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Research Article

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Erythrocytes in Sickle Cell Anemia Are Heterogeneous in Their Rheological and Hemodynamic Characteristics

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ABSTRACT To understand the contribution to the pathophysiology of sickle cell anemia of the different erythrocyte density types present in the blood of these patients, we have studied the viscosimetric and hemodynamic characteristics of four major classes of hemoglobin SS erythrocytes. We have isolated reticulocytes, discocytes, dense discocytes, and irreversibly sickled cells (fractions I–IV) on Percoll-Renografin density gradients. Bulk viscosity was studied in a cone-plate viscosimeter and the hemodynamic studies were performed on the isolated, artificially perfused mesoappendix vasculature of the rat (Baez preparation).

Bulk viscosity measurements at shear rates of 230 s^{-1} demonstrate that when the cells are oxygenated, fraction I (reticulocyte rich) has a higher viscosity than expected from its low intracellular hemoglobin concentration. The rest of the fractions exhibit moderate increases in bulk viscosity pari-passu with the corresponding increases in density (mean corpuscular hemoglobin concentration). When deoxygenated, all cell fractions nearly doubled their bulk viscosity and the deoxy-oxy differences remained constant. The Baez preparation renders a different picture: oxygenated fractions behave as predicted by the viscosimetric data, but, when deoxygenated, cell fractions exhibit dramatically increased peripheral resistance and the deoxy-oxy difference are directly proportional to cell density, thus, the largest increases were observed for fractions III and IV. The differences between the rheological and the hemodynamic measurements are most probably due to the different sensitivity of the two methods to the extent of intracellular polymerization.

These results also demonstrate that the hitherto unrecognized fraction III cells (very dense discocytes that change shape very little on deoxygenation) are as detrimental to the microcirculation as the irreversibly sickled cell-rich fraction IV. They may, however, induce obstruction by a different mechanism.

As the extent to which these fractions are populated by erythrocytes varies considerably from patient to patient, the distribution function of cell densities in each sickle cell anemia patient might have consequences for the type of pathophysiological events occurring in their microcirculation.

INTRODUCTION

The blood of patients with sickle cell anemia is comprised of erythrocytes with a shortened life span, an increased number of reticulocytes, and, in addition, dehydrated erythrocytes called irreversibly sickled cells (ISC),[1] which are generated by the sickling process. By the use of Percoll-Renografin density gradient fractionation, we have isolated four different subpopulations of erythrocytes in sickle cell anemia patients

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[1] *Abbreviations used in this paper:* cP, centipoise; Fv, venous outflow; Hb, hemoglobin; Hct, hematocrit; ISC, irreversibly sickled cells; MCHC, mean corpuscular hemoglobin concentration; Ppa, arterial perfusion pressure; PRU, peripheral resistance units; Pv, venous outflow pressure; RSC, reversibly sickled cells; SEM, scanning electron microscopy; Tpf, pressure-flow recovery time.

characterized by enrichment in either reticulocytes, discocytes, dense discocytes, or ISC.

In this paper we have systematically characterized the rheological (bulk viscosity) and hemodynamic (peripheral resistance in an ex vivo vascular preparation) properties of each of these classes of cells. The knowledge of these characteristics will allow a better understanding of their individual contributions to the pathophysiology of the disease.

The pathophysiological effects of these different classes or subpopulations of sickle cells is important because they are unequally represented in a given patient (1, 2) and the distribution function varies from patient to patient (1).

The data presented here make particular use of the Baez preparation, an isolated denervated mesenteric vasculature of the rat (3). This preparation resembles the human microcirculation in vascular dimensions and branching patterns and is a physiologically relevant system.

METHODS

Fresh heparinized samples were obtained by venipuncture from normal adults (n = 4) and from sickle cell patients (n = 6) who were out of crises and had not received blood transfusion in the preceding 4 mo.

Density gradient fractionation. Hemoglobin (Hb)SS erythrocyte subpopulations were separated on Percoll (colloidal silica coated with polyvinylpyrrolidone; Pharmacia Fine Chemicals, Piscataway, NJ)-Renografin-76 (66% distrizoate meglumine and 10% meglumine sodium; E. R. Squibb & Sons, Inc., Princeton, NJ) density gradients. There gradients were prepared using a ratio of Percoll/Renografin-76/water/0.9% NaCl of 3.5:1.7:4.1:0.7, as described by Vettore et al. (4). The resulting osmolarity was 320±5 mosmol and the pH, before the addition of cells, was 7.50±0.05. The hematocrit (Hct) was adjusted to 40–45% before the cells and plasma were added to the gradient mixture. In these preparative studies, 1.0 ml of whole heparinized blood was added to 9 ml of gradient mix. The tubes were spun for 20 min at 17,000 rpm at 5°C and the cells were harvested within 0.5 h after centrifugation. No significant change in mean corpuscular hemoglobin concentration (MCHC) or other cellular properties (enzyme levels, ATP, O_2 equilibrium, K^+ efflux) was noted after fractionation of cells. No changes in density were observed when washed, fractionated cells were subjected to density gradient centrifugation a second time and compared with the densities of the original whole blood.

The whole blood was separated into four major fractions (I–IV) according to cell density as determined by their depth on Percoll-Renografin gradients. The original division into four fractions was guided by the observation that most sickle cell patients have a bimodal distribution of cells on density gradients. One major population consists of cells with a density similar to that of AA cells (fraction II); the other major population is very dense (fraction IV) and contains mainly ISC (Table I). The cells lighter than fraction II (fraction I) contain a high population of reticulocytes (Table I). The fraction between II and IV is called fraction III and the characteristics of this fraction are described in this paper. The choice of four fractions was guided by the desire to define fractions that have a morphologically dominant species and are easily identifiable on inspection of the gradient. Typical cell distributions on Percoll-Renografin gradients are illustrated in Fig. 1 of reference 1 and characterized in Table I of this paper.

