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Short communication

Surface properties of irreversibly sickled cells differ from those of the bulk of sickle cells Studies by partitioning in aqueous phase systems

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Abstract

Counter-current distribution (CCD) of red blood cells (RBC) from individuals with homozygous sickle cell (HbSS) disease in a charge-sensitive aqueous dextran-poly(ethylene glycol) phase system, which fractionates cells on the basis of surface properties, indicates that the percentage of irreversibly sickled cells (ISC) increases and the percentage of reticulocytes decreases with increasing cell partition ratios. The high partition ratios of ISC correspond to those of older RBC when RBC from normal individuals are subjected to CCD. Our results thus indicate that ISC differ in surface properties from those of the bulk of sickle RBC (including reticulocytes) in the population and that the difference is, most likely, charge-related. While the question as to whether ISC are indeed old cells has not yet been unequivocally answered, this view finds support in the fact that the independent parameters of ISC surface properties, as reflected by partition ratios, and densities correlate as they do in older RBC from normal individuals.

Keywords: Partitioning; Homozygous sickle cells; Irreversibly sickled cells; Counter-current distribution; Dextran; Poly(ethylene glycol)

1. Introduction

Even when oxygenated, red blood cells (RBC) from individuals with homozygous sickle cell (HbSS) disease exhibit morphologic heterogeneity [1]: (a) reversibly sickled cells which have the normal biconcave shape; (b) irreversibly sickled cells (ISC) which have an elongated, boat-shaped form with elevated membrane rigidity and increased membrane viscosity.

Reports that ISC have membrane abnormalities (for a summary see Ref. [2]) prompted us to test by a purely physical method, cell partitioning, whether alterations are detectable at the membrane surface. If so, could information be derived from the ISC partitioning

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behavior relevant to that of normal RBC as well to the age of ISC relative to the rest of the sickle cell population?

Partitioning in dextran-poly(ethylene glycol) (PEG) aqueous phase systems is an established method for the fractionation of cells on the basis of differences in surface properties [3,4]. Depending on the polymer concentrations and on the salt composition and concentrations, phase systems can be either charge-sensitive (i.e., have a Donnan potential between the phases) or non charge-sensitive [3]. Counter-current distribution (CCD), a multiple extraction procedure, in a charge-sensitive dextran-PEG phase system, of RBC from normal individuals [5] and from sickle cell donors indicated that cells from the latter fall within the distribution range associated with normal RBC. However, microscopic examination of RBC from sickle cell donors revealed that the percentage of ISC increases markedly with cell partition ratio.

2. Experimental

2.1. Reagents and chemicals

The aqueous two-phase system contained 5% (w/w) dextran T500 (lot. no. 11648, Pharmacia LKB, Piscataway, NJ, USA), 4% (w/w) PEG 8000 (Union Carbide, Long Beach, CA, USA), and 0.11 *M* Na-phosphate buffer, pH 6.8 (for preparation see Refs. [3,7]). The system, at 4–5°C, was mixed and permitted to settle in a separatory funnel overnight. Top and bottom phases were then separated with the material at the interface being discarded.

2.2. Samples

Blood samples were obtained from six adult patients with homozygous HbSS disease, with approval of the University of Southern California Human Subjects Research Committee. Patients were defined by the usual hematologic criteria [6]; excluded were patients in sickle cell crisis and those who had received transfusions within the prior three months. Blood from sickle donors was collected by venipuncture into heparin (5 IU/ml) and stored at 4°C until used. All procedures, except microscopic examination of stained or fixed cells (see below), were completed within 24 h of collection. All cell suspensions and polymer phase systems were equilibrated with room air (oxygen tension ca. 150–155 mmHg, ca. $2 \cdot 10^4$ Pa). RBC were washed three times with phosphate-buffered saline (0.15 *M* NaCl + 0.01 *M* Na-phosphate buffer, pH 6.8) prior to use in the experiments outlined below.

2.3. Apparatus and procedure

CCD of erythrocytes was carried out on a thin-layer unit [8] as previously described [3,7] (see Fig. 1 for operational details). After CCD, cells in each of three adjacent cavities were pooled (four or five cavities at each end of the distribution curve) for analysis. Aliquots of the washed cells in each pool were (a) lysed for determination of the cell CCD curve in terms of hemoglobin absorbance [3,7], (b) glutaraldehyde-fixed and ISC counted microscopically as a percentage of total RBC [1,9], or (c) stained with new methylene blue and reticulocytes counted microscopically as a percentage of total RBC [10]. Aliquots of the original, unfractionated cells were also analyzed as in (b) and (c).

