RED CELL MEMBRANE ABNORMALITIES IN β-THALASSAEMIA MAJOR

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1. Introduction

The thalassaemia syndromes are defined by the decreased or absent production of a specific globin chain. In β-thalassaemia major the decrease in β-chain synthesis leads to a relative excess of unstable α-chains which precipitate as intracellular inclusions [1]. It is thought that these inclusion bodies are one of the major factors leading to premature destruction of the cell in the bone marrow or spleen [2]. Interaction between the inclusion bodies and membrane components may result in membrane damage. There is no evidence to suggest a primary defect in the red blood cell membrane structure in this blood disorder.

The red cells of patients with β-thalassaemia major have been characterised by their dacryocytic morphology [3], altered surface sialic acid [4,5], a decreased number of titratable sulphydryl groups and an increased ratio of saturated to unsaturated membrane lipids, both induced by oxidative stress as a result of the increased production of 'activated oxygen' leading to lipid peroxidation and the formation of the secondary lipid breakdown product malonyldialdehyde [6,7].

Our studies on the β-thalassaemic membrane show that the hydrophobic lipid interior on the β-thalassaemic membrane becomes more rigid than in normal red cell membranes and although the membrane lipid and cholesterol contents are considerably enhanced in this disorder, there is no change in the cholesterol:phospholipid ratio. A significant factor may be the observed decreased number of free sulphydryl groups. Our results also show a lowering of ATP levels in β-thalassaemic red cells. Decreased ATP levels may be indicative of defects of red cell metabolism in the glycolytic pathway on which the production and reduction of oxygen free radicals partially depend, and may be one of the important factors leading to the observed membrane changes in β-thalassaemia major.

2. Experimental

Fresh human erythrocytes were obtained from normal, healthy donors and from patients with β-thalassaemia major. Samples were removed for the determination of total phospholipid content [8] and for cholesterol extraction [9] and analysis by gas chromatography. ATP levels were assayed using the UV-Method Test–Combination Kit from Boehringer. Ghosts were prepared within 24 h essentially by the method in [10] but using Tris buffers. Normal erythrocyte ghost preparations were haemoglobin free as judged by A_{414}. Thalassaemic ghosts were pink as expected due to haemoglobin retention but the absorbance was negligible at the concentrations used ($A_{414} < 0.06$ at 53.3 μg/ml). The ghost protein concentration was measured by the Lowry method [11] using crystalline bovine serum albumin (Sigma) as standard.

Fluorescence polarisation measurements were performed on the Elscint MV-1a Microviscosimeter (Elscint Ltd, Haifa) using the apolar fluidity probe 1,6-diphenyl-1,3,5-hexatriene (Aldrich) which penetrates the hydrophobic interior of the membrane and samples were labelled as in [12] to give a probe:lipid ratio of 0.007 [13]. Excitation was from polarised light (366 nm) from a mercury arc. Correction for light scattering was carried out by successive dilutions of the sample with Tris-buffered saline until a plateau value of the degree of fluorescence polarisation was obtained [14]. The observed polarisation of fluorescence value, p, the averaged response from a particu-

Table 1
The membrane microviscosity, cholesterol and total phospholipid contents, ATP levels and free sulphydryl contents of erythrocyte membranes from normal donors and patients with β-thalassaemia major

Mean ± SEM	Normal	β-Thalassaemics
Microviscosity (poise)η at 25°C	6.70 ± 0.19 (6)	7.90 ± 0.24 (11)
Cholesterol (μmol/10^{10} cells)	3.22 ± 0.15 (3)	4.49 ± 0.34 (8)
Total phospholipid (μmol/10^{10} cells)	4.42 ± 0.40 (3)	6.90 ± 0.53 (8)
Cholesterol:Phospholipid ratio	0.75 ± 0.08 (3)	0.67 ± 0.07 (8)
Sulphydryl group content (nmol/mg protein)	81.8 ± 1.5 (5)	65.0 ± 2.2 (4)
ATP levels (μmol/ml cells)	1.40 ± 0.12 (5)	0.74 ± 0.14 (9)
ANS binding constant K_d (μM)	22.6 ± 2.0 (3)	20.9 ± 2.0 (3)
No. ANS molecules bound (μmoles/g)	95.0 ± 3.0 (3)	70.0 ± 2.0 (3)

The figures in brackets represent the no. samples

lar membrane sample under specific conditions [15], is transposed into the fluorescence anisotropy r, $[r = 2p/(3 - p)]$ which can be converted to the microviscosity, η, according to the Perrin equation for rotational depolarisation of a nonspherical fluorophore [13]. The value of the excited state life-time was taken as 10 ns at 25°C [16].

Fluorescence measurements using the magnesium salt of 1-anilino-8-naphthalene sulphonate (ANS) (Eastman) were performed on a Perkin Elmer Hitachi 204 spectrofluorimeter at 25°C. This was essentially as outlined in [17] except that the membrane samples contained 53.3 μg ghost protein/ml and 10 μM ANS with a probe:lipid ratio of 0.143; the binding experiments involved varying probe and membrane concentrations. Appropriate blanks were included. The extrinsic fluorescence of ANS in the membrane was excited at 350 nm and the emission observed at 460 nm.

