STUDIES ON THE ALTERED MEMBRANE CHARACTERISTICS OF SICKLE CELLS

C. RICE-EVANS, K. R. BRUCKDORFER and G. DOOTSON

Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, Hunter Street London, WC1N 1BP, England

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1. Introduction

It has been shown that sickle cell anaemia causes abnormalities of the red cell membrane which are reflected in altered red cell deformability, permeability, surface area [1], elevated calcium content [2], altered protein patterns and reduced membrane sialic acid content [3], potassium loss and cellular dehydration [4]. The major feature of this disease is the tendency of erythrocytes to sickle when exposed to decreased oxygen tensions and to unsickle when reoxygenated. The sickle cell membrane is deformed by polymerised fibres of deoxygenated Hb-SS which adhere to the internal surface of the membrane [5]. This polymerisation is essentially reversible with oxygenation, although some of the cells do maintain their original sickle shape. These irreversibly sickled cells constitute 4–44% of cells in oxygenated capillary blood of individuals with sickle cell disease [6–8].

In this communication we have investigated alterations in the fluidity of the hydrophobic lipid region and changes at the surface of the sickle cell membranes compared with those of normal erythrocytes.

The results indicate that, in comparison with normal erythrocyte membranes:

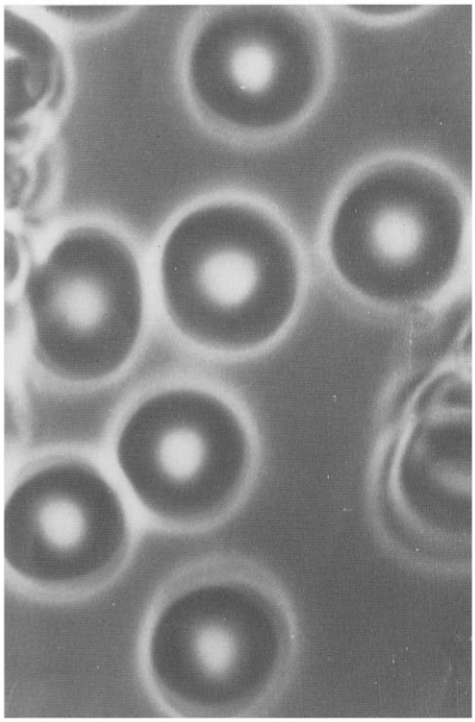
(i) The hydrophobic lipid region of sickle cell membrane is less fluid.
(ii) This is not a consequence of alterations in the cholesterol/phospholipid ratio or fatty acid content.
(iii) The negative potential at the surface of the sickle cell membrane is decreased.

2. Experimental

Erythrocyte membranes were prepared from freshly-drawn blood from haematologically normal donors and homozygous sickle cell disease patients, within 24 h, essentially applying the method in [9] but using Tris buffers. Normal erythrocyte ghost preparations were haemoglobin free as judged by $A_{414\ nm}$. Sickle cell ghosts were slightly pink, as expected due to haemoglobin retention, but the absorbance was negligible (<0.06) at the concentrations used (53.3 μg/ml). The ghosts were finally suspended in Tris–HCl buffer in isotonic saline, at pH 7.4. The ghost protein concentration was assayed by the Lowry method [10] using crystalline bovine serum albumin (Sigma) as standard.

Ghost and cell morphology were examined (unfixed) by phase contrast microscopy using the Zeiss WL standard research microscope.

Fluorescence polarisation measurements were performed on the Elscint MV-1a Microviscosimeter at 25°C. The membranes were labelled with 1,6-diphenylhexatriene (DPH) (Aldrich) according to the method outlined in [11] to give a probe : lipid ratio of 0.007. When interpreting data on the apparent 'bulk' microviscosity of the lipid region of the membrane using fluorescent molecules such as DPH, it is important to note that the probe may occupy different sites in the membrane interior in which the true local viscosities opposing the rotation of the probe may be very different [12]. The observed value is therefore an averaged response.



