 \) with a coefficient of correlation \( r^2 = 0.80 \), and for AS RBC suspended in PBS is: \( \eta = 2.2454 \cdot PF + 1.7201 \) with \( r^2 = 0.65 \). These results confirm our hypothesis that from a
rheological viewpoint it is not the type of hemoglobin, *per se*, that is important but the amount of polymer formed inside the cell.

Therefore, data for AS and SS cells obtained as a function of polymer fraction can be combined. Fig. 4.5 summarizes the results performed in PBS for AS and SS cell suspensions, the correlation between the apparent viscosity and polymer fraction is given by: \( \eta = 3.9269 \cdot \text{PF} + 1.7907 \) with \( r^2 = 0.75 \).

**Figure 4.4.:** Polymer Fraction vs. Apparent viscosity: Effect of Hemoglobin Type
4.1.3. Effects of Drugs

The effect of Hydroxyurea (patients SS 3 and SS 4) is shown in Figs. 4.1 and 4.6. The effect of having a high Hb F concentration can also be observed with patient SS 2 who has 20% of Hb F. The polymer fraction profile of patient SS 4 is below the two other patients because donors SS 4 has a higher proportion of fetal hemoglobin. A high level of fetal hemoglobin results in a lower intracellular polymerization and therefore apparent viscosity.

Figure 4.5.: Correlation Between Polymer Fraction and Apparent Viscosity
Clotrimazole does not produce a significant reduction in the apparent viscosity (Fig. 4.7). Experiments are done at 50 % hematocrit to enhance the effect of the drug but no statistically significant difference is observed. It should be noted that at 50 % hematocrit, the viscosity increase under deoxygenation is much higher than at 25 % hematocrit. For SS patients, the viscosity increases from about 5 cP to 25 cP. For AS patients, the viscosity increases from about 4.5 cP to 8 cP. These results stress the importance of hematocrit and clearly indicate that the difference in viscosity between AS and SS donors becomes important when the hematocrit increases. This difference comes from the difference of sickle deoxyhemoglobin polymerization between AS and SS RBC at zero oxygen saturation.
4.1.4. Discussion

The results show that the apparent viscosity varies linearly with polymer fraction for AS, SS and SC red blood cell suspensions. This confirms our hypothesis that polymer fraction is the major parameter in sickle cell disease. It is not the type of hemoglobin per se that is critical from a rheological viewpoint but the amount of polymer inside the cells.

The difference in viscosity seen between RBC suspended in plasma and in PBS comes from two factors: first, there is an intrinsic viscosity difference between plasma and PBS. The viscosity values of PBS and plasma measured in the microrheometer are found to be 1.12±0.02 cP and 1.43±0.02 cP at 37° C, respectively. When the data are compensated for the difference in the suspending fluid viscosity, a good agreement is found between the data. Second, plasma proteins may play a role in the aggregation of erythrocytes. Usami et al. [1975] noted a
difference of viscosity between SS RBC suspended in plasma and Ringer solution. The viscosity difference observed in the present study is not as pronounced as in the study by Usami et al., because of the reduction in hematocrit. At 25 % hematocrit, there is less interaction between the erythrocytes than at 45 % hematocrit. The higher scattering observed in the plasma data is due to the aggregation of RBC. Since aggregation is another variable that affects the apparent viscosity, a better correlation between apparent viscosity and polymer fraction is obtained with the PBS data.

As expected, hemoglobin polymerization is the major factor in the increase in blood viscosity. Measurements of intact SS, SC, AS suspensions in PBS are comparable although Fig. 4.4 shows that AS cells have viscosity values that are slightly lower than for SS cells. The viscosity difference between AS and SS donors may come from the cell membrane. SS patients have cells with membrane abnormalities and irreversible sickle cells (ISC) whereas AS subjects do not [Platt et al., 1985; Hiruma et al., 1995 a]. However, the observed difference in viscosity is small. When AS and SS data are regrouped together, the coefficient of correlation does not vary too much, indicating that AS and SS viscosity data follow the same trend as the polymer fraction increases. Polymer fraction is a parameter that contains vital information about the patient, particularly, the proportion of sickle hemoglobin. It is shown that polymer fraction is a good parameter for characterizing the rheology of sickle cell blood. In contrast, pO\textsubscript{2} and SO\textsubscript{2} also affect the blood rheological properties but cannot be used for evaluating sickle cell disease.

