SC Erythrocytes Have An Abnormally High Intracellular Hemoglobin Concentration

PATHOPHYSIOLOGICAL CONSEQUENCES

MARY E. FABRY, DHANANJAYA K. KAUL, CARMEN RAVENTOS-SUAREZ, HENRY CHANG, and RONALD L. NAGEL, *Department of Medicine, Division of Hematology; Department of Anesthesiology and Department of Physiology, Albert Einstein College of Medicine, Bronx, New York 10461*

ABSTRACT We have examined 20 SC patients on Percoll-Stractan continuous density gradients and find that they have an elevated mean corpuscular hemoglobin concentration (MCHC). Reduction of the MCHC to normal values results in amelioration of four physiologically important blood abnormalities: decreased oxygen affinity, viscosity of deoxygenated erythrocyte suspensions, rate of sickling, and deoxygenation induced K^+ efflux. These observations suggest that the rehydration of SC cells to normal values should be considered a potential approach in the therapeutic manipulation of this disease.

INTRODUCTION

The clinical expression of SC disease is moderately severe. It cannot be predicted from the in vitro interactions of HbS and C. The minimum gelling concentration of 50-50 mixtures of HbS and HbC is only 2 g per 100 dl lower than equivalent mixtures between HbA and HbS (1). On the other hand, the sickling rates of SC cells are similar to SS cells and not AS cells, (2) (even when compared with a theoretical AS cell with 50% S) and follow closely the differences in clinical severity.

The work presented here resolves this paradox and illustrates that cellular factors, as well as the solution behavior, have to be considered in order to understand the sickling process.

METHODS

Blood was collected, after informed consent, in heparinized Vacutainer tubes (Becton Dickinson Co., Rutherford, NJ) from donors characterized as SC at our Heredity Unit and used within 24 h for all studies.

Density gradients. Percoll (collodial silica coated with polyvinylpyrrolidone; Pharmacia, Inc., Piscataway, NJ)-Stractan (arabino-galactan polysaccharide; St. Regis Paper Co., West Nyack, NY) gradients were formed as previously described (3). The hematocrit of each blood sample was adjusted to 40 and 0.1 ml was added to 5.9 ml of the gradient mixture and mixed by inversion. Samples were centrifuged in a Sorvall SS-34 rotor at 17,000 rpm for 45 min at 38°C (Sorvall Instrument Co., Newtown, CN).

Percoll-Stractan gradients can also be used to determine the osmolarity at which the density (mean corpuscular hemoglobin concentration [MCHC])[1] of SC cells becomes equal to that of AA cells. The osmolarity of the gradient can be adjusted without altering the shape (density vs. depth in the tube) of the gradient by increasing or decreasing the amount of 10× balanced salts added. Pharmacia Density Marker Beads can be used to monitor the shape of the gradient. Osmolarity was measured with a Micro-osmette (Precision Systems, Sudbury, MA).

Viscometry. Viscosity measurements were made at 37°C on oxygenated and deoxygenated erythrocyte suspensions in Ringer's solution at a hematocrit of 30%. A Wells-Brookfield micro cone-plate viscometer (model LVT, Brookfield Engineering Laboratories, Brookfield, Inc., MA). was used as described previously (4). Samples taken before and after viscometric measurements showed <5% change in oxygen saturation. Osmolarity of the supernatant was checked both before and after viscosity measurements.

Sickling rates. The rate of sickling was measured by a syringe technique as previously described (2) with the exception that the 0.1 ml whole blood in 5 ml of pH 7.4 sodium phosphate-buffered saline adjusted to either 235 or 295 mosM was added to 5 ml of the same buffer containing 1 mg/ml of sodium dithionite.

Potassium efflux. The method of Roth et al. (5) was used with the exception that carbon monoxide treated, rather than oxygenated cells were used as the control. The pH and os-

This work was presented at the 95th Annual Meeting of the Association of American Physicians, Washington, DC, 7–11 May 1982.

Received for publication 30 June 1982 and in revised form 7 September 1982.