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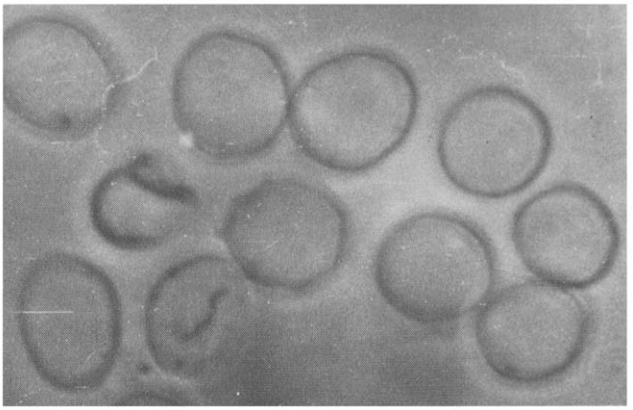

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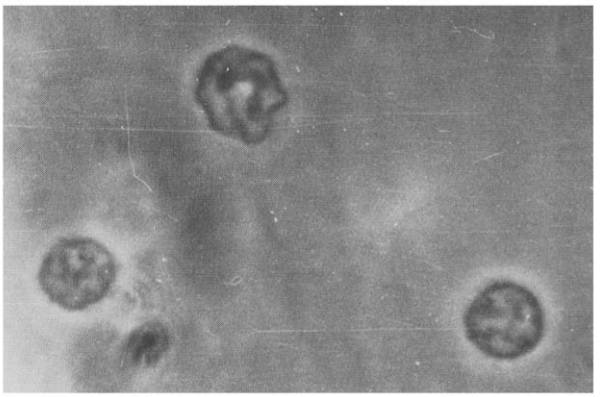

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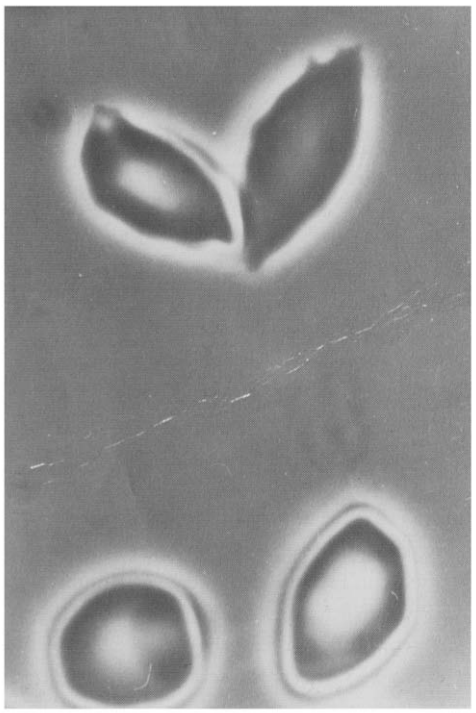

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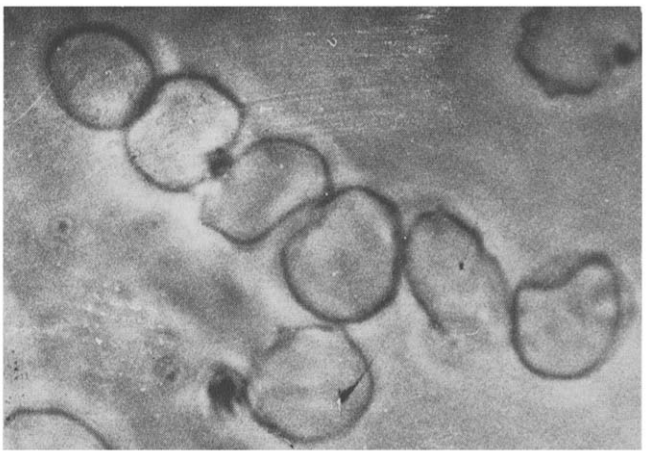

e

Fig.1

The membrane microviscosity (η) was calculated from the formula:

$$\frac{r_o}{r} = 1 + C(r)\frac{T_\tau}{\eta}$$

where r is the degree of fluorescence anistropy obtained from the polarisation value ($r = \frac{2P}{3-P}$), r_o a limiting anistropy for DPH, 0.362 [13]; $C(r)$ is a structural parameter of the probe which varies slightly with r, and was calibrated for DPH [14] by using paraffin as a reference oil, τ is the excited-state lifetime and T the absolute temperature.

Fluorescence measurements using the magnesium salt of 1-anilino-8-naphthalene sulphonate (ANS) (Eastman) were carried out as outlined in [15] using a probe : lipid ratio of 0.143.

Total phospholipid content was determined by the colorimetric method [16] and cholesterol was extracted by the method in [17] and analysed by gas chromatography.

