There is wide variation in the clinical manifestations of sickle cell disease (SCD) from one affected individual to another. Many investigators have sought to discern parameters that would explain this variability. In the present studies we have attempted to correlate the frequency of painful events and the extent of end organ failure in SCD with rheologic properties of packed suspensions of sickle cells, using a magneto-acoustic ball microrheometer developed in our laboratory. Using this device we have measured the steady-state viscosity, and the viscous and elastic moduli of cell suspensions in 18 individuals with hemoglobin SS disease who were untransfused and in their steady state. The rheologic parameters were then correlated with clinical parameters. The clinical parameters measured were emergency department visits, hospitalizations, hemoglobin, reticulocyte count, age, and end organ failure (nephropathy, avascular necrosis of bone, stroke, retinopathy, resting hypoxemia after acute chest syndrome(s), leg ulcer, and priapism with impotence). The $P$ value for the correlation between the steady state viscosity and end organ failure was .001 with a correlation coefficient ($R$ value) of .73. The $P$ value for the correlation between the viscous modulus of viscosity and end organ failure was .00006 with an $R$ value of .83. The $P$ value for the correlation between the elastic modulus of viscosity and end organ failure was .0006 with an $R$ value of .76. However, there was no significant correlation between any component of packed cell rheology and emergency department visits or hospitalizations for pain.

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MATERIALS AND METHODS

Patients. All patients were selected from the population of individuals attending the adult clinic of the Duke University Comprehensive Sickle Cell Center. The individuals chosen for the study had Hb SS confirmed by cellulose acetate and citrate agar, Hb F less than 10% by column chromatography, and 4 $\alpha$-globin genes by Southern blot analysis. These individuals were all at least 4 months from their last blood transfusion and had no symptoms of vaso-occlusion at the time of sample withdrawal. Multiple determinations of the rheologic parameters were made and the average was used in the statistical analyses reported in Results.

Frequency of painful events. Painful crises in this study were defined as episodes of pain sufficiently severe to prompt a visit to the emergency department or an inpatient hospitalization in which the individual received parenteral narcotic analgesia. An emergency department visit that led to an inpatient hospitalization was scored as a hospitalization, but not as an emergency department visit in the statistical analyses. Most of the patients in the study

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received all of their care at Duke Medical Center. However, due to distance from the medical center, some individuals received care at their local hospital. All information regarding emergency department visits and/or hospitalizations was collected retrospectively by a data coordinator and confirmed by a physician who was not aware of the results of the rheologic studies. Documentation of painful events covers a period of 1 year before the time that samples were withdrawn for the rheologic studies. A painful vaso-occlusive episode of SCD was presumed to be the correct diagnosis if the painful episodes lasted for 4 hours or longer, the patient felt that the pain was secondary to vaso-occlusion (typical pain for them), and no other etiology of the pain was apparent. Exacerbations of chronic painful conditions such as avascular necrosis of bone or pain in a leg ulcer were not counted as a vaso-occlusive episode. End organ failure was a cumulative score obtained at the time of sample collection (count of all end organ events over the life of the patient up to the time of the rheologic studies).

**End organ failure score.** In gathering these data, no attempt was made to generate a clinical severity score. The patients’ charts were reviewed to ascertain whether or not they had any of the following complications of their SCD: (1) central nervous system (CNS) stroke, (2) avascular necrosis of femoral or humeral head, (3) nephropathy (serum creatinine >1.5 mg% or proteinuria >1.5 g in 24-hour urine), (4) retinopathy, (5) acute chest syndrome with resting hypoxemia (arterial PO2 of <70 mm Hg), (6) leg ulcer, and (7) priapism with impotence. The individual patient’s end organ score was one point for each manifestation present in that individual. Therefore, the potential range of scores was from 0 to 7. These determinations were made by the same physician in all cases who was not aware of the results of the packed cell rheologic studies.

**Hematologic data.** Hb and reticulocyte counts were made by routine methods (Coulter Electronics, Hialeah, FL). The Hb electrophoresis was performed on citrate agar and cellulose acetate using established methods.25 Hb F and Hb A2 determinations were made using the alkaline resistance and microchrotophromatography methods.25

**Determination of α-globin genotype.** α-Globin genotypes were determined by Southern blot analysis on peripheral blood leukocytes using plasmid JW101 as a probe.25