3. Results

Fig. 1 depicts the CCD curves, in a charge-sensitive aqueous phase system (for composition see Fig. 1), of the RBC from two patients (FR, top; WD, bottom) having 8% and 13% ISC, respectively. The mean partition ratios of these cell populations fall within the range associated with normal RBC [5,11]. However, microscopic examination of RBC from different cavities along the extraction train indicates an increase in the percentage of ISC, from about 2 to 21% (FR) and 1 to 28% (WD), with increasing partition ratio (i.e., from left to right through the curve). Note that the ISC results shown are typical for all six donors. For these patients there was a $110 \pm 30\%$ (mean ± S.D., $p < 0.001$) in-

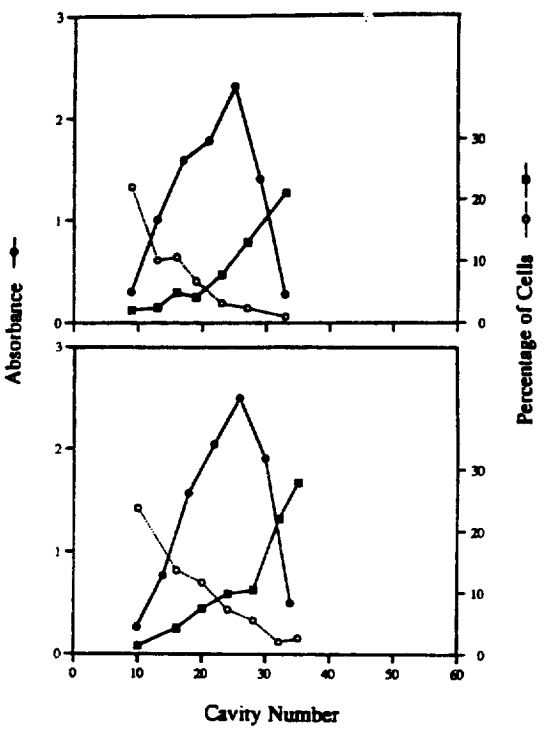

Fig. 1. Counter-current distribution (CCD) patterns of red blood cells (RBC) from two patients with homozygous sickle cell (HbSS) disease. The unfractionated RBC of FR (top) contained 8.1% ISC and 6.2% reticulocytes, those of WD (bottom) 12.7% ISC and 9.0% reticulocytes. CCD was carried out in a charge-sensitive dextran-poly(ethylene glycol) (PEG) aqueous phase system [3,7]. Phase system composition: 5% (w/w) dextran T500, 4% (w/w) PEG, 0.11 *M* Na-phosphate buffer, pH 6.8. Fifty-nine transfers were completed at 4–5°C using a settling time of 6 min and a shaking time of 22 s; (●) total cell distribution in terms of hemoglobin absorbance at 540 nm, (■) the percentage of irreversibly sickled cells (ISC), and (○) percentage of reticulocytes through the distribution. For additional details as well as procedural references see text.

crease of percentage ISC in the RBC population with the highest partition ratio (i.e., at the right end of the CCD curve) when compared to the unfractionated RBC. Microscopy also reveals that the percentage of reticulocytes [6% (FR); 9% (WD) of total RBC] decreases, from 22 to 1% (FR) and 24 to 3% (WD), with increasing partition ratio (Fig. 1).

It should be mentioned that correcting the percentage of ISC in each cavity for reticulocytes has virtually no effect on the percentage reported for ISC in Fig. 1.

4. Discussion

Previous analyses of cells under different parts of the CCD curve, obtained in a charge-sensitive phase system with human normal RBC or with RBC from patients having a reticulocytosis, have shown that reticulocytes have the lowest partition ratio of any RBC in the peripheral blood [3,12]. Thus the low partition ratio of reticulocytes in RBC populations obtained from HbSS patients is in accord with these earlier findings.

In normal individuals, young mature RBC also have low partition ratios while old mature RBC have higher partition ratios (i.e., the former are to the left while the latter are to the right under the CCD curve) [3,11]. It is not known whether this age-related change in partition ratios for human RBC is sequential, which would reflect systematic alterations in surface properties with cell age [3,4], as has been established in rat [3,10].