Clotrimazole does not significantly decrease the blood apparent viscosity (Fig. 4.7) even though it is reported to have some effect on other cell mechanical properties. Stuart et al., 1994, studied in vitro the effect of Clotrimazole on the deformability of sickle cells using filterability techniques. They showed that Clotrimazole has some positive effect in decreasing the red cell transit time (RCTT) through 5 \(\mu\)m after a 15 hour period of oxygenation-deoxygenation cycle. The differences between control and Clotrimazole tests noted by Stuart et al., although
significant, are small. Since filterability techniques provide information on the rheology of individual cells, they are more sensitive to changes in the cell membrane properties and morphology than viscometric devices. It has also been shown by Brugnara et al. [1996] that Clotrimazole reduces intracellular hemoglobin concentration. This should reduce the polymer fraction. But the reduction in intracellular hemoglobin concentration should mainly affect the kinetics of polymerization in vivo as pointed out by Eaton and Hofrichter [1994]. By reducing the hemoglobin intracellular concentration, the polymerization delay time in vivo is increased, thus the red blood cells have time to go through the capillaries without polymerizing. However, this difference in polymerization kinetics, if detectable, cannot be observed in the present experiments because they are performed at equilibrium.

The viscosity augmentation under deoxygenation for RBC suspended at 25 % is low: the viscosity increases from about 2 cP to 5 cP. Previous studies, done at higher hematocrit (mainly 45 % and 90 %) have reported higher viscosity values under deoxygenation [Usami et al., 1975; Chien et al., 1982]. However, their data are in agreement with the present study. For example, in the Clotrimazole experiments, the viscosity of the control samples increases from 5 cP (oxygenated, pO₂ = 160 mm Hg) to 25 cP (deoxygenated, pO₂ = 0 mm Hg) at 50 % hematocrit and 37°C. Usami et al. measured the viscosity of SS RBC suspensions in Ringer Solution at 45 % hematocrit and 37°C as a function of pO₂ in a coaxial cylinder viscometer. For a shear rate of 208 s⁻¹, which corresponds to the magnitude of the shear rates generated in the microrheometer (in the present experiments, the maximum shear rate varies between 400 and 1500 s⁻¹ depending on the viscosity), Usami et al. found an apparent viscosity of 4 cP at pO₂ = 120 mm Hg and 18 cP at pO₂ = 10 mm Hg.

This study shows that hematocrit has a large influence on the blood rheology. However at 25 % hematocrit, which corresponds to the physiological condition in SCD, the contribution of RBC sickling on the overall blood viscosity is small. Therefore, in order to better understand the
pathophysiology of sickle vaso-occlusive crisis, it is also indispensable to consider the rheology of individual blood cells and of white blood cells in particular.

4.2. Rheology of White Blood Cells in Sickle Cell Disease

4.2.1. Comparison between Normal and Sickle Leukocytes

4.2.1.1. Aspiration experiments

The aspiration experiments are performed on neutrophils from two different patients, sickle patient SS 6 and control donor AA 1, under similar conditions (i.e. similar aspiration pressure, $\Delta P$, and pipette radius, $R_p$). The cell viscosity is calculated from the rate of entry into the pipette as seen in Figs. 4.8 and 4.9. Aspiration experiments on sickle neutrophils are done at $\Delta P=28$ Pa and $R_p=2.6\ \mu m$. The cell apparent viscosity for the cell presented on Fig. 4.8 is equal to $\eta=427$ Pa.s with a coefficient of correlation $r^2=0.9952$. The average viscosity value for patient SS 6 is $436\pm68$ Pa.s for 6 cells. For the control neutrophils, the experiments are performed at $\Delta P=30$ Pa and $R_p=2.4\ \mu m$. The cell apparent viscosity for the cell presented on Fig. 4.9 is equal to $\eta=422$ Pa.s with a coefficient of correlation $r^2=0.9968$. The average viscosity value for the control is $414\pm57$ Pa.s for 6 cells. The difference in cell viscosity between the two populations is not significant.