**Rheologic measurements.** The blood samples were drawn into EDTA anticoagulant vacuum tubes and quickly centrifuged at 670g for 20 minutes. The plasma and buffy coat were removed and the cells washed twice in isotonic HEPES-buffered saline solution (132 mmol/L NaCl, 4.7 mmol/L KCl, 2.0 mmol/L CaCl2, 1.2 mmol/L MgSO4, 20 mmol/L HEPES, 0.1% gelatine, 0.2% albumin, 100 U/mL penicillin, 100 μg/mL streptomycin, pH 7.4, mOsm/kg = 290). Finally, the hematocrit (Hct) was adjusted to 42% ± 10% of hematocrit (Hct) was adjusted to 90% in HEPES-buffered saline. The bulk viscosity and the viscous and elastic components of the complex viscosity of the cell suspensions were measured with the magneto-acoustic ball micro rheometer described elsewhere.24 The viscous and elastic components of the complex viscosity are also called the viscous modulus and elastic modulus, respectively. The balls used with the instrument in the studies reported here had a ball-to-tube diameter ratio of 0.8. The viscous and elastic moduli were measured at an oscillatory frequency of 1 Hz and the amplitude of the ball displacement was approximately 0.050 mm in all experiments. Blood was stored at 5°C after venipuncture for at most 36 hours before experimental measurement. All measurements were made at 25°C with cells equilibrated with room air for at least 12 hours at 5°C before performing the rheologic measurements. The magneto-acoustic ball micro rheometer measures viscosity of bulk suspensions of the erythrocytes by the falling ball method. The ball is tracked by ultrasound echo location. The rate of fall of the ball is related to the viscosity of the suspension by Stokes drag equation. The elastic and viscous components of rheology of the suspensions are made by suspending the ball in an electromagnetic field and oscillating the ball back and forth at 1 Hz. The viscous and elastic moduli of the cell suspensions are calculated using the published equations.24

**Statistical analysis.** The statistical analyses were performed on the Clinfo System (using the Bolt Beranek and Newman Software Products Corporation, Cambridge, MA). Correlations were performed using the Pearson R correlation. A correlation P value of .05 is considered statistically significant.

**RESULTS**

**Clinical and hematologic parameters.** Table 1 shows the results of the clinical characteristics. The mean age for the studied population was 28.7 ± 5.5 years. Five of the 16 individuals were female. The mean Hb for the group was 8.2 ± 1.4 g/dL. The mean reticulocyte count for the group was 429 ± 221 × 109/L. The mean number of emergency department visits and hospitalizations for pain were 10.88 ± 13.6/yr and 3.13 ± 3.03/yr, respectively. The mean end organ failure score was 2.44 ± 1.5 events (Table 1).

**Rheologic parameters.** The mean steady-state viscosity (SSV) (measured from the falling ball experiments) for the group was .26 ± .13 P (for comparison, the average for normal AA suspensions is .16 ± .02 P). The group mean of the viscous modulus (VI C) and elastic modulus (EL C) (measured from the oscillating ball experiments) was .55 ± .44 P and .88 ± .07 P/s, respectively. (For comparison, the average for normal AA suspensions is .41 ± .07 P and .49 ± .17 P/s, respectively) (Table 1).

**Statistical correlations.** Pearson R coefficients for univariate analysis were performed for several parameters. The P value for the correlation between the SSV and end organ failure was .001 with a correlation coefficient (R value) of .73 and an R2 of .54, using the equation: end organ score = 0.1 + 8.8 × SSV (Fig 1). The P value for the correlation

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Mean SSV = .26 ± .55 g/dL; SSV = .88 ± 28.6; Mean P = 10.87; Mean M = 2.43; Mean E = 8.2; Mean R = 429; Mean SD = .13 ± .44 ± .07 ± .54 ± 3.03 ± 13.6 ± 1.5 ± 1.38 ± 221
PACKED CELL MICRORHEOLOGY IN SCD

The present studies have attempted to determine whether there is a correlation between the rheologic properties of suspensions of RBCs with SCD and painful events and end organ failure of individuals with SCD.

Previous studies have shown that rheologic aspects of Hb

The correlation between end organ failure score and SSV shown graphically. The correlation coefficient (R value) is .73 with an R² of .54. The P value for the regression is .001. The equation describing the correlation line is end organ failure = 0.14 + 8.8 × SSV. (—) Predicted.

Fig 1.

When multivariate regression analysis was performed using the equation: end organ failure = -1.7 + 7.5 × VI C (Fig 2). The P value for the regression between the EL C and end organ failure was .006 with an R value of .76 and an R² of .577 using the equation: end organ score = -0.8 + 3.6 × EL C (Fig 3). When multivariate regression analysis was performed using the SSV and the VI C and EL C, the P value for the regression was .0018 with an R value of .88 and R² of .70, using the equation: end organ failure = -1.9 + 18.0 × SSV + 6.6 × VI C + 1.3 × EL C. However, there was no statistically significant correlation between any component of packed cell rheology (SSV, VI C, and EL C) and painful events (emergency department visits or hospitalizations for pain). Nor was there a significant correlation between the patients' Hb, Hct, or reticulocyte count and parameters of packed cell rheology. There was also no statistically significant correlation between age and end organ damage. Univariate and multivariate regression analyses also failed to show any significant correlation between Hb, Hct, and reticulocyte count and painful episodes (emergency department visits or hospitalizations for pain) or end organ failure.