Membrane free sulphydryl groups were assayed spectrophotometrically at 412 nm using Ellman method [18] involving 5,5′-dithiobis-(2-nitobenzoic acid) (Sigma) as the colour reagent and using glutathione (reduced form) (Sigma) as standard. Cell morphology was examined unfixed using the Zeiss WL standard research microscope.

3. Results

The dynamic properties of the erythrocyte membrane lipid core in patients with β-thalassaemia major were studied by the polarisation of fluorescence of the apolar probe for fluidity, 1,6-diphenyl-1,3,5-hexatriene (DPH) and the polarisation and therefore the microviscosity of the membrane lipid region were markedly increased at 25°C (table 1). It remains to be established whether it is the bulk lipid fluidity which is decreased or whether distinct lipid domains are

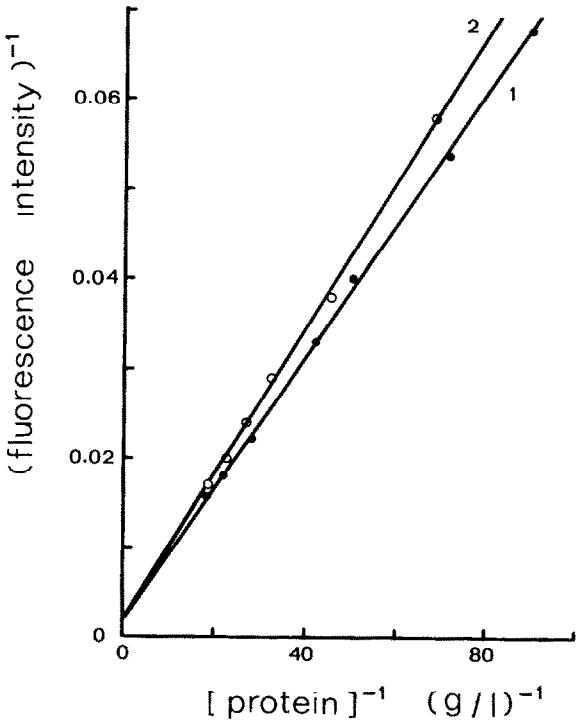

Fig.1. Double reciprocal plots of ANS fluorecescence (10 μM) as a function of membrane protein concentration: (1) normal erythrocyte membranes; (2) β-thalassaemic membranes.

involved. Measurements of the total phospholipid and the cholesterol contents were performed and both were shown to be elevated compared with normal, although there was no significant change in the cholesterol:phospholipid ratio.

Measurements of the sulphydryl group content showed a 20% decrease in free-SH groups suggesting protein crosslinking via disulphide bridge formation in the thalassaemic membranes or links between membrane proteins and haemoglobin, but this effect, though significant, is much less pronounced than those mentioned in [6]. Furthermore, our experiments show that ATP levels are decreased in thalassaemic cells down to 50% of the values obtained for normal erythrocytes.

The anionic fluorescent probe ANS was used to seek information on the changes in charge at the membrane surface [19]. The quantum yield of the bound ANS was unaltered in the thalassaemic membranes (fig.1) as shown by the limiting ANS fluorescence enhancement estimated by varying the membrane protein concentration at a fixed concentration of ANS and extrapolating the plot to infinite protein concentration. No change in the emission maximum wavelength was observed. To determine the number of binding sites for ANS and the binding constants the data were analysed by a Scatchard plot [20] using

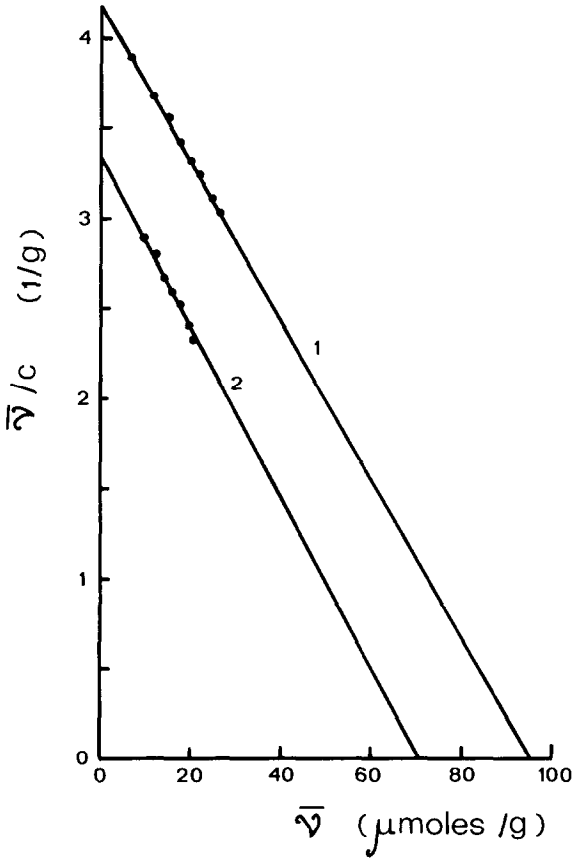

Fig.2. Scatchard plots for the binding of ANS (0–10 μM), membrane protein 0.0533 mg/ml: (1) normal erythrocyte membranes; (2) β-thalassaemic membranes.