The cortical tensions of sickle and normal neutrophils are also found to be comparable, the average value measured in the present study is $2.5\pm0.5\times10^5$ N/m. This value is used to interpret the recovery data to follow.
Figure 4.8.: Typical Aspiration Experiment: Sickle Neutrophil \( \eta = 427 \pm 11 \text{ Pa.s}, \Delta P = 28 \text{ Pa}, R_p = 2.6 \mu \text{m}, \text{donor SS 6} \)

Figure 4.9.: Typical Aspiration Experiment: Control Neutrophil \( \eta = 422 \pm 13 \text{ Pa.s}, \Delta P = 30 \text{ Pa}, R_p = 2.4 \mu \text{m}, \text{donor AA 1} \)
4.2.1.2. Recovery experiments

Typical cell recovery shapes, as a function of the time after expulsion from a 5.0 \( \mu m \) diameter pipette, are presented in Figs. 4.10 and 4.11 for sickle and normal neutrophils, respectively. The best fit between the experimental data and the shape equations derived by Tran-Son-Tay et al. [1991] (see Material and Methods section 3.2.3) gives the value of the apparent viscosity. For long holding times \( (T_{\text{holding}} > 10 \text{ s}) \), the Newtonian liquid drop model gives a good fit. On Fig 4.10, the best curve fit for the presented cell gives a viscosity of 239 Pa.s with a deviation value from the curve fit of \( \pm 11 \text{ Pa.s} \). On fig 4.11, the best curve fit for the presented cell gives a viscosity of 147 Pa.s with a deviation value from the curve fit of \( \pm 8 \text{ Pa.s} \).

Table 4.3 summarizes the apparent viscosity measurements done on 8 different neutrophils from 2 sickle cell and 2 healthy donors \( (T_{\text{holding}} > 10 \text{ s}, R_p=2.5 \mu m) \). There is great variability within a single subject, the standard deviation is about 30 Pa.s, and also between subjects. Neutrophils from patient SS 6 have viscosity values similar to those from control donors. However, neutrophils from patient SS 9 show a slightly higher, but statistically significant, apparent viscosity than control cells.
Figure 4.10: Typical Recovery Experiment: Sickle Neutrophil ($\eta=239\pm11$ Pa.s, $T_{\text{holding}}=10$s, $R_p=2.5$ $\mu$m, SS 9)
Figure 4.11.: Typical Recovery Experiment: Control Neutrophil ($\eta=147\pm8$ Pa.s, $T_{\text{holding}}=10s$, $R_p=2.5\ \mu m$, AA 1)

Table 4.3.: Apparent Viscosity of Sickle and Normal Neutrophils: Recovery Experiments. The viscosity values are in Pa.s. The asterix, *, denotes statistically significant difference (ANOVA one way).

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<td>139</td>
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<tr>
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<td>Average=158±34</td>
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4.2.1.3. Effect of holding time

In this experiment, a neutrophil is aspirated into a 5.2 $\mu m$ pipette radius at an aspiration pressure of 500 Pa. The cell is then held inside the pipette for a period of time, characterized as holding time, before being expelled from the pipette. The subsequent recovery of the cell is analyzed. Experiments with holding times of 15 and 2 seconds on the same cell are investigated. The experiment with long holding time is performed first, then the same cell is reaspirated for short holding time experiment.

The apparent viscosity values are significantly different between long and short holding times as observed by Tran-Son-Tay et al. [1991]. The 2 sec holding time leads to a smaller apparent viscosity than the 15 sec holding time, indicating of non-newtonian behavior of neutrophils.
Table 4.4.: Effect of the Holding Time on the Recovery of Normal Neutrophils. Values for the cell viscosity are in Pa.s. The symbol, *, denotes significant difference (paired t-test).

<table>
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<th>Normal neutrophil #1</th>
<th>15 sec holding time</th>
<th>2 sec holding time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal neutrophil #2</td>
<td>154</td>
<td>93</td>
</tr>
<tr>
<td>Normal neutrophil #3</td>
<td>190</td>
<td>110</td>
</tr>
<tr>
<td>Normal neutrophil #4</td>
<td>162</td>
<td>96</td>
</tr>
<tr>
<td>Normal neutrophil #5</td>
<td>123</td>
<td>76</td>
</tr>
<tr>
<td>Average</td>
<td>154.2±26</td>
<td>92.6±12*</td>
</tr>
</tbody>
</table>

4.2.1.4. Summary of WBC experiments

Table 4.5. summarizes the experimental data from aspiration and recovery experiments. Only slight differences between normal and sickle neutrophils were reported in the recovery experiments. The differences of viscosity between aspiration and recovery experiments, and between short and long holding time confirm that the Newtonian liquid drop is not an adequate model to describe the hydrodynamics of the leukocytes. If the liquid drop model is to have any validity, the rheological parameter (i.e. apparent viscosity in this case) should be constant and not function of extent and rate of deformation.