Fig 2. The correlation between end organ failure score and VI C at 1 Hz shown graphically. The correlation coefficient (R value) is .83 with an R² of .69. The P value for the regression is .00063. The equation describing the correlation line is end organ failure = -1.7 + 7.5 × VI C. (—) Predicted.

Fig 3. The correlation between end organ failure score and the EL C shown graphically. The correlation coefficient (R value) is .76 with an R² of .577. The P value for the regression is .00063. The equation describing the correlation line is end organ failure = -0.75 + 3.6 × EL C. (—) Predicted.

The acoustic ball microrheometer measurements have the advantage that the measurements are made on washed RBCs suspended in buffer, the osmolality of which has been adjusted to that of normal plasma. These measurements were also made on suspensions that were adjusted to an Hct of 90%. Thus, these experiments avoid the variation in viscoelastic measurements made in patients' plasma and those variations caused by differences in the Hct of the suspensions. The remaining variations are related to intrinsic properties of the RBC membrane and the Hb in suspension inside the RBCs. This method does not allow us to delineate the component of bulk viscosity or of the VI C and EL C that result from membrane visco-elastic properties from those due to the Hb protein inside the RBCs. Thus, the rheologic properties of a washed, packed cell suspension reflect the elasticity and deformability of the cells making up the suspension, as well as the interactions between the cells. Cells with less deformable membranes, more adherent cells, and cells with more viscous cytoplasmic proteins would be expected to increase the rheologic properties measured. The cell-cell adhesion effects measured by the magneto-acoustic ball microrheometer are not reflected in measurements made by ektacytometry or micropipette aspiration, but may play a role physiologically in SCD. This difference, along with the procedures that eliminate plasma protein and Hct variation, may explain why previous measurements of rheologic parameters in SCD have not shown as significant a correlation as the present studies.

We did not perform density separation on these cells to assess the components of the visco-elastic properties created by the cells of various densities. All studies reported here were performed on unfractionated samples of RBCs suspended in HEPES buffer to an Hct of 90%.

The present studies have attempted to determine whether there is a correlation between the rheologic properties of suspensions of RBCs with SCD and painful events and end organ failure of individuals with SCD.

Fig 3.

DISCUSSION
S-containing erythrocytes are influenced by α-globin gene number and Hb F levels. Therefore, we have studied a population with 4 α-globin genes and Hb F less than 10%.

We have found no significant correlation between retrospective painful events and any component of packed cell rheology. This finding is somewhat surprising because the studies of other investigators\(^4\)\(^,\)\(^9\)\(^,\)\(^10\) suggest that there is a positive correlation between RBC deformability, as measured in an ektacytometer, and painful events. The difference in the findings might be explained by the different methods used or by the inherent subjectiveness in evaluating a painful crisis.

The correlation between end organ failure and each of the measured rheologic properties (SSV, VI C, and EL C) was statistically significant. The strongest correlation was between the VI C and end organ failure, with an R value of .83 and an R\(^2\) of .69, suggesting that 69% of the variation in end organ failure in this population of untransfused Hb SS individuals could be explained by this component. It remains to be determined what component of these parameters of packed cell micro rheology are due to membrane alterations in SCD as opposed to that due to the abnormal properties of the Hb contained in the RBCs. The correlation between the EL C and end organ failure, and the correlation between the SSV and end organ failure were similarly significant. It is also important to note that there was no correlation between age and end organ failure or age and viscosity.

A possible explanation for the results found in this study could be that the higher viscosity of the packed cell suspension measured in vitro may also be a factor in the microcirculation of the patients with SCD. Such highly viscous suspensions flow less readily through the microcirculation and result in a chronic low oxygen delivery state and end organ damage. The compensatory mechanisms of the vascular system may be sufficient to prevent painful episodes in the face of continuously high viscosity.\(^2\)\(^,\)\(^3\) However, this compensation may not be sufficient to prevent slow, constant end organ damage.

Should the strong correlation between packed cell rheology and end organ damage hold for larger populations of sickle cell individuals with SCD in a prospective manner, rheologic instruments such as the microrheometer might prove to be a valuable tool for providing prognostic information. Such information would be most beneficial in choosing patients for prophylactic interventional trials and risky but curative therapies such as marrow transplantation.

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Relationship of clinical severity to packed cell rheology in sickle cell anemia

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