Deformability Measurements on Individual Sickle Cells Using a New System With pO₂ and Temperature Control

By Toshiyuki Itoh, Shu Chien, and Shunichi Usami

Although the rheologic behavior of sickle erythrocytes (SS cells) is highly dependent on oxygen tension (pO₂) and temperature, very little data exist regarding the effects of deoxygenation and reoxygenation on the rheology of "individual" SS cells at body temperature. We have devised and assessed a new experiment system, in which micropipette aspiration can be performed on individual cells in a constant-temperature chamber that has ports for changing media with different pO₂ (effected in 30 to 120 seconds) and sensing probes for monitoring pO₂ and temperature. This system enabled us to simultaneously alter and monitor pO₂ at 37 ± 0.5°C, and to monitor and study a single cell under microscopic observation. The static rigidity (E) and dynamic rigidity (η) of individual SS cells were determined by repeated aspirations of the same cell under various pO₂. With stepwise reductions in pO₂, E and η showed no significant changes before sickling, but once sickled, their values markedly increased by 10³- to 10⁴-fold concomitantly with morphologic alteration of the cell. Thus, the deformability of a single SS cell behaves in an "all or none" manner at a critical pO₂, and earlier studies on the effect of deoxygenation on the rheology of SS cell suspensions probably reflect the overall behavior of SS cells with widely distributed critical pO₂.

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sandwiched with a microscope glass slide and a coverslip. The chamber had dimensions of 12×30×7 mm (outside) and 9×12×4 mm (inside). Five small holes were drilled for the insertions of pO2 measuring electrode, reference electrode, thermocouple, micropipette, and fluid exchange tubing. The electrodes and the thermocouple were mounted in and glued into plastic connectors for tight fitting into the holes. The fluid exchange port was connected to a medium exchanger as described below. These holes, except the one for the micropipette, were sealed with vacuum grease to minimize increases in pO2 in the low pO2 experiments. A diagrammatic sketch of this system is shown in Fig 1.

The chamber itself was mounted on a copper plate (dimension 40×70 mm, thickness 3 mm), the temperature of which was regulated at 37°C by a Peltier-thermoelement and a temperature controller (TS-4 and TH-6D, respectively; Physitemp, Clifton, NJ). The temperature of the medium was monitored by a teflon-coated thermocouple (IT-21; Physitemp), which was sufficiently thin to eliminate time delay in feed-back regulation.

Alterations of pO2 were performed by replacing the medium in the chamber with another medium deoxygenated to a desired pO2 level in advance. The medium exchanger was composed of a gas equilibration apparatus, a pumping device, and a water bath (Fig 2). The water bath was heated deliberately at 38 to 38.5°C to compensate for the temperature decrease in the fluid transferred from the bath to the chamber. Aliquots of a phosphate buffer solution containing potassium and glucose (BSKG, see Cell Preparation below) were bubbled in the bath with different gas mixtures (O2 20%, 10%, 5%, or 0%; CO2 5%; N2, balance) in advance. Fifty milliliters of a gas-equilibrated solution was drawn into a 60-ml syringe, mixed through a stopcock with 1 ml of 5% bovine serum albumin solution contained in another syringe, and then delivered into the chamber with a syringe pump (Model 22; Harvard Apparatus, South Natick, MA) through the fluid exchange port. By varying the pumping rate, we could control the rate of fluid exchange in the chamber.

As the new medium was introduced, an equal volume of the original medium overflowed through the micropipette port, which was removed with the addition of a gentle vacuum. The rate of medium exchange was 0.5 to 2.0 ml/min. Under relatively high pO2 before sickling, i.e., when the cell was highly deformable, the medium was exchanged sufficiently slowly to prevent the loss of the cell. When the deformability had decreased in low pO2, we could increase the exchange rate up to 2 ml/min, while the cell was held to the micropipette tip by applying a small negative pressure. By the use of this system, we could simultaneously change the pO2 and study a single cell without either losing the cell or interrupting the microscopic observation. The medium exchanger, including the water-bath heater, was turned off during the micropipette aspirations to eliminate unnecessary vibrations.