Table 4.5.: Summary Table: the values of the mean viscosity are given in Pa.s. Th designs the holding time

<table>
<thead>
<tr>
<th>Viscosity (aspiration)</th>
<th>Viscosity (recovery)</th>
<th>Viscosity (recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>SS</td>
<td>AA</td>
</tr>
<tr>
<td>414±57</td>
<td>436±68</td>
<td>146±34</td>
</tr>
<tr>
<td>146±34</td>
<td>189±29</td>
<td>154±26</td>
</tr>
<tr>
<td>long T_h (AA)</td>
<td>short T_h (AA)</td>
<td>93±12</td>
</tr>
</tbody>
</table>
4.2.2. Nucleus Visualization

In order to completely describe the rheological properties of leukocytes, it is necessary to characterize the rheological properties of the nucleus [Kan, 1997]. Visualization of the nucleus during leukocyte aspiration and recovery provides valuable information on the nucleus viscoelastic properties. A new fluorescent assay has been developed for that purpose.

A UV excited, blue fluorescent bisbenzimidazole dye designated as Hoechst is used. This dye is reported to be cell-permeant and acts as a DNA-specific fluorochrome when bound to sequences of three A-T base pair in DNA. Hoechst 33342 (Sigma chemical) has been specifically chosen because it has slightly higher membrane permeability than Hoechst 33258 [Arndt-Jovin and Jovin, 1989]. Both dyes are water soluble and relatively non toxic. Stoichometric vital staining of DNA by Hoechst 33342 generally requires exposure of the cells to the dye solution (5-10 µM) for at least 30 min. [Shapiro, 1995]. Lower concentrations (1-2 µM) or shorter incubation periods can be used to demonstrate differences in rates of dye uptake between cell types [Lalande et al., 1980]. This technique has been used to differentiate resting T lymphocytes (low uptake) from resting B cells and activated T cells (high uptake) [Loken, 1979; Shapiro, 1982].

A stock solution of Hoechst 33342 is made by dissolving 0.1 mg of the dye into 1 ml of Hanks’ balanced salts solution. A working solution (cc=10µg/ml, pH=7.3) is freshly made by diluting 10 X the stock solution. The cells are incubated for 30 min. at room temperature in a solution with a final concentration of 5µg/ml or 8.5 µM (50% working solution - 50% cells solution).

After incubation, the cells are placed in the chamber under an inverted microscope equipped with fluorescent capability (Zeiss Axiovert 100, 100X oil immersion objective). To excite the Hoechst 33342 dye, a variable intensity mercury arc lamp (Zeiss, HBO 100 W AttoArc) is used. The cells are first excited with a low light intensity (10 %), then the intensity is
progressively increased during the experiment to allow visualization of the nucleus. Cells are only illuminated for short periods to prevent quenching and cell damage due to long exposure to UV excitation.

Images are obtained with a high sensitivity (0.0003 lux) television camera (CCD Video camera system, Model ZVS-47 DEC) and displayed on a color TV monitor (Sony). The experiments are also recorded with a VCR (Panasonic, AG 1730) and pictures are grabbed through a Power Macintosh 7100/80 AV, and analyzed with the NIH image 1.61 software. A sequence of pictures showing the aspiration of a 7.5 µm lymphocyte into a 3.7 µm pipette is presented in Fig. 4.12. The deformation of the nucleus and contour of the cell can clearly be seen.
4.2.3. Discussion