Some variation (±0.3 cm out of 8 cm) in the depth at which tubes are cut occurs from one preparation to the next. However, these variations should have only a small effect on the average MCHC of the individual fractions and probably most of the variation in the average MCHC of each fraction is the result of the inherent errors (±1.5 g%) in MCHC determinations. The low MCHC of fraction IV for S.M. and O.E. probably reflects an absence of very dense ISC in these patients' blood. The leukocytes and platelets were removed by aspirating the layer that contains these cells with no loss of reticulocytes, which is a major advantage of density gradient fractionation as opposed to aspirating a buffy coat after centrifugation. MCHC was measured using handspun Hct and Drabkin's reagent. Reticulocytes were stained by methylene blue and ISC counts were made in the neutral formalin (10%)-fixed cells.

For rheologic and hemodynamic studies, complete removal of the leukocytes and platelets from fraction I (top) and unfractionated blood was verified by microscopic examination of stained smears. All the samples were washed three times in Ringer's solution of either bicarbonate (154.3 mM NaCl, 5.63 mM KCl, 2.16 mM $CaCl_2$, and 0.6 mM $MgCl_2$ or nonbicarbonate (118 mM NaCl, 5 mM KCl, 27 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 0.6 mM $MgCl_2$) composition, each containing 0.5 or 1% bovine albumin. The pH of bicarbonate Ringer's solution was adjusted to 7.4 by equilibration with gas mixtures containing 5.6% CO_2. The pH of nonbicarbonate Ringer's was adjusted by NaOH or HCl (0.05 M each). The osmolarity of these solutions was adjusted to 295–300 mosmol with the use of a Microosmette (Precision Systems, Inc., Sudbury, MA). The use of Ringer's solution of either composition yielded the same results in viscosity and hemodynamic measurements. Samples were deoxygenated by alternate application of a moderate vacuum and water-saturated nitrogen and were rotated to ensure a large surface area for gas exchange. The percentage of oxy, carbon monoxy, and methemoglobin present in the samples were determined with the use of a Instrumentation Laboratory Co-oximeter model 282 (Instrumentation Laboratory, Inc., Lexington, MA).

Scanning electron microscopy (SEM). Samples were fixed in 3% gluteraldehyde in phosphate-buffered saline pH 7.35 and layered on glass coverslides coated with 0.1% poly-L-lysine hydrobromide type VI (Sigma Chemical Co., St. Louis, MO) before dehydration. Series of increasing ethanol concentration followed by Freon 113 were used for the dehydration. Freon 13 was used as transitional fluid for critical point drying (Bomar SPC-900/EX: The Bomar Co., Tacoma, WA) and gold sputtering for coating time (EMS-41 Minicoater Film-Vac, Inc., Englewood, NJ). Samples were examined on a mini scanning electron microscope (International Scientific Instruments, Inc., Santa Clara, CA).

Viscosimetry. Viscosity measurements were made at 37°C on oxygenated and deoxygenated erythrocyte suspensions in Ringer's containing 0.5% bovine albumin at a Hct of 30%. A Wells-Brookfield microcone-plate viscosimeter (model LVT, Brookfield Engineering Laboratories, Inc., Stoughton, MA) was used. Calibration of cone and cup was done frequently using a silicone oil standard (viscosity-5 centipose, cP). Each time 1.10 ml of sample was pipetted into the center of the cup and viscosity measurements were taken in duplicate at shear rates ranging from 11.5 to 230 s^{-1}. During the study of deoxygenated samples, the cone-plate

chamber was gassed with N_2 via the gas inlet. The mean percent hemoglobin oxygen saturation (±SD) value (HbO_2) for deoxygenated fractions and unseparated cells were 4.1±2.0 (I), 11.7±2.6 (II), 12.8±3.3 (III), 12.2±3.1 (IV), and 8.2±1.0 (unseparated). Samples taken before and after viscosimetric measurements showed <1% change in oxygen saturation.

Hemodynamic studies. These studies were performed in an isolated, acutely denervated, artificially perfused rat mesoappendix vasculature. Isolation, perfusion, and maintenance of the tissue and microvasculature were done by the method of Baez (3, 5). Briefly, in anesthetized (sodium pentabarbitol 30 mg/kg) rats of the Wistar strain, 120–150 g, the right ileocolic artery and vein were cannulated with heparinized (100 μl/ml) silastic tubing at a site 3 cm distant from the ileocolic junction. Under a steady-state perfusion with Ringer's containing 1% bovine albumin, the ascending colon and terminal ileum (3 cm each) were sectioned between ties. After hemostatic ties of all vascular connections was achieved, the tissue was isolated. The isolated mesoappendix was gently spread on an optically clear lucite block on a special microscope stage. The entire preparation was covered with a plastic Saran wrap or, when required, enclosed in a plastic capsule, except for outlets of cannulas and the microscope objective.