The pO2 in the chamber was continuously monitored using a pO2 measuring electrode, reference electrode, and fluid exchange tubing. The pressure was removed in a step. Three to five aspirations were desired for low pO2, and six to ten aspirations were desired for high pO2. The first aspiration was performed at 10 to 20 mm Hg/h. The next major uptake was performed at a rate of 60 to 120 seconds for sickled cells, the pressure was removed in a step. Three to five aspirations were

**Fig 1.** Schematic drawing of the set-up used for micropipette aspiration and play-back analysis. A cell-holding chamber is mounted on a Peltier thermoelement for temperature regulation that, in turn, is placed on the stage of an inverted microscope. The chamber has ports for changing media with different pO2 and sensors for monitoring temperature (T) and pO2 (E). The micropipette (MP) is hydraulically connected to a pressure control system.

**Fig 2.** Schematic view of the medium exchange system, which is composed of a gas-bubbling apparatus (B) with warming coil (C), a pumping device, and a water bath kept at 37°C. The chamber (CH), fabricated from an acrylic resin block, is sketched in the inset. Alterations of pO2 are performed by replacing the medium in the chamber with a different medium deoxygenated to a desired pO2 level in advance. As the new medium is injected, an equal volume of the original medium overflows through the micropipette port, which is removed with the aid of a gentle vacuum (V). In the inset, abbreviations E, R, T, and F denote the ports for pO2 electrode, reference electrode, thermocouple, and fluid exchange tubing, respectively.
performed on a cell in the fully oxygenated state to obtain the control data, then the pO2 was modified in steps by replacing the medium; after the pO2 was stabilized at each step, the aspiration was repeated on the same cell. The morphology of the cell under study was continuously monitored, and the pO2 at which sickling occurred was recorded. Sickling was defined as a morphologic alteration of the cell with roughened surface and rim, usually with an elongated shape. In most cells, the experimental pO2 covered a range down to less than 40 mm Hg, even if sickling had already taken place. In some cells, however, the reduction in pO2 was terminated at 60 to 80 mm Hg because the cells had already become too rigid to be aspirated.

Once sickling had occurred, the SS cell exhibited a very long time for relaxation (sometimes > 3 minutes) for morphologic recovery after its release from the micropipette tip. In such cases, we selected another portion of the rim for the next aspiration. Because 3 to 5 minutes were required to complete the pO2 modification and the aspiration test for each step, the aspirations were always performed after the cell had completely recovered from the deformation. We did not pursue the numerical analysis of the recovery phase and focused on the deformation process. After the deoxygenation, the aspiration procedure and morphologic observation were repeated after step increases in pO2.

After the completion of an experimental run on a single cell, the pO2 electrode was calibrated with the cell-free buffer. A new SS cell suspension was reloaded in the chamber, a new cell was selected under microscopic observation, and the above procedures for pO2 modifications and the aspiration tests were reiterated.

Data acquisition and numerical analysis. The aspirational entry of the SS cell was recorded with a charge-coupled device (CCD) camera and a video tape recorder. The relevant data (ie, time, frame number, aspiration pressure, pO2, and temperature) were simultaneously recorded on the video tape by the use of a video timer (VTG-33; FOR-A, Tokyo, Japan) and a video multiplexer (Model 401; Vista Electronics, La Mesa, CA). The tape was played back through a time-base corrector (FA-200; FOR-A) and a dimension analyzer (Instrument for Physiology and Medicine, La Jolla, CA). The time-base corrector was used to obtain higher quality images. The electrical output from the dimension analyzer, corresponding to the aspirated length of the cell membrane, was transmitted to an A/D converter (System 570; Keithley Instruments, Cleveland, OH) and stored in a desktop microcomputer. The data storage into the microcomputer was performed with the use of Keithley's software (RTMDS, Version 1.0).