There is a good agreement between the experimental data and the Newtonian liquid drop model as seen on Figs. 4.8 to 4.11. It has been shown by Tran-Son-Tay et al. [1991], that if the holding time is long enough (>8 seconds), and the pipette diameter between 4 and 6 µm, the neutrophil recovers as a Newtonian liquid drop. The values for the cortical tension and the apparent viscosity measured in the present studies for normal neutrophils, long holding time and large deformation are comparable to those measured by Tran-Son-Tay et al. [1991]. In their recovery experiments, Tran-Son-Tay et al. measured an apparent viscosity of 152±40 Pa.s, with a cortical tension of 0.024 dyn/cm.
From the experimental results, particularly the recovery tests, there is a slight difference in the apparent viscosity between sickle and normal neutrophils in their passive state. However, there is a great variability from donor to donor. Although one SS patient (SS 9) shows consistently a higher apparent viscosity, it is not clear if this is due to a natural difference between SS and control cells or because of the variability between subjects. SS population is not homogenous and the proportion of sickle hemoglobin greatly varies between sickle patients (Table 4.1). This explain why clinical manifestations in sickle cell disease are extremely variable. In addition to this heterogeneity factor, the patient condition at the moment of blood collection (i.e., steady state period without crisis or period after crisis) also influences the leukocyte mechanical properties. Abnormalities in the leukocyte rheological behavior and the number of inflammatory cells depend on the patient condition. Boghossian et al. [1991] observed that the leukocyte adhesive and rheological properties depend on the steady state or crisis condition of the patient. In the present experiments, only neutrophils in their passive state are studied. Pseudopods or uneven recovery are direct indicators of neutrophils activation. However, a slight neutrophil activation could have occurred and not been detected through the optical microscope, and could explain the higher apparent cell viscosity for patient SS 9.

Patient SS 9 is sickle β<sup>0</sup>-Thalassemia, which is characterized by a high proportion of sickle hemoglobin and a low hematocrit [Serjeant, G.R., 1982]. Recent studies [Kauschau et al., 1996; Hofstra et al., 1996] have shown the role of plasma factors in activating neutrophils in SS patients. From the present experiments, it is not clear whether SS neutrophils in their passive state behave differently from controls or were slightly activated producing a different rheological behavior.

The values obtained for the apparent viscosity with the aspiration experiments are in agreement with those measured by Tsai et al. [1993] at comparable aspiration pressures. They reported apparent cell viscosities of 450±93 Pa.s for an aspiration pressure of 90 Pa. These
different apparent viscosities between aspiration and recovery experiments evidence the non-
newtonian behavior of neutrophils. In this study, the shear rate between aspiration and recovery
experiment is different, aspiration experiments are performed at lower shear rates and lead to
higher apparent viscosities than recovery experiments. This shear thinning behavior has been
observed in other studies [Tsai et al., 1993; Zhelev and Hochmuth, 1994]. In addition, the
recovery experiments done at two different holding times confirm the non-newtonian behavior of
the neutrophil.

By taking into account the nucleus, the three layer model developed by Kan et al. [1997]
can explain the results in term of the energy stored in the deformed nuclear and cellular
interfaces. The proposed fluorescent technique for visualizing the nucleus under deformation is
shown to be effective. It allows staining of the nucleus without activating the leukocytes.
Absence of activation is evidenced by the absence of pseudopods, and the corresponding recovery
times are comparable to those obtained using standard light microscopy. Under fluorescent
illumination conjugated with visible light, the nucleus, the cell membrane and the pipette tip can
be visualized at the same time.
CONCLUSIONS

5.1. Summary

The present study on the rheology of blood cells in sickle cell disease provides the following insights into the understanding of this complex disease.

(1). The apparent viscosity measured with the microrheometer is shown to be a good parameter to assess the clinical severity of the disease.

(2). There is a linear relationship between the apparent viscosity and polymer fraction. This relationship does not depend on the sickle trait of the patient, indicating that hemoglobin polymerization within the erythrocytes is a dominant factor in the rheology of sickle cell disease.

(3). At physiological hematocrit in SS disease, the increase of the apparent blood viscosity due to the hemoglobin polymerization is small. This leads to the conclusion that vaso-occlusive crisis cannot be explained in term of blood viscosity increase alone. Other factors are implicated in the disease and the study of individual cells is necessary to characterize the pathophysiology of the disease.

(3). Whereas Hydroxyurea displays a positive effect in reducing hemoglobin polymerization, the benefit of Clotrimazole has not been evidenced in this study.

(4). The contribution of leukocytes in the initiation of vaso-occlusive disease has been highlighted.

(5). Sickle neutrophils appear to be slightly more resistant to deformation than control cells but this difference is small.

(6). A fluorescence assay can be used to simultaneously observe the nucleus and cytoplasmic membrane during the leukocyte deformation process. This procedure will be useful in characterizing the rheological properties of the cell.
5.2. Future Work

Below, is a list of observations and suggestions that should be considered in order to improve our understanding of the biorheological behavior of blood cells in sickle cell disease.