The increase in the percentage of ISC through the distribution curve (Fig. 1) indicates that this sickle cell subpopulation has surface properties which differ from those of the bulk of the population and that this difference is, most likely, membrane charge-associated [3,4]. In fact, preliminary CCD studies using a non-charge-sensitive phase system [3] indicate that the percentage of ISC remains essentially constant with increasing RBC partition ratios (data not shown). While sickle red cells have been reported to have the same electrophoretic mobility as normal RBC [13] and also appear to have the same mean partition ratios as normal RBC [5], it is established that the surface charge measured by electrophoresis and that reflected by partitioning need not be the same [14]. Electrophoresis gauges charge at the plane of shear [15] while partitioning also assesses charge deeper into the membrane [14].

Since the percentage of ISC increases through the curve while that of reticulocytes decreases, it

appears that ISC and reticulocytes have different surface properties. Furthermore, the position of the ISC under the curve is reminiscent of that of older cells in CCD curves of normal RBC [3,11]. That ISC are among the most dense cells, as are the older RBC in erythrocyte populations from normal individuals, has previously been established [1]. It is thus tempting to conclude that the ISC are among the older cells in the RBC population. Since there is evidence for a direct reticulocyte origin of ISC [16], the basis for the similar partitioning behavior of ISC and old normal RBC remains to be addressed. While the question as to whether ISC are indeed old cells has not yet been unequivocally answered, the independent parameters of their surface properties, as reflected by their partition ratios, and densities do correlate as they do in older RBC from normal individuals.

5. Conclusions

Based on their partitioning behavior in a charge-sensitive dextran-PEG aqueous phase system, we conclude that ISC have surface properties which differ both from reticulocytes and the bulk of mature sickle RBC in the cell population. Their partition ratios relative to the other RBC under the CCD curve are most akin to those of old mature erythrocytes in normal RBC populations.

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References

[1] G.B. Nash, C.S. Johnson and H.J. Meiselman, Blood, 63 (1984) 73.
[2] A.N. Schechter, C.T. Noguchi and G.P. Rodgers, in G. Stamatoyannopoulos, A.W. Nienhuis, P. Leder and P.W. Majerus (Editors), The Molecular Basis of Blood Diseases, W.B. Saunders, Philadelphia, PA, 1987, p. 179.
[3] H. Walter, in H. Walter, D.E. Brooks and D. Fisher (Editors), Partitioning in Aqueous Two-Phase Systems. Theory, Methods, Uses, and Applications to Biotechnology, Academic Press, Orlando, FL, 1985, p. 327.
[4] H. Walter, in H. Walter and G. Johansson (Editors), Methods in Enzymology, Academic Press, San Diego, CA, 1994, p. 299.
[5] H. Walter and E.J. Krob, Biochem. Biophys. Res. Commun., 120 (1984) 250.
[6] D.R. Powars, W.A. Schroeder, J.N. Weiss, L.S. Chan and S.P. Azen, J. Clin. Invest., 65 (1980) 732.
[7] H. Walter and C. Larsson, in H. Walter and G. Johansson (Editors), Methods in Enzymology, Academic Press, San Diego, CA, 1994, p. 42.
[8] T.E. Treffry, P.T. Sharpe, H. Walter and D.E. Brooks, in H. Walter, D.E. Brooks and D. Fisher (Editors), Partitioning in Aqueous Two-Phase Systems. Theory, Methods, Uses, and Applications to Biotechnology, Academic Press, Orlando, FL, 1985, p. 131.
[9] G.B. Nash, C.S. Johnson and H.J. Meiselman, Blood, 72 (1988) 539.
[10] H. Walter, A. Miller, E.J. Krob and G.S. Ascher, Exp. Cell Res., 69 (1971) 416.
[11] H. Walter and E.J. Krob, Cell Biophys., 5 (1983) 205, 301.
[12] H. Walter, E.J. Krob and G.S. Ascher, Biochim. Biophys. Acta, 641 (1981) 202.
[13] G.V.F. Seaman and B.A. Pethica, Biochem. J., 90 (1964) 573.
[14] H. Walter, R. Tung, L.J. Jackson and G.V.F. Seaman, Biochem. Biophys. Res. Commun., 48 (1972) 565.
[15] G.V.F. Seaman, in D.M. Surgenor (Editor), The Red Blood Cell, Academic Press, New York, NY, 1975, p. 1135.
[16] R.M. Bookchin, O.E. Ortiz and V.L. Lew, J. Clin. Invest., 87 (1991) 113.