[1] *Abbreviations used in this paper*: ISC, irreversibly sickled cells; MCHC, mean corpuscular hemoglobin concentration; P_{50}, oxygen pressure at half saturation of hemoglobin; SEM, scanning electron microscopy.

molarity of the supernatant were verified at the end of each experiment.

Scanning electron microscopy (SEM). Samples were fixed in 10% buffered formaline and layered on glass cover slides coated with 0.1% poly-L-lysine hydrobromide type VI (Sigma Chemical Co.) before dehydration. Series of increasing ethanol concentration, followed by Freon 113 were used for the dehydration step; Freon 13 as transitional fluid for critical point drying (Bomar SPC-900/Ex, The Bomar Co., Tacoma, WA) and gold sputtering for coating (EMS-41 minicoater, Film-Vac Inc., Englewood, NJ). Samples were examined on a mini-SEM (International Scientific Instruments, Santa Clara, CA).

Oxygen equilibria. The Imai cell technique was used, as previously described (6).

RESULTS

Density distribution in SC erythrocytes. We have examined the blood of 20 SC patients using Percoll-Stractan continuous density gradients and find that their cells are much more dense than AA cells (Fig. 1) with an average MCHC of 36.4±0.76 g/dl (mean and standard deviation) determined from the depth on the gradient, a procedure described in detail in ref. 7. The cells are symmetrically distributed around the average density. In somewhat less than half of the cases, a small number of very dense cells are found, some of which are similar to irreversibly sickled cells (ISC). They usually represent <5% of the total number of cells and are slightly more dense than the ISC found in SS patients. The reticulocytes of SC patients are more dense than those found in AA individuals (Fig. 1). AC cells were also found to be more dense than AA cells (Fig. 1) but the increase in density is less pronounced (average MCHC = 34 g/dl).

Effect of osmolarity on density distribution in SC cells. The effect of extracellular osmolarity, ranging between 295 and 195 mosM, was examined for SC erythrocytes (Fig. 2): at 240 mosM they acquire approximately the same erythrocyte density as AA cells. The SC cells retain discocyte morphology at 240 mosM despite the significant increase in cell volume (Fig. 3).

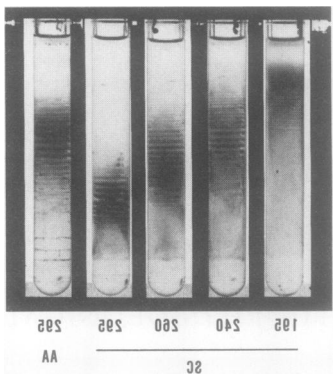

FIGURE 2 Percoll-Stractan continuous density gradients pH 7.4, 38°C, and osmolarity as indicated under the tube. The reference tube on the right with AA cells under isotonic conditions also contains density marker beads. The four left-hand tubes contain SC cells.

Effect of the correction of the MCHC of SC cells on oxygen pressure at half saturation of hemoglobin (P_{50}). The reduction in osmolarity of the suspending medium was accompanied by a reduction of the P_{50} of SC cells from 31.2±0.2 mm Hg in isotonic media (295 mosM) to a value of 27.5 mm Hg in 237 mosM (Fig. 4). Intermediate osmolarities yield intermediate P_{50} (e.g., P_{50} = 29.0 at 276 mosM). In other words, the correction of the increase in MCHC was followed by almost total normalization of the abnormal P_{50}.

Effect of the correction of the MCHC of SC cells on viscosity. In two separate experiments on different patients, we found that oxygenated SC cells at 300 mosM and a hematocrit of 30 are more viscous at all shear rates than either oxygenated or deoxygenated (93–94% deoxy-Hb) SC cells at 240 mosM (Fig. 5).