The control arterial perfusion pressure (Ppa) and venous outflow pressures (Pv) were kept constant at 80 and 3.8 mmHg, respectively, and monitored via Statham-Gould P-50 pressure transducers (Statham Instruments, Inc., Oxnard, CA). The venous outflow (Fv) rate was monitored using a photoelectric dropcounter and expressed in milliliters per minute. A lapse of 10–12 min was allowed for tissue equilibration and stabilization of Fv. Only preparations exhibiting mesoappendix microvasculature free of host blood cells and with a steady Fv of 4.6±0.5 (mean±SD) were used. The experiments were done at 37°C. After control measurements of Ppa and Fv, oxygenated or deoxygenated gradient fraction samples (0.2 ml; Hct 30%) were gently delivered via an injection port, 15 cm distal to site of arterial cannulation, and the changes in Ppa and Fv were recorded on the strip chart of a Grass polygraph (Grass Instrument Co., Quincy, MA). The tissue preparations were perfused for 10–15 min before the infusion of samples with Ringer's solution to allow stabilization of the tissue and clear the vasculature of the remaining blood cells of the host animal. Each mesoappendix and tissue vasculature preparation was sufficient for the evaluation of four to five samples. The resulting obstruction after the infusion of deoxygenated SS cells was cleared and the flow restored, in most instances, by briefly (2–3 min) perfusing the vasculature with fully oxygenated Ringer's solution at high pressure (100 mmHg). The mean percent HbO_2 (±SD) in deoxygenated samples (fraction I–IV and unseparated cells) was as follows: 14.0±4.9 (I), 18.1±4.1 (II), 18.8±4.1 (III), 18.0±2.6 (IV), and 17.8±5.2 (unseparated). In selected infusions (n = 4) determination of HbO_2% made before and after the passage of deoxygenated samples showed mean values of 18.4±5.2 (arterial) and 23.8±6.0 (venous). The slight increase in the percent oxygenation on the venous side occurred because the sealed capsule contained atmospheric levels of oxygen to maintain tissue viability. At the end of each experiment the entire tissue preparation (free of cannules and luminal content) was weighed. Peripheral resistance units (PRU) were calculated as previously described (6) and expressed as PRU = ΔP/Q = mmHg/ml per min per g; where ΔP (mmHg) is the arteriovenous pressure difference and Q (ml/min per g) is the rate of venous outflow per gram of the tissue.

In each experiment, pressure-flow recovery time (Tpf) was determined following the bolus infusion of samples. Tpf is defined as the time (seconds) required for Ppa and Fv to return to their base-line levels following the delivery of a given sample, and it represents total transit time throughout the mesoappendix vasculature.

Perfusion of the isolated vasculature with Ringer's solution (1% albumin) did not result in any visible changes in the morphological characteristics of the vascular endothelium as seen by scanning as well as transmission electron microscopy of randomly chosen tissue samples.[2]

RESULTS

SEM

When SS whole blood is separated on a Percoll-Renografin density gradient, four distinct fractions, each with characteristic morphological features, can be isolated (Table I, Fig. 1). Fraction I has a large percentage of reticulocytes; in the oxygenated state, many of these cells appear lobulated while others appear flat and ovalocytic. Interestingly, when the count of lobulated cells by SEM is compared with the count of reticulocyte by supravital stain in the same aliquot, it appears that erythrocyte retaining reticulum can be of either the lobulated or flat ovalocytic shape. This observation is in complete agreement with the findings of Coulombel et al. (7). In addition, a very small percentage of cells in this fraction appear to be morphologically ISC. In each patient, the percent reticulocytes recovered in fraction I is related to the total count in the unfractionated samples. Fraction II is composed mainly of cells of discocyte morphology in the oxygenated state or reversibly sickled cells (RSC) but also has a variable but small number of reticulocytes and ISC. Fraction III are mainly dense discocytes, in the oxygenated state, with some ISC. The bottom fraction IV is comprised mainly of dense ISC (60–85%) with an average MCHC in the group of patients studied in the range of 41.2–47.0 g/dl. Following deoxygenation, typical sickle forms are seen in fraction I and II. Although we have not observed dynamic morphological aspects of sickling, the multilobulated reticulocytes of fraction I appear to assume multispiculated forms (Fig. 1) rather than a typical sickle shape. The dense discocytes of fraction III show minimal transformation, displaying only tiny spicules projected from a more or less circular morphology, while no significant changes are observed in the ISC-rich fraction (IV), which also contains the densest discocytes.

Of six patients studied, five had fraction II as the predominant component; the percentage of total cells recovered in this fraction ranged from 42 to 65. Only in one case (V.S.) were fraction I and IV larger than

[2] Burns, E. R. Personal communication.

TABLE I

Hematological Data on HbSS Erythrocyte Subpopulations Separated on Percoll-Renografin Density Gradients (Fraction I–IV) and on Unseparated Blood (UB)

	Total cells				MCHC					Reticulocytes					ISC				
Patient	I	II	III	IV	I	II	III	IV	UB	I	II	III	IV	UB	I	II	III	IV	UB
	%				g/dl					%					%				
B.W.	10	62	11	17	32.2	35.2	38.1	46.5	37.5	53	20	8	0	25	7	6	14	71	19
O.E.	12	49	24	15	31.5	33.8	37.8	43.5	35.9	60	11	3	3	15	11	5	26	60	23
E.M.	7	65	13	15	32.8	36.1	40.2	46.2	35.9	31	8	0	0	11	7	5	12	65	22
D.L.	12	42	14	32	33.3	35.2	38.5	46.8	35.1	13	11	6	0	9	6	3	13	68	32
S.M.	12	50	18	20	31.7	35.3	38.1	41.2	35.4	43	5	0	0	12	6	2	21	73	15
V.S.	26	18	10	46	32.7	34.8	40.8	46.9	35.7	64	38	21	16	35	9	15	49	84	46

others. Analysis of unseparated blood of this patient revealed a smaller discocyte population but high counts of reticulocytes (35%) and ISC (46%).