The constitutive equation that governs the rheologic behavior of aspirated red blood cell membrane and its integration have been introduced previously.7 In this model, the erythrocyte was postulated to possess a viscoelastic membrane and its deformability was characterized by two parameters: the shear elastic modulus and the shear membrane viscosity. The elastic modulus (E) was derived from the aspiration pressure (P), the micropipette radius (R), and the maximal entry of the cell into the micropipette (Dpm); the cellular viscosities for the initial rapid deformation phase (phase I) and the subsequent slow phase (phase II) were calculated as the product of E and the corresponding time constants of deformation. In the present study, as noted in the previous section, the behavior of some cells in the sickled state and even in the fully reoxygenated state seemed to deviate from that of an ideal viscoelastic material. Thus, to express the effective deformability of the cells, we used the nomenclature of "static rigidity" for E (dyne/cm) and "dynamic rigidity" for η and η' (dyne·s/cm), as Evans et al.16 did. The mathematical formulation is as follows:

\[ E = \frac{P \cdot R}{2 \cdot Dpm / R - 1 + \ln (2 \cdot Dpm / R)} \]

\[ \eta = E \cdot \tau \] for phase I,

\[ \eta' = E \cdot \tau' \] for phase II,

where τ and τ' are cell-dependent time constants, which were determined by fitting the integration of the constitutive equation to the actually acquired data points by the least square method.

Cell preparation. Blood samples were obtained from five patients with homozygous SS disease, with the approval of the Institutional Review Board, Health Sciences, Columbia University. Patients were not in crisis and had not undergone transfusion therapy within the preceding 3 months. Fresh blood (8 to 10 mL) was collected via venipuncture into an EDTA vacutainer. After centrifugation at 2,000 rpm for 10 minutes, the plasma and buffy coat were removed. The erythrocytes were washed three times in a buffered saline containing potassium and glucose (BSKg: 7.808 g NaCl, 0.373 g KCl, 0.194 g NaH2PO4·H2O, 1.220 g NaHPO4·2 H2O, 2.0 g glucose in 1,000 mL distilled water). After the adjustment of pH to 7.4 and osmolality to 290 mOsm/Kg H2O, bovine serum albumin (0.2%) was added before usage. Osmolality was determined by the freezing-point depression method (Model 3D II; Advanced Instruments, Needham Heights, MA). As the control, normal AA blood obtained from healthy volunteers was prepared in a similar manner. The AA cells, however, were not subjected to deoxygenation and served only for comparison with the SS cells in the oxygenated state, because it has been reported that AA cell suspensions19 and individual AA cells20 do not exhibit significant rheologic changes even under deoxygenation to below 20 mm Hg.

RESULTS

The unique feature of our new system is that rheologic measurements and microscopic observation can be made on given erythrocytes under a constant temperature and controlled levels of pO2. The temperature of the chamber was found to be held at the set value of 37°C within a range of ±0.5°C, and the electrically insulated Peltier-thermoelement had no interference on the pO2 electrodes. The system allowed us to make a step change of pO2 in 30 to 120 seconds, to continuously monitor the pO2, and repeatedly study the rheologic properties of an individual SS cell at various levels of pO2.

With the use of this new system, we studied 16 SS cells from five patients. Because six of the cells were lost during the procedures of pO2 modification, 10 cells were subjected to analysis. Seven cells were discoid and three were oval-shaped (aspect ratio between 1.2 and 1.3). All of these cells had smooth edges and could be clearly differentiated from irreversible sickle cells on a morphologic basis. The rheologic parameters of the 16 SS cells in the control state, ie, before deoxygenation, are displayed in Table 1, together with those of the normal AA erythrocytes. In the current

<table>
<thead>
<tr>
<th>Group</th>
<th>Static Rigidity (10-1 dynes/cm²)</th>
<th>Dynamic Rigidity (dyne·s/cm)</th>
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</thead>
<tbody>
<tr>
<td>SS (n = 16)</td>
<td>5.05 ± 0.59*</td>
<td>54.3 ± 8.37</td>
</tr>
<tr>
<td>AA (n = 17)</td>
<td>3.06 ± 0.17</td>
<td>32.2 ± 3.90</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for [n] cells. The terms phase I and phase II denote the rapid deformation process and the subsequent slow deformation process, respectively.