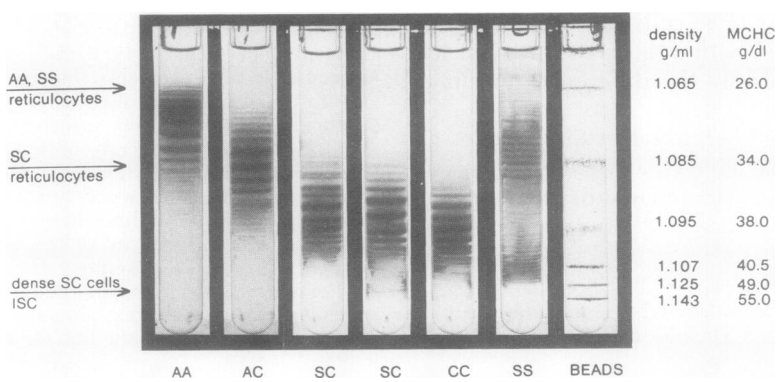

FIGURE 1 Percoll-Stractan continuous density gradients pH 7.4, 290 mosM, 38°C. The AA, AC, CC, and SS individuals are typical. The two SC individuals illustrate the presence and absence of dense cells. Reticulocytes were identified by methylene blue. The beads refer to Pharmacia Density Marker Beads.

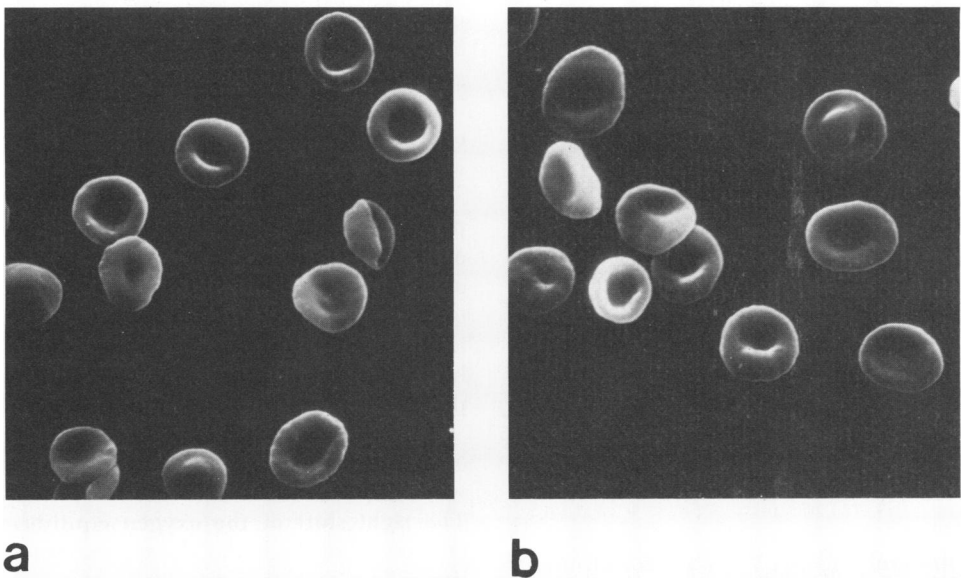

FIGURE 3 SEM of SC cells. Panel a shows cells at 295 mosM and panel b shows cells at 243 mosM. ×2,000.

There is a slight increase in viscosity when SC cells are deoxygenated at 240 mosM. Both oxy and deoxy AA cells have viscosities that are identical to those of oxy SC cells at 240 mosM. In contrast, at 300 mosM, the viscosity at high shear rates of deoxygenated SC cells nearly doubles over the viscosity of comparable oxygenated cells. Note that the more highly deoxygenated sample (90% deoxy Hb) is significantly more viscous than the less deoxy (85% deoxy Hb) sample.

Sickling rates. The time required for half of the SC cells to sickle ($t_{1/2}$) at 235 mosM was compared with the $t_{1/2}$ for AS cells at 295 mosM. Since AS cells contain differing ratios of HbS and HbA, samples from two different AS individuals were examined (Fig. 6). Because the $t_{1/2}$ of SC is too fast to measure with the manual syringe method, the determination was made in the apparatus of Harrington and Nagel (2) where the rate was found to be of the order of 18 s. The rehydration of SC cells to a normal MCHC (33 g/dl) increases the $t_{1/2}$ of sickling drastically to 4.0 min. One AS individual (E.R., S = 33.1%, A = 58.1%) was found to have a $t_{1/2}$ of 4.0 min; the second AS subject (M.E., S = 30.2%, A = 60.0%) was found to have a $t_{1/2}$ of 5.8 min.