Viscosimetric observations

Oxygenated fractions. Viscosimetry (shear rate, 230 s^{-1}) of the isolated oxygenated fractions (I–IV) of six patients at matching Hct (30%) establishes that each fraction has different rheologic characteristics (Fig. 2). Fraction II, composed mainly of discocytes, is the least viscous of all the fractions and its bulk viscosity is comparable to that of unseparated AA samples. Of special interest is the elevated bulk viscosity of fraction I in each case over that of fraction II in spite of lower MCHC in the former. At the shear rate of 230 s^{-1}, the difference between the viscosity of fraction I and II are significant but at a $P < 0.05$. However, a strong correlation was evident between fraction I viscosity values of each patient and the percent reticulocytes present in the same samples ($y = 2.1299 + 0.006x$, $r = 0.89$). The lowest viscosity of 2.2 cP was obtained for a fraction I sample (D.L.) containing 13% reticulocytes, while another sample (V.S.) with highest percent of reticulocytes (64%) was also the most viscous (2.68 cP). The elevated MCHC and presence of ISC in fraction III are responsible for its higher viscosity than that of fraction I and II. Fraction IV, with its preponderance of ISC and consequently high density (MCHC) is significantly most viscous at all shear rates. Individual samples from fraction III–IV revealed an excellent correlation ($y = 2.467 + 0.0088x$, $r = 0.84$) between bulk viscosity (2.40–3.40 cP) and percent ISC (13–84%). The viscosities of unseparated samples fall in between those of fraction I and II. Clearly, these values reflect a balance between various subpopulations and cannot, in any simple way, be predicted from the varied percentages of rheologically different subclasses of cells. In this context, it may be of significance to note that whole blood MCHC values in different individuals are strikingly similar (36.01±0.5 g/dl) despite significant differences in how the cells were apportioned among the four subpopulations.

Deoxygenated fractions. Deoxygenation of isolated fractions (I–IV) results in almost twofold increase in bulk viscosity over the respective oxy values at the shear rate used (Figs. 2 and 5). It appears that the viscosity of deoxygenated fractions is linearly dependent on MCHC. At the comparable deoxy levels the least dense fraction (I) is also the least viscous, while the maximum viscosity is obtained for fraction IV. The viscosities of the unseparated samples fall in between those of fractions III and IV. The O_2 partial pressure at which 50% of hemoglobin is oxygenated (P_{50}) of sickle cells increases with MCHC (1), which means that there is no simple relation between deoxy whole blood viscosities and the deoxy viscosities of the individual fractions. This is because the viscosity of the SS cell is sensitive to the degree of deoxygenation and in unseparated samples the discocytes will have a higher percentage of oxyhemoglobin than the ISC because of the difference in P_{50}.

Hemodynamic observations

In each case the isolated vasculature is first perfused with Ringer's solution (control) at a constant Ppa of 80 mmHg to be followed by a bolus infusion of a given cell sample (oxy or deoxy). The resulting changes (Ringer's solution vs. cells) in hemodynamic parameters (Ppa and Fv) are determined along with Tpf for each fraction (Table II). Fig. 3 is representative polygraph recordings of hemodynamic responses of the mesoappendix vasculature to the bolus infusion of oxy and deoxy cells from each fraction of a single patient (O.E.). The percent changes in the calculated peripheral resistance (PRU) for cells as compared with the control (Ringer's) values reveal different flow characteristics for each density gradient fraction (Fig. 4).