*p < .01

*p < .05 (SS v AA).

Table 1. Comparison of Rheologic Properties Between Oxygenated SS and AA Cells
study, we selected the smooth edge of the rim as aspiration site, because the sickled SS cells no longer had the dimple region. In the normal cells, the mean value of the static rigidity, 3.06 (±0.17 SEM, n = 17) × 10^{-3} dyne/cm, was slightly lower than those obtained for the dimple region, 3.58 (±0.20 SEM, n = 18) × 10^{-3} dyne/cm. In the SS cells, the static rigidity and the dynamic rigidities were significantly higher than the corresponding values for the AA cells (Student’s t-test, P < .05).

Each cell underwent sickling when deoxygenated to below its threshold PO, level. Typical time courses of aspirational deformation and recovery for a single cell under control, deoxygenated, and fully reoxygenated states, are displayed in Fig 3A through C, together with recorded temperature and PO,. In the control oxygenated state (Fig 3A), the deformational entry and the recovery occurred quickly without leaving persistent distortion.

With deoxygenation, in this particular cell, sickling took place at the PO, of 55 mm Hg. Micropipette aspiration of the cell after sickling is displayed in Fig 3B. The deformation was very slow (note different time scale from Fig 3A and C) and some residual deformation persisted even after 1 minute. The same tendency was also observed in other SS cells, ie, the sickled cells exhibited some plastic properties. Because our chamber was not a completely closed system due to the presence of a port for the micropipette, PO, showed a slight drift during the lower PO, aspiration tests that required a longer aspiration time. Thus, we defined the PO, value at which 80% of the Dpm was attained as the PO, level of the aspiration test, because this level of Dpm usually corresponds to the point of inflection that makes the transition from the initial rapid deformation phase to the subsequent slow phase. In the case shown in Fig 3B, for example, the PO, was set to be 44 mm Hg at the beginning of aspiration, but it increased to 51 mm Hg during 180 seconds of aspiration and recovery. The defined PO, value was 45 mm Hg.

The time courses of deformation and relaxation for the same cell after full reoxygenation are presented in Fig 3C. In this particular cell, the morphologic change of sickling was reversible, and the cell resumed its discoid shape as in the control state. The rheologic behavior also returned to the control state. Eight of the 10 cells studied, however, did not revert to the unsickled discoid shape even after full reoxygenation, and their deformability also remained highly impaired.

Repeated aspirations of the same SS cell under various PO, showed that the rheologic properties of the SS cells underwent a sharp transition in association with their morphologic alteration. The deformability of the cells did not show significant changes before sickling, but once sickled, the rheologic parameters increased steeply to high levels that were approximately 2 orders of magnitude greater than those before sickling. Some cells became so rigid with a further reduction in PO, that they did not respond to the maximal suction pressure (350 mm Hg), and behaved like solid bodies. When the cell did not deform at all after more than ~30 seconds under the maximal negative pressure, we considered E and η as infinite and the cell behaved essentially as a “solid.”

Figures 4 and 5 illustrate such sharp changes in E and η, respectively, during deoxygenation. The recovery upon...
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failed to attain the control values; even under full reoxygenation, the values were still 2 to 10 times higher than those in the initial oxygenated state. The deformation-recovery path showed hysteresis after sickling. Cell A, originally discoid SS cell, exhibited not only a morphologic recovery from sickled state but also a rheologic recovery. On the other hand, cell B, originally oval-shaped, showed a rather poor rheologic recovery (Figs 4 and 5). The other cells showed similar behavior as the two examples shown in Figs 4 and 5.

The extent of cellular stiffening in association with morphologic alteration, together with the critical pO2 levels, is summarized in Table 2. Because we could not make aspiration tests during sickling process, the rheologic variables very shortly before (<90 seconds) and after (<45 seconds) sickling are compared. One cell behaved as a "solid" after sickling, and hence mean values were computed from the data obtained on the other nine cells.