Potassium efflux in SC cells and the effect of MCHC correction. The deoxygenation-dependent potassium

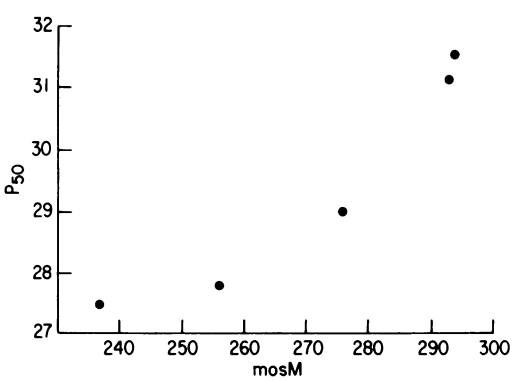

FIGURE 4 P_{50} of SC cells as a function of osmolarity as determined by the Imai cell.

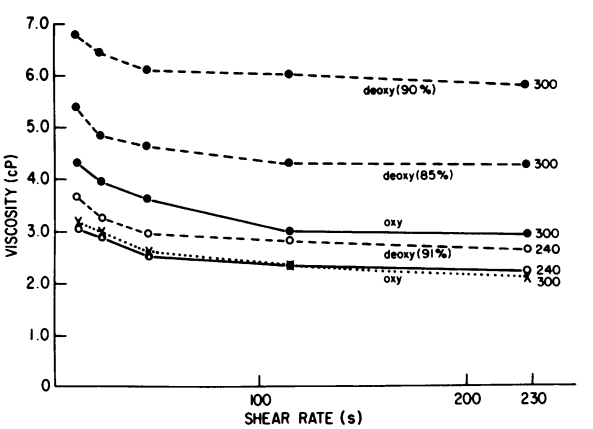

FIGURE 5 Relationship between viscosity (centipoise, cP) mean values and shear rate at hematocrit 30% and temperature 37°C for SC cells at two osmolarities (300 and 240 mosM) with the percent deoxy Hb indicated in parentheses. The dotted line represents average values for oxy and deoxy AA cells at 300 mosM.

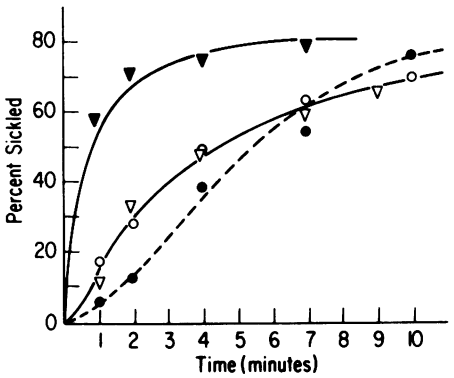

FIGURE 6 Effect of osmolarity on the percent sickled cells at 22°C as a function of time in minutes. (SC cells at 295 mosM, ▼; SC cells at 240 mosM, ▽; and AS cells for two different patients at 295 mosM, ●, ○.)

efflux at 295 mosmol and pH 7.1 was 15.2±2 meq/h per 1 RBC, which is more than twice that of SS cells (6.5 meq/h per 1 RBC) under the same conditions. When the osmolarity was decreased to 240 mosM, the potassium efflux decreased to 7.0±2 meq/h per 1 RBC (Fig. 7).