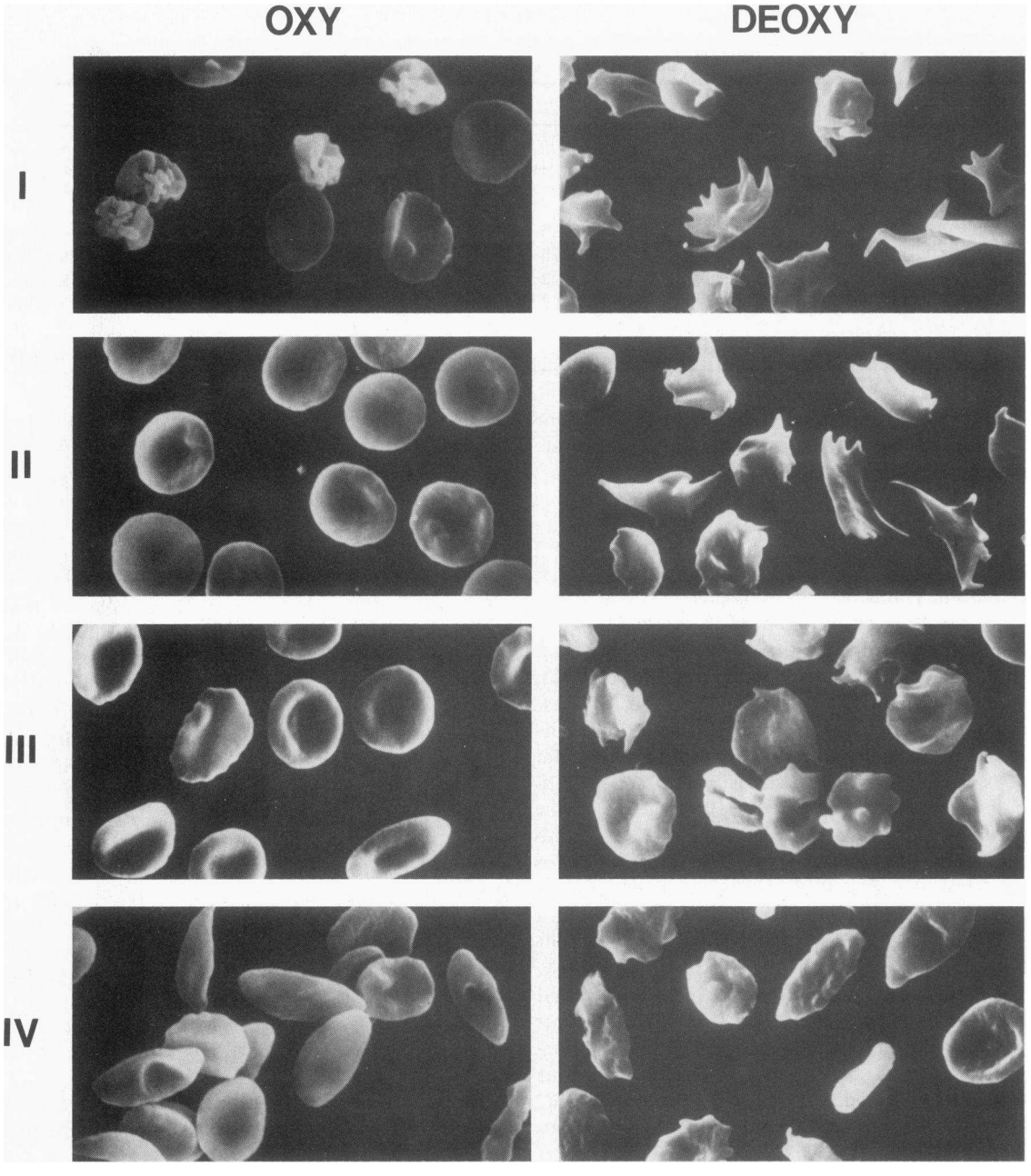

FIGURE 1 SEM of the four major HbSS erythrocyte subpopulations (fractions I-IV) of a single patient (S.M.) separated on Percoll-Renografin density gradients. Oxy fractions: Fraction I is characterized by the presence of a large number of reticulocytes; young reticulocytes appear multilobulated (7). Fraction II is composed mainly of discocytes. Fraction III contains dense discocytes and some ISC. Fraction IV is comprised mainly of ISC. Deoxy fractions: Following deoxygenation typical sickle forms are seen in fraction I and II. The multilobulated reticulocytes appear to assume multispiculated appearance. Little or no transformation is seen in fractions III and IV.

Oxygenated fractions. Although the passage of oxygenated subpopulations from fraction I–IV is essentially rapid and unimpeded, fraction II results in minimal rise in Ppa and decrease in Fv as compared with control (Ringer's) level, which indicates a smoother transit of these cells (mainly discocytes) through the

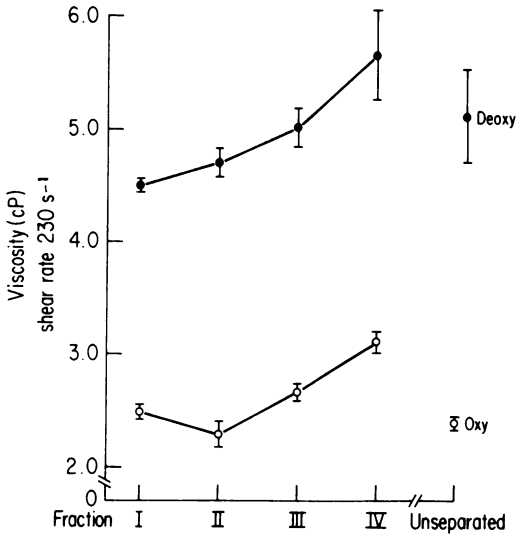

FIGURE 2 Representation of mean viscosity values (±SE) of oxy and deoxy density gradient fractions (I–IV) and of unseparated blood (Hct 30%) from six patients at a shear rate of 230 s^{-1}. During oxygenated conditions fraction II (discocytes) is the least viscous and fraction IV (ISC) the most. The elevated viscosity values of deoxygenated fractions are dependent on the MCHC of each fraction. The viscosities of unseparated erythrocyte suspensions are plotted for comparison.

microvasculature. The calculated mean PRU shows only a slight increment (6.9%) over the control (Ringer's) value. In each case the infusion of fraction I cells results in higher PRU (mean 14.6%). Tpf (Table II) shows an increase for fraction I as compared with that for fraction II. Tpf for fraction II is closer to that for unseparated AA cells than for unseparated SS cells. The percent changes in PRU with the infusion of fraction III cells (dense discocytes) are closer to that observed with fraction II than with fraction I. However, the passage of fraction III cells through the isolated vasculature is somewhat prolonged as demonstrated by an extended Tpf. When the calculated changes in PRU and Tpf for each oxy fraction are compared for each patient, the flow behavior of fraction II is found to be most normal and that of ISC-rich fraction IV the least.