DISCUSSION

Although abnormal mechanical behavior of the SS cell has generally been recognized as a fundamental pathophysiologic disturbance in sickle cell anemia, there is still a lack

Fig 4. The static rigidity $E$ of a single SS cell as a function of pO2. Two examples are shown, but similar behavior was observed for all the cells studied. Open and filled symbols represent deoxygenation and reoxygenation processes, respectively. $E$ did not show any significant augmentation as long as the cells stayed unsickled. After the occurrence of sickling at critical pO2 levels (indicated by arrows), $E$ suddenly increased 20- to 50-fold and continued to increase up to 100- to 500-fold with further reductions in pO2. Some SS cells attained hyper-rigid "solid-like" states, displayed with the symbol X. $E$ showed gradual decrease with reoxygenation, but it failed to attain the control values even under full reoxygenation.

reexpression is less sharp. The changes of $\eta'$ were omitted, because they were similar to those of $\eta$. To avoid complexity, only two examples were shown in each figure, but similar behavior was observed for all the cells studied. Cell A (O) had smooth discoid morphology at the control state and resumed the smooth discoid shape after full reoxygenation. Cell B (\(\triangle\)), the oval-shaped erythrocyte in the control state, did not recover its initial smooth shape and had roughened surface even after full reoxygenation.

Figure 4 shows that the static rigidity $E$ remained essentially unchanged when pO2 was lowered, as long as the cells stayed unsickled. After the occurrence of sickling at critical pO2 levels (55 mm Hg in A and 114 mm Hg in B), the values of $E$ suddenly increased to values 20 to 100 times higher and it continued to increase up to 100- to ~500-fold as the pO2 was further reduced. Hyper-rigid "solid-like" states were displayed with the symbol X connected by dashed lines. $E$ decreased with reoxygenation, but they failed to attain the control values; even under full reoxygenation, the values were still 2 to 10 times higher than those in the initial oxygenated state. The deformation-recovery path showed hysteresis after sickling. Cell A, originally discoid SS cell, exhibited not only a morphologic recovery from sickled state but also a rheologic recovery. On the other hand, cell B, originally oval-shaped, showed a rather poor rheologic recovery (Figs 4 and 5). The other cells showed similar behavior as the two examples shown in Figs 4 and 5.

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Fig 5. The dynamic rigidity $\eta$ as a function of pO2 in the same two SS cells as in Fig 4. Open and filled symbols represent deoxygenation and reoxygenation processes, respectively. Sharp changes in association with sickling at critical pO2 levels (indicated by arrow) and hysteresis during recovery with reoxygenation can be seen. Some cells attained hyper-rigid "solid-like" states, displayed with the symbol X.
of sufficient information on the correlation of the abnormal mechanical behavior to cellular morphology and on its role in the actual circulation. In an attempt to obtain a better understanding of the cellular basis of the disease, we have devised a new experimental system to maintain a constant temperature of 37°C in the chamber, to alter and monitor PO₂, to follow a single cell as a function of PO₂. Thus, we were able to track the "personal" history of the rheology of a single SS cell as PO₂ was varied.

The most striking finding in the present study was the suddenness of the onset of rheologic alteration in each SS cell (Figs 4 and 5). In previous studies using SS cell suspensions and HbS solutions, two smooth nonlinear relationships have been observed between the rheologic properties of the single cell as a function of PO₂. Thus, we were able to track the "personal" history of the rheology of a single SS cell as PO₂ was varied.

In contrast to the observed sharp onset of the reduction in individual cell deformability upon deoxygenation, the rheologic variables showed more gradual decrease after reoxygenation. It should be noted that the responses to deoxygenation and reoxygenation showed wide variety among individual cells. For example, in Figs 4 and 5, while cell A underwent sickling at a PO₂ of 55 mm Hg and recovered morphologically and rheologically upon reoxygenation, cell B underwent sickling at a much higher PO₂ (114 mm Hg) and showed incomplete recovery.