DISCUSSION

The data presented here demonstrate that cells containing both HbS and HbC have an elevated MCHC of ~37 g/dl, higher than both normal cells (MCHC = 33 g/dl) and the reversibly sickled cell fraction of SS cells (average MCHC 33-34 g/dl) (3). The increase in density is an early development in the life of the cell, since SC reticulocytes are more dense than either normal or SS reticulocytes. Elevated MCHC can be attributed to the presence of HbC since the MCHC of cells from homozygotes for HbC of both reticulocytes and mature erythrocytes is even higher (MCHC = 38 g/dl) as demonstrated by Fabry et al. (8). Because SC cells maintain discoid morphology even after their MCHC is lowered to 33 g/dl, we propose that their elevated MCHC is the result of water loss and not primarily due to an abnormal ratio of membrane area to hemoglobin or loss of membrane.

Because of the presence of HbS, these cells also have properties that result from the intracellular polymerization of HbS (*a*) they sickle on deoxygenation with a rate similar to SS cells (2), (*b*) there is a dramatic deoxygenation-linked increase in the efflux of K^+ (reported here for the first time), that is even higher than the well known effect found in SS cells (9) (Fig. 7), (*c*) cell viscosity increases after deoxygenation, and (*d*) oxygen affinity is decreased.

As stated earlier, the magnitude of these abnormalities is surprising when the modest differences in minimum gelling concentration for HBS and HbC solutions are compared with solutions containing HbS and HbA (1). Moreover, Bunn et al. (10) using an equilibrium method for the study of polymerization, find even less difference between SC and AS hemoglobin mixtures.

The results presented here resolve one aspect of this paradox: the abnormalities that result from the presence of HbS are magnified by the significant increase of the MCHC found in these cells. This is the consequence of the exquisite dependency of HbS polymerization on initial concentration for both the extent and kinetics of the phase change as demonstrated by the pioneer work of Hofrichter and Eaton (11).

This hypothesis is confirmed here by the correction of four of the HbS-dependent abnormal properties when the elevated MCHC of SC cells is normalized. The right shift of the oxygen equilibrium is almost entirely abolished, the deoxygenation-dependent increase in viscosity is drastically reduced (Fig. 5), and the sickling rates are reduced to values near to those of AS cells (Fig. 6). In addition, the deoxygenation-dependent K^+ efflux is reduced but not abolished (Fig. 7). An understanding of why the K^+ efflux is not abolished will have to await the elucidation of the mechanism of potassium loss as well as an explanation for the enhanced rate of loss in SC cells, beyond that observed for SS cells, under isotonic conditions.

Two factors that also contribute to enhanced sickling of SC cells, are the higher proportion of HbS (50 vs. 40%) compared with AS cells and the lower minimum

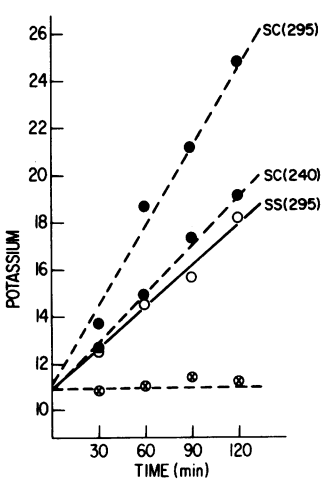

FIGURE 7 Potassium efflux in milliequivalents per liter as a function of time after deoxygenation in minutes at pH 7.1, 38°C. The osmolarity is indicated in parentheses to the right. (SC cells deoxy at 295 and 240 mosM, ●; SS cells deoxy at 295 mosM, ○; SS and SC cells equilibrated with CO used as controls, averaged for both SS and SC cells at 235 and 295 mosM, ⊗).

gelling concentration of 50% mixtures of HbS and C when compared with 50% mixtures of HbS and A. However, the efficacy of normalizing the MCHC in correcting the four abnormal properties described above indicates the dominant role played by MCHC in the SC cell.

These observations have practical implications. Interest has been generated recently in treatment of sickle disease by decreasing intracellular HbS concentration. Our in vitro results with SC cells demonstrate the potential usefulness of such an approach since four physiologically important properties (oxygen affinity, deoxy viscosity, sickling rates and deoxy-induced K+ efflux) have been restored to more normal values by reducing the MCHC (12). Furthermore, discocyte morphology, which is crucial for successful transit of the microcirculation in vivo, was maintained. This approach appears promising because of two factors: (*a*) the change in MCHC needed to correct the abnormalities is small (only 37 to 33 g/dl) and (*b*) the elevated MCHC was due to dehydration, which means that the cell volume could be increased with retention of normal morphology.