Deoxygenated fractions. The resulting changes in Ppa and Fv following the infusion of cells from deoxy fractions become successively more pronounced from fraction I to IV (Table II). Consequently, PRU for fraction I, II, III, and IV increases linearly (Figs. 3 and 4). In contrast to the nearly uniform rise (about twofold) in viscosity recorded for each fraction following deoxygenation, the ratios of deoxy to oxy PRU (percent) for fraction I–IV clearly demonstrate the profound adverse influence of deoxygenation on the flow behavior of cells from dense fractions (III and IV) in the isolated microvascular preparation (Fig. 5). Fraction I results in about twofold increase in PRU (percent) over the oxy value (Fig. 5). The change in calculated mean PRU (percent) for deoxy fraction II is about fivefold greater than the mean oxy PRU value, due to the particularly low PRU values observed in the oxygenated condition for this fraction. Significantly, fraction III exhibits

TABLE II
Hemodynamic Parameters in the Isolated Microvasculature After Bolus Infusion of HbSS Gradient Fractions

Fraction	Ppa*	Fv decrease	PRU increase	Tpf
	mmHg		*%*	*s*
Oxygenated				
I	82.7	9.3	14.6	90.0
II	81.0	2.9	6.9	78.0
III	82.4	6.1	8.3	98.0
IV	83.4	10.4	18.2	150.0
SS, unseparated	82.8	5.2	9.8	82.0
AA, unseparated‡ (n = 9)	80.8±0.2	2.8±0.5	6.0±1.2	76.8±3.1
Deoxygenated§				
I	88.0	12.0	26.1	
II	92.1	17.1	35.8	
III	95.7	36.0	91.6	Partial recovery
IV	100.0	50.0	158.0	
SS, unseparated	92.0	29.2	63.4	

* Control Ppa was 80 mmHg.
‡ Values are mean±SD.
§ Initial mean percent HbO_2 ranged from 14.0 to 18.8 (see text for details).

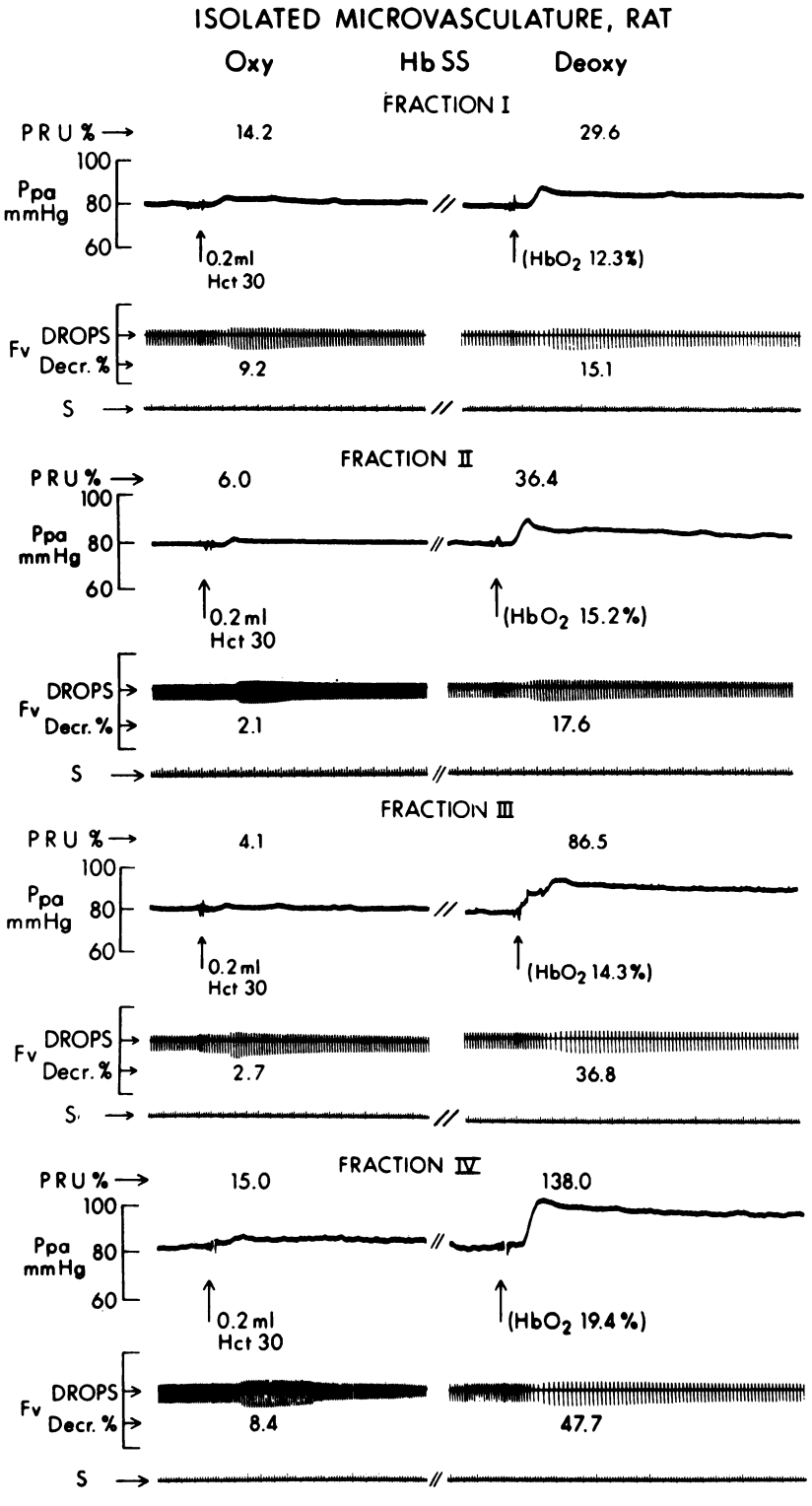

FIGURE 3 An example of hemodynamic response of the isolated mesoappendix vasculature of the rat to the infusion of oxy and deoxy HbSS cells from fraction I (mainly reticulocytes), II (discocytes), III (dense discocytes), and IV (ISC) of a single patient (O.E.). The vasculature is