3. Results

Phase contrast micrographs of oxygenated normal (A.A.) and oxygenated sickle cell erythrocytes (S.S.), and ghost samples from normal and sickle cell blood are shown in fig.1a–e. Sickle-shaped cells (fig.1b) are apparent among the oxygenated Hb-SS erythrocytes, showing the presence of irreversibly sickled cells. Ghosts prepared from samples of oxygenated Hb-SS erythrocytes fall into two categories designated group A and group B. Figure 1d shows the typical morphology of SS-ghosts of which the original blood sample contained a significant proportion of irreversibly sickled cells, as observed in the microscope (group A). The whole population of ghosts has the same contracted appearance, shows increased haemoglobin retention in the form of membrane-bound micro-aggregates and has a tendency towards the echinocytic shape compared with haematologically normal ghosts (fig.1c). The SS-ghost membranes of which the original erythrocytes apparently contained a very low proportion of irreversibly sickled cells (group B) are less contracted (fig.1e), though significantly distorted from normal, and retain less haemoglobin than group A samples.

The fluorescent hydrocarbon DPH is increasingly becoming established as an efficient fluorescent polarisation probe for monitoring the fluidity of hydrophobic lipid regions [11]. When DPH is incorporated into sickle cell membranes the degree of polarisation and therefore the microviscosity is enhanced compared with membranes of normal erythrocytes (table 1). The results seem to fall into the two distinct groups, designated on the morphological basis above. Group A SS-membranes have much more rigid lipid regions than the group B SS-membranes which are less fluid

Table 1
The membrane microviscosity and cholesterol and total phospholipid contents of erythrocyte membranes from normal donors and sickle cell patients

Sample	η (25°C)	Mean ± SEM	
		Cholesterol (μmol/10^{10} cells)	Total phospholipid (μmol/10^{10} cells)
Normal	6.22 ± 0.061 (5)	3.03 ± 0.12 (6)	3.42 ± 0.23 (6)
SS group A	10.63 ± 0.33 (5)	3.35 ± 0.25 (10)	3.67 ± 0.34 (10)
SS group B	8.1 ± 0.44 (8)		

Fig.1. Phase contrast photographs of: (a) oxygenated normal erythrocytes; (b) oxygenated sickle cell erythrocytes in plasma; (c) normal erythrocyte ghosts; (d) sickle cell erythrocyte ghosts – group A (see text); (e) sickle cell erythrocyte ghosts – group B, in Tris buffer in isotonic saline, pH 7.4. Magnification 1250 ×.

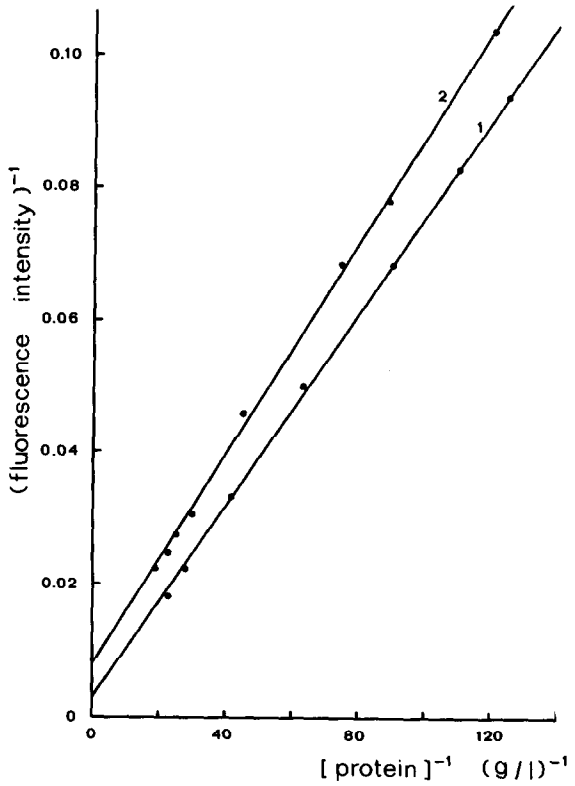

Fig.2. Double reciprocal plots of ANS fluorescence (10 μM) as a function of membrane protein concentration: (1) normal erythrocyte membranes; (2) sickle cell membranes.