This approach is less likely to be successful for sickle cells because (*a*) the change in MCHC needed to significantly delay polymerization in homozygous cells is large (reduction to 25 g/dl), and (*b*) because the reversibly sickled cells have an average MCHC to 33–34 g/dl (ref. 3 and Fig. 1), large volume increases will convert these discocytes into near spherocytes, which are much less deformable. This could turn a good approach into an exchange of equally unwelcome properties.

We can conclude that the modification of intracellular hemoglobin concentration is an attractive avenue of approach, when considering therapeutic modalities intended for SC disease.

ACKNOWLEDGMENTS

We wish to acknowledge the excellent technical assistance of Gertrude Neuman, Joyce Johnson, and Sandra Suzuka and the assistance of Elizabeth Ezzone in the preparation of the manuscript.

This work was supported by National Institutes of Health grant HL21016.

REFERENCES

1. Fabry, M. E., J. Harrington, H. Chang, and R. L. Nagel. 1982. Critical contribution of cell density to the pathophysiology of SC cells. *Clin. Res.* **30:** 559a (Abstr.).
2. Harrington, J. P., and R. L. Nagel. 1977. The effects of alkylureas and nitrogen mustards on the kinetics of red cell sickling. *J. Lab. Clin. Med.* **90:** 863–872.
3. Fabry, M. E., and R. L. Nagel. 1982. Heterogeneity of red cells in the sickler. A characteristic with practical clinical and pathophysiological implications. *Blood Cells.* **8:** 9–15.
4. Kaul, D. K., S. Baez, and R. L. Nagel. 1981. Flow properties of oxygenated HbS and HbC erythrocytes in the isolated microvasculature of the rat. *Clin. Hemorh.* **1:** 73–86.
5. Roth, E. F., Jr., R. L. Nagel, and R. M. Bookchin. 1981. pH dependency of potassium efflux from sickled red cells. *Am. J. of Hematol.* **11:** 19–27.
6. Ueda, Y., R. M. Bookchin, and R. L. Nagel. 1978. A decreased effect of organic phosphates in hemoglobin S at low concentration. *Biochem. Biophys. Res. Commun.* **85:** 526–531.
7. Fabry, M. E., and Nagel, R. L. The effect of deoxygenation on red cell density: significance for the pathophysiology of sickle cell anemia. *Blood.* In press.
8. Fabry, M. E., D. K. Kaul, C. Raventos, S. Baez, R. Rieder, and R. L. Nagel. 1981. Some aspects of the pathophysiology of homozygous HbCC erythrocytes. *J. Clin. Invest.* **67:** 1284–1291.
9. Tosteson, P. C., E. Carlson, and E. T. Dunham. 1955. The effects of sickling on ion transport. I. Effect of sickling on potassium transport. *J. Gen. Physiol.* **39:** 31–42.
10. Bunn, H. F., W. A. Eaton, C. T. Noguchi, D. A. Torchia, and A. N. Schechter. 1982. Interaction of S and C hemoglobins in the intact cell. *Clin. Res.* **30:** 558A (Abstr.).
11. Hofrichter, J., P. D. Ross, and W. A. Eaton. 1976. A physical description of hemoglobin S gelation. Proceedings of the Symposium of Molecular and Cellular Aspects of Sickle Cell Disease, Dallas, Texas, 10 December 1975. (DHEW Publication No. [NIH] 76-1007). J. I. Hercules, G. L. Cottam, M. R. Waterman, editors. Bethesda, MD. pp. 145–181.
12. Clark, M. R., N. Mohandas, and S. B. Shohet. 1981. Hydration of sickle cells using the Na ionophore monensin. A model for therapy. *Blood.* **58:** 58a (Abstr.).