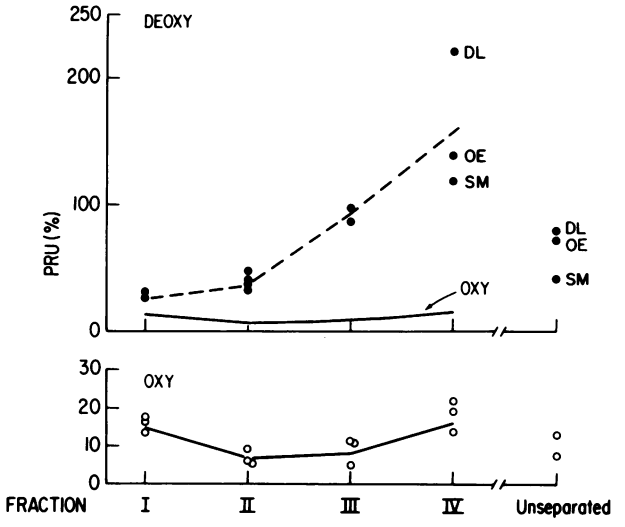

FIGURE 4 Representation of percent changes in the calculated peripheral resistance (PRU) in the mesoappendix vasculature following the infusion of oxy and deoxy cells from gradient fractions (I-IV) and of unseparated SS cells.

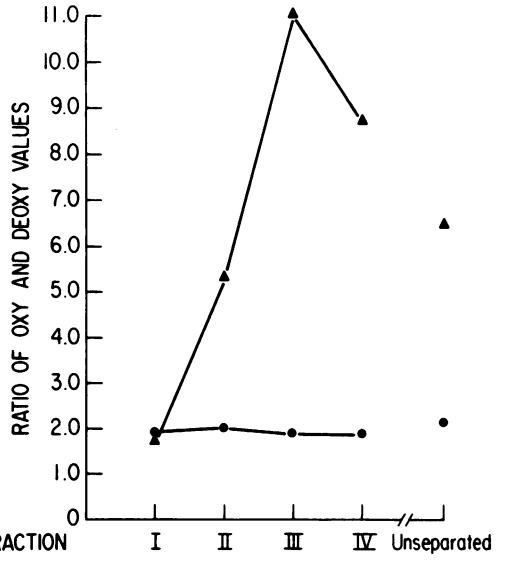

FIGURE 5 Representation of the ratios of oxy and deoxy values (PRU [▲] and viscosity [●]) of each gradient fraction (I–IV) and unseparated SS cells. Nearly uniform increase (about twofold) in viscosity is recorded upon deoxygenation of each fraction and unseparated cells. In contrast, in the isolated vasculature, infusion of deoxy cell populations results in varying degree of increases in PRU over their oxy counterparts. The changes in the ratios of oxy and deoxy PRU (percent) are most dramatic for fraction III (dense discocytes) and IV (ISC), which demonstrates profound vasoocclusive behavior of dense cell classes with deoxygenation.

maximum rise in PRU with deoxygenation that is more than 10-fold over that of its oxy counterpart. Fraction IV shows a similarly large effect (more than eightfold) upon deoxygenation. Only partial recovery of pressure and flow is seen following the infusion of deoxy samples, which indicates persistent microvascular obstruction. The PRU values for the unseparated samples lie between those for fraction II and fraction III. Again the difference in P_{50} for the various fractions makes the comparison of PRU values for the unseparated samples with those of the individual fractions a complex but not unapproachable problem.

DISCUSSION

The differences reported here in the rheologic and hemodynamic characteristics of four different erythrocyte fractions (as defined by gradient separation) have pathophysiological significance due to the marked density heterogeneity of erythrocytes in the blood of sickle cell patients. The heterogeneity of erythrocyte density has been previously reported by Fabry and Nagel (1) and is in accordance with observations of Clark et al. (2). In addition to this heterogeneity in the density distribution within each patient, Fabry and Nagel (1) have detected a marked patient-to-patient variation in the extent in which each range of density is populated by erythrocytes.

The results depicted in Fig. 2 demonstrate significant differences in bulk viscosity (at a shear rate of 230 s^{-1}) between the four fractions in the oxygenated state. Moreover, the bulk viscosity almost uniformly doubles in each fraction following deoxygenation (Fig. 5). In other words, the deoxy-oxy viscosity difference

first perfused with oxy or deoxy Ringer's solution at Ppa of 80 mmHg, which is followed by a bolus infusion of a given fraction (0.2 ml, Hct 30, vertical arrows). The changes in Ppa and Fv are recorded and the peripheral resistance calculated. Oxygenated fractions (*left*): Fraction I results in larger increases in Ppa decrease in Fv as compared with that observed for fraction II cells. Though fraction III resulted in smaller increase in PRU, the Ppa-Fv recovery time is prolonged as compared with that observed for fraction I and II. The passage of ISC-rich fraction IV results in higher PRU (15%) and there is delayed recovery of Ppa and Fv. Deoxygenated fractions (*right*): There is a progressive increase in PRU for fraction I-IV. Fraction I and II, respectively, result in 29.6 and 36.4% rise in PRU. On the other hand, dense discocytes (fraction III) and ISC (fraction IV) cause a much pronounced rise in percent PRU, i.e., 86.5 and 138%, respectively. In each case there is only partial recovery of Ppa and Fv.

remains constant in fraction II, III, and IV. A different picture emerges from the Baez preparation: the fractions also exhibit differences in peripheral resistance in the oxygenated state but more significantly, a dramatic progressive increase in their differences in the deoxygenated state (Fig. 4). Consequently, the deoxy-oxy differences increase progressively in fractions II, III, and IV (Fig. 5). This finding demonstrates that bulk viscosity of deoxygenated sickle erythrocytes at high shear rate is sensitive to the presence of polymerized Hb S but not very sensitive to changes in the amount of intracellular polymer formed, since it has been well established (8) that the size of the polymer phase increases drastically with increasing MCHC. On the other hand, in the Baez preparation, the peripheral resistance of deoxy cells exhibit progressive increases with erythrocyte density (MCHC) and consequently with the amount of the polymer phase. This is in accordance with the major contribution of the cytosol viscoelastic properties on erythrocyte deformability (9). These results should discourage the attempt to derive pathophysiological conclusions in sickle cell anemia from bulk viscosity data alone.