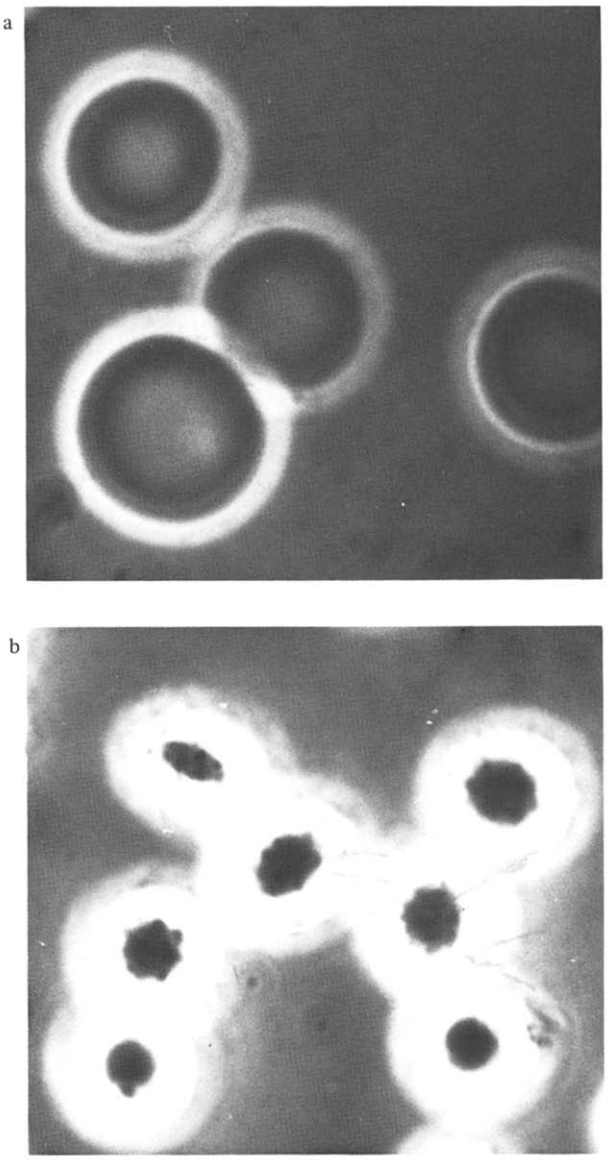

Fig.3. Phase contrast photographs of: (a) normal human erythrocytes in plasma; (b) erythrocytes from a patient with homozygous β-thalassaemia major. Magnification 1350 ×.

the values of the fluorescence of the ANS bound determined from the double reciprocal plot, as outlined in [17]. This data analysis involves the assumptions that all the binding sites are independent and identical in probe-binding properties. The results (fig.2) show that although the affinity of the membrane for the probe is unaltered less probe is actually bound to the thalassaemic membranes. Phase contrast photographs of normal and homozygous β-thalassaemic erythrocytes are shown in fig.3a,b. The thalassaemic cells appear contracted and show the presence of membrane-bound micro-aggregates of precipitated haemoglobin chains.

4. Discussion

The normal operation of the glycolytic and the pentose-phosphate pathways is one of the important factors in the prevention of haemoglobin precipitation and haemolysis as it is linked to the production and reduction of oxygen radicals [21]. The decreased levels of ATP observed here in the red cells of patients with β-thalassaemia major are possibly a result of a defective red cell metabolism in the glycolytic pathway. The production of activated oxygen radicals leads to lipid peroxidation and the consequence of the increased oxidative stress is an increased ratio of saturated to unsaturated lipid fatty acyl chains which is presumably one of the contributing factors towards the observed increase in the membrane rigidity in this blood disorder.

Changes in the charge at the membrane surface are deduced from the ANS binding experiments. Our findings that less anionic ANS molecules are bound at the thalassaemic membrane surface are consistent with the report [5] that the membrane sialic acid level of glycophorin in unsplenectomised patients with β-thalassaemia major is elevated, since the major source of the negative surface charge on human red cells is the carboxyl group of membrane-bound *N*-acetyl neuraminic acid. Other workers have reported a 25% decrease in the sialic acid content of the thalassaemic membranes but the majority of these patients had been splenectomised [6] and these data are consistent also with the results of thalassaemic patients who had undergone splenectomy [5].

Our work here suggests that disulphide crosslinking of the membrane proteins has occurred in this blood disorder but less extensively than has been reported by others. The contribution that the membrane protein crosslinking makes to the increased lipid rigidity is not clear. However it has been shown [22] that the rigidity of phospholipid bilayers is increased by lipid peroxidation. It is probable that lipid peroxidation is the major factor involved in the increased rigidity of the thalassaemic membrane lipids and this may be further enhanced by membrane protein crosslinking.

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