(1). Effects of other drugs should be investigated using the microrheometer. For example, stereospecific agents binding to Hb S that block the intermolecular Hb S subunit contacts required for polymer formation, should have a detectable effect on the apparent viscosity measured with the microrheometer.

(2). A device that allows the study of the influence of polymerization kinetics will be useful to characterize in vitro the effects of drugs. This device should permit rapid blood deoxygenation or reoxygenation. For example, blood flow resistance through gas-permeable hollow fiber could provide additional information on the kinetics of polymerization.

(3). Concerning the fluorescence techniques, preliminary results have shown that this method is effective in staining the nucleus. Quantitative data on the rheological parameters of the nucleus need to be obtained. These experimental results could then be used to verify the numerical analysis developed by Kan [1997].

(4). Study of the flow of leukocytes into constricted or tapered geometries (see Fig 5.1) using the fluorescence method will also be essential for modeling the leukocyte rheological behavior. Flow of leukocytes into these geometries allows a more accurate interpretation of the entry flow conditions into/inside the pipette.
Figure 5.1.: (a) Flow of Leukocyte into a Constricted Tube, (b) Flow into Tapered Tube
APPENDIX A

Polymer Fraction Profiles for AS, SC, and SS Patients

The abscisse $x$ represents the oxygen saturation, the ordinate $y$ the polymer fraction

**AS 1**  $y = -0.4167x^3 + 0.1132x^2 - 0.4839x + 0.2835$

**AS 2**  $y = -0.4046x^3 + 0.1047x^2 - 0.4947x + 0.3393$

**AS 3**  $y = -0.4130x^3 + 0.1029x^2 - 0.4676x + 0.2752$

**AS 4**  $y = -0.4398x^3 + 0.1397x^2 - 0.5063x + 0.3258$

**AS 5**  $y = -0.4306x^3 + 0.1360x^2 - 0.5054x + 0.3161$

**AS 6**  $y = -0.4361x^3 + 0.1390x^2 - 0.4998x + 0.2970$

**AS 7**  $y = -0.4769x^3 + 0.1728x^2 - 0.5276x + 0.3509$

**SS 1**  $y = -0.5030x^3 + 0.4181x^2 - 0.8354x + 0.6324$

**SS 2**  $y = -0.2480x^3 - 0.1885x^2 - 0.2816x + 0.4658$

**SS 3**  $y = -0.2501x^3 - 0.0773x^2 - 0.4472x + 0.5045$

**SS 4**  $y = -0.2965x^3 - 0.0717x^2 - 0.3314x + 0.3771$

**SS 5**  $y = -0.4351x^3 + 0.2706x^2 - 0.6957x + 0.5195$

**SS 6**  $y = -0.3896x^3 + 0.1899x^2 - 0.6387x + 0.5101$

**SS 7**  $y = -0.5167x^3 + 0.3364x^2 - 0.6900x + 0.4745$

**SS 8**  $y = -0.4669x^3 + 0.3508x^2 - 0.7924x + 0.6291$

**SS 9**  $y = -0.2966x^3 + 0.1107x^2 - 0.6738x + 0.6264$

**SC 1**  $y = -0.5167x^3 + 0.3001x^2 - 0.6420x + 0.4066$

**SC 2**  $y = -0.3778x^3 + 0.1126x^2 - 0.5526x + 0.4369$
A) LIST OF REFERENCES


B) BIOGRAPHICAL SKETCH

Emmanuelle Damay was born on November 22, 1973, in the city of Amiens, France. She received her “Diplôme d’Ingénieur” in biomedical engineering from the University of Technology of Compiègne, France, in November 1996. Her motivation to learn more and to get experience from a stay in a foreign country led her to come for graduate work at University of Florida in the Department of Aerospace Engineering, Mechanics and Engineering Science in Fall 1995. She graduated with a Master of Science in engineering mechanics, in August 1997.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Roger Tran-Son-Tay, Chair
Associate Professor of Aerospace Engineering, Mechanics and Engineering Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Wei Shyy
Professor of Aerospace Engineering, Mechanics and Engineering Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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Professor of Materials Science and Engineering

This thesis was submitted to the Graduate Faculty of the College of Engineering and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Master of Science.

August, 1997

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