It is the pronounced increases in the peripheral resistance of deoxygenated cells from fractions III (dense discocytes) and IV (ISC and some very dense discocytes), which suggests that the patient-to-patient distribution of cells among the four density classes may be significant in the pathophysiology of the disease.

This is the first time that it has been demonstrated that the dense discocytes (fraction III) may pose a severe challenge to flow in the microcirculation. This hitherto unrecognized fraction, is formed by those cells that are morphologically discoid, but have an inherently elevated MCHC. Even under oxygenated conditions these cells induce a prolonged Tpf. The pronounced increase in PRU of deoxygenated fraction III cells demonstrates the greatly enhanced vasoocclusive behavior of these cells as compared with fraction I and II cells that have a lower MCHC. SEM of oxy and deoxy cells from the four classes (Fig. 1) offers insight into the possible origin of the effects of fraction III cells on the microcirculation. It is well known that following deoxygenation most discoid cells (from fraction I and II) assume characteristic sickled shapes and that ISC (fraction IV) remain unchanged (11). Significantly, the dense discocytes of fraction III, like ISC, undergo minimal shape change on deoxygenation which suggests that they are converted to rigid disk-shaped cells. Thus, this study provides evidence that in the whole blood of sickle cell patients there are two functionally different classes of discocytes. Earlier studies of Clark et al. (10) have shown that in vitro (nystatin-sucrose-induced) dehydration of HbS discocytes before deoxygenation results in retention of discoid morphology after deoxygenation. We observe that dehydrated discocytes occur in whole sickle blood and that they cause pronounced hemodynamic changes in the isolated vasculature; this suggests a significant role for these cells in the vasoocclusive phenomena.

The question arises then, why are dense discocytes (fraction III) particularly incompetent in traversing the microcirculatory bed in the Baez preparation. One hypothesis, that deserves consideration, is that the shape of the dense discocyte might represent a bigger obstacle to capillary flow than the elongated ISC. Pertinent to this is the observation of La Celle (11) that deoxygenated ISC once in the capillary microcirculation of mice orient themselves along the vessel axis, posing no significant problem to the capillary flow. Hence, the ISC-induced obstruction occurs mainly at the junction of precapillary arterioles and true capillaries (11–14). We hypothesize that dense discocytes might, in fact, reach the capillaries more frequently (as they are less likely to be stopped in the junction point) but once deoxygenated would fail to deform appropriately for the requirements of flow, thus resulting in an effective capillary blockage. We are in the process of experimentally testing this hypothesis.

The similarity of the effects of fraction III and fraction IV on the hemodynamic characteristics studied might partly explain the persistent controversy that exists (15, 16) over the contribution of morphologically defined ISC in the clinical severity of the disease.

The results presented here also demonstrate that each fraction has distinctive viscosity and hemodynamic characteristics even under oxygenated conditions. The present observations on the viscosity characteristics of discocytes and ISC are in complete agreement with those of Chien et al. (17) and Murphy et al. (18). The reticulocyte-rich fraction I shows increased viscosity as well as PRU over those observed for fraction II, which behaves more like AA cells. Reduced deformability of reticulocytes in sickle cell and other hemolytic anemias has been noted earlier using micropipette and nucleopore filtration techniques (19–21) and interpreted as a consequence of lobulated shape of immature reticulocytes (19) or increased rigidity of the reticulocyte membrane (20). It should be recognized, however, that this increase in rigidity does not necessarily imply a change in the reticulocytes membrane intrinsic material properties, as the effect could be derived exclusively from extrinsic (shape) factors contributing to deformability (9, 22); a corrugated sheet of metal is more rigid than a flat sheet of the same material.

When deoxygenated, the rheological and hemodynamic characteristics of fraction I are governed by the changes in intracellular viscosity rather than the shape and the effects of low reticulocyte MCHC become ap-

parent. Therefore, the suggestion of Leblond and Coulombe (21) that reticulocytosis may significantly affect the flow characteristics of the whole blood in hemolytic anemias, is only relevant to the oxygenated condition in sickle cell anemia. In deoxygenated blood, the reticulocyte is the hemodynamically most competent erythrocyte as it produces the least degree of peripheral resistance in the Baez preparation and has the highest oxygen affinity.

Another interesting aspect to be kept in mind, is that antisickling agents might differentially affect each category of cells, and that a substance effective in interfering with polymerization might not be capable of initially reducing the fraction III and IV count.

We can conclude then, that due to the variability from patient to patient in the distribution of cells in the four categories defined by density gradients, the pathophysiology of sickle cell anemia might vary considerably among affected individuals. Depending on which fraction predominates, or which fraction is represented over a critical threshold, the sequences of events in the microcirculation might be different (precapillary or intracapillary plugging, for example). Future work will be needed to define the exact nature of these events and their relation with the clinical course of sickle cell anemia.

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