Wide variabilities of morphology and intracellular Hb concentration are a characteristic feature of HbSS erythro-

### Table 2. Effects of Deoxvgenation on Rheology of Individual SS Cells

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>Initial Shape</th>
<th>Critical PO₂ (mm Hg)</th>
<th>Static Rigidity (10⁻² dyne/cm²)</th>
<th>Dynamic Rigidity (10⁻² dyne·s/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Presickling</td>
<td>Post-sickling</td>
</tr>
<tr>
<td>1</td>
<td>Discoid</td>
<td>55</td>
<td>0.50</td>
<td>57</td>
</tr>
<tr>
<td>2</td>
<td>Discoid</td>
<td>61</td>
<td>0.39</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>Discoid</td>
<td>61</td>
<td>0.53</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>Discoid</td>
<td>66</td>
<td>0.31</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>Discoid</td>
<td>75</td>
<td>0.86</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>Discoid</td>
<td>81</td>
<td>0.39</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Discoid</td>
<td>86</td>
<td>0.35</td>
<td>46</td>
</tr>
<tr>
<td>8</td>
<td>Oval</td>
<td>97</td>
<td>0.62</td>
<td>*</td>
</tr>
<tr>
<td>9</td>
<td>Oval</td>
<td>114</td>
<td>0.90</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>Oval</td>
<td>118</td>
<td>0.36</td>
<td>77</td>
</tr>
</tbody>
</table>

Mean ± SEM

Post-/Pre-Ratio (mean ± SEM) | 81 ± 7 | 0.52 ± 0.07 | 30 ± 8 | 0.056 ± 0.011 | 15.5 ± 8.2 | 0.91 ± 0.12 | 214 ± 64

The rheologic coefficients very shortly before (<90 seconds) and after (<45 seconds) sickling are compared. Both static and dynamic rigidities showed highly significant increases in association with morphologic alteration (P < .001). The terms phase I and phase II denote the rapid deformation process and the subsequent slow deformation process, respectively.

*Cell No. 8 behaved as "solid-like" body upon the first aspiration after sickling, and it was not possible to obtain postsickling data for this cell. Therefore, the postsickling mean values and post-/pre-ratios were computed from only nine cells.

Intracellular gelation or polymerization of HbS, which is the major determinant of rheologic alteration in the SS cells, is ~10 times faster at body temperature than at room temperature. In the present study, we used a temperature-controlled chamber to provide a physiologic level of temperature. The performance of the experiments at 37°C led to an accelerated intracellular gelation of HbS and hence made it possible to observe the acute reduction in deformability upon deoxygenation.

SS erythrocyte suspensions do not exhibit discontinuous changes in rheologic behavior; the PO₂ and viscoelastic variables show a continuously smooth relationship. The profile of the polymer fraction of HbS has been reported to be a smooth function of PO₂. This is probably because the critical PO₂ levels at which sickling takes place differs from cell to cell; while the rheologic behavior of a single SS cell changes in an "all or none" manner at a critical PO₂, studies on the rheology of cell suspensions reflect the overall behavior of SS cells with widely distributed critical PO₂.
cytes. This variability, particularly the existence of dense SS cells, has long been deemed to be an important factor for the severity of the disease, because the difference in Hb concentration is known to affect the rigidification of the SS cells upon deoxygenation. In this regard, our present investigation is somewhat limited in that we did not have measurement of the Hb concentration of the cells studied. Although the oval-shaped SS cells studied here were likely dense cells on the basis of their morphology, we were not able to correlate the individual histories of the cells to their Hb concentration. Thus, the role of Hb concentration in responses to modified pO₂ deserves further experimental study. By using SS cells separated according to their density, our new experiment system will allow us to explore the relationship between the cell rheology upon deoxygenation and the density of cell fractions, and hence to differentiate the responses of subpopulations of SS cells to varying pO₂.

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