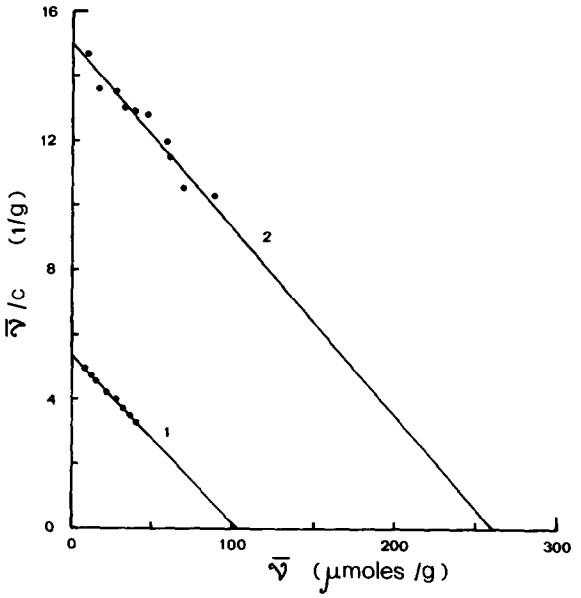

Fig.3. Scatchard plots for the binding of ANS (0–10 μM), membrane protein 0.0533 mg/ml: (1) normal erythrocyte membranes; (2) sickle cell membranes.

than the lipid region of normal erythrocyte membranes.

The membrane surface charge is a major determinant in the binding of the anionic probe of the membrane surface ANS [18]. Titrations of varying concentrations of normal and sickle cell membranes with a fixed concentration of ANS were carried out and the double reciprocal plot extrapolated to estimate the limited ANS fluorescence enhancement when all the probe is bound to the membrane [19]. Bound ANS in sickle cell membranes is less fluorescent than in normal erythrocyte membranes (fig.2) indicating different characteristics in the binding site. This observed decreased fluorescence is accompanied by a red shift of the emission maximum wavelength of magnitude 7 nm, implying a more polar environment for the bound probe molecules. All the sickle cell samples responded in the same way to interaction with ANS.

A Scatchard analysis [20] was performed on the data (fig.3) as outlined in [15]. The results show that the binding of ANS to sickle cell membranes is much enhanced (n = 230 μmol/g) compared with that of normal erythrocytes (n = 100 μmol/g) but the affinity of the membrane for the probe remains unaltered.

In order to clarify whether the differences in the degree of membrane fluidity in the normal and sickle cell membranes is partially controlled by alterations in the cholesterol/phospholipid ratio or fatty acid composition, total phospholipid, cholesterol and fatty acid analyses were performed. The results (table 1) showed no significant differences, using 10 homozygous sickle cell samples and 6 haematologically normal erythrocyte samples.

4. Discussion

It has been demonstrated [21] that even fully oxygenated Hb-SS erythrocytes have reduced membrane elasticity culminating finally in the formation

of irreversibly sickled cells. Our results show that it is the hydrophobic lipid region in sickle cell membranes which is more rigid. The factors responsible are not altered cholesterol:phospholipid ratio or the degree of unsaturation of the fatty acyl chains. It is not clear whether the decreased fluidity of the lipid region of the sickle cell membrane is due to changes in bulk lipid fluidity or the formation of distinct lipid domains nor to what extent the membrane proteins are involved.

Possibly, changes in the orientation or arrangement of the phospholipid headgroups occur as suggested by the results of the ANS-binding experiment. The binding of ANS to membranes may be used to indicate changes in surface potential [22]. The lowered quantum yield of the bound ANS and the red shift in the ANS emission maximum in the sickle cell membrane suggest that the binding sites have different characteristics and are more accessible to the aqueous environment, that is, the naphthalene ring system of the probe molecule penetrates less deeply into the hydrophobic region of the membrane. The increased binding of the probe is consistent with the increased Ca^{2+} concentration [2,21] in sickle cell membranes, causing a decrease in the negative potential in the plane of the lipid headgroups at the cytoplasmic surface [23] were calcium has been preferentially located [24,25].

Whether elevated Ca^{2+} concentration is a cause [21] or a consequence [26] of the membrane alterations leading to irreversible sickling is still a matter to be resolved. It is currently considered that the increased level of Ca^{2+} in the sickle cell membrane may also be involved in specific associations with one or more membrane proteins, changing their state in such a way as to cause the contraction of the membrane [21]. This would contribute towards the increased rigidity.

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