ERYTHROCYTE MEMBRANE ABNORMALITIES IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY OF THE MEDITERRANEAN AND A-TYPES

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1. Introduction

The red cells of patients with inherited deficiencies of glucose-6-phosphate dehydrogenase (G-6-PD) are sensitive to the haemolytic effects of a wide variety of drugs such as phenylhydrazine, primaquine, acetylsalicylic acid and fava bean [1]. The oxidative damage induced by such haemolytic agents may be caused by the generation of hydrogen peroxide, superoxide and hydroxyl radicals from the autoxidation of the active drug or its metabolites, or via a linked oxidation between oxyhaemoglobin and the drug or its metabolites [2–4]. These reactive species have the capacity to initiate the peroxidation of unsaturated fatty acids in the erythrocyte membrane phospholipids [5,6]. Oxidation of reduced glutathione is the major pathway of hydrogen peroxide metabolism in intact erythrocytes [7]. Continuous reduction of the oxidised glutathione is dependent on glutathione reductase, which itself is ($NADPH + H^+$)-dependent. The reduced form of this coenzyme is in short supply in individuals deficient in glucose-6-phosphate dehydrogenase and the resulting failure to maintain normal concentrations of reduced glutathione and the accompanying intracellular accumulation of hydrogen peroxide and oxidising radicals leads to the oxidation of free sulphydryl groups.

The red cell membranes from G-6-PD-deficient individuals with accompanying chronic haemolytic disease may contain polypeptide aggregates involving spectrin, dissociable by disulphide reducing agents [8,9]. These aggregates are considered to be indicators of oxidant damage to the red cell membrane leading to decreased deformability and haemolysis.

This work involves the erythrocytes from patients with the less severe and more common G-6-PD mutant of the A- and Mediterranean-types which are characterised clinically by intermittent haemolysis associated with drug treatment or infection; their catalytic activity is ~20–40% of the average activity of normal fresh human red cells. The results indicate the following membrane changes in such erythrocytes:

(i) The bulk lipid fluidity is increased;
(ii) The cholesterol:phospholipid ratio is decreased;
(iii) The lipid peroxidation level is elevated.

2. Materials and methods

Fresh human erythrocytes were taken from haematologically normal donors and from patients with G-6-PD deficiency (Mediterranean- and A-types). Samples were removed for the assay of glucose-6-phosphate dehydrogenase and ATP levels using the UV-method test-combination kits from Boehringer. Further samples were collected for the extraction of total lipid [10] and for phospholipid and cholesterol analysis [11].

Erythrocyte membranes were prepared within 24 h essentially applying the method in [12] but using Tris buffers and ghosts were finally suspended in Tris–HCl buffer in isotonic saline (pH 7.4). The ghost protein concentration was measured by the Lowry method [13] using crystalline bovine serum albumin as standard. The content of free sulphydryl groups in the membrane proteins was determined spectrophotometrically at 412 nm using the Ellman method [14] involving 5,5′-dithiobis-(2-nitrobenzoic acid) (Sigma) as standard. Red cell membrane lipid peroxidation was assayed as in [15] using the thiobarbituric acid method for measuring malonyldialdehyde, a secondary breakdown product of polyunsaturated fatty acid peroxides.

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Polyacrylamide gel electrophoresis was performed following the standard procedures [16,17] using 5% acrylamide gels. Gels were run at 5 mA/gel for 2 h, stained with Coomassie brilliant blue and scanned using a densitometer.

Measurements of the fluidity of the lipid region of the membrane were performed using the apolar fluorescent polarisation probe, 1,6-diphenyl-1,3,5-hexatriene (Aldrich) on the Elscint MV-la Microviscosimeter (Elscint Ltd, Haifa) as outlined in [18]. Excitation was from polarised light (366 nm) and corrections were made for light scattering by successively diluting the samples until the values for fluorescence polarisation reached a plateau. The observed polarisation p, the average response under specific conditions [19], was transposed into fluorescence anisotropy r, ($r = 2p/3-p$ and then converted into the relative microviscosity parameter $[(r_o-1)/r]^{-1}$, which is directly proportional to the microviscosity, where r_o is the limiting anisotropy for DPH, 0.362 [20].

Fluorescence measurements using the magnesium salt of 1-anilino-8-naphthalene sulphonate (ANS) (Eastman) were performed on a Perkin-Elmer Hitachi 204 spectrofluorimeter as outlined in [21].

3. Results

The G-6-PD deficient erythrocytes in the study contained an average level of glucose-6-phosphate dehydrogenase of ~25% of normal red cells (table 1). The lipid peroxidation level as measured by the production of malonyldialdehyde, formed during the breakdown of peroxidised fatty acid side chains of the phospholipids, was enhanced as shown in table 1. This was accompanied by the crosslinking of some of the sulphydryl groups of the membrane proteins to a small extent as shown by measurements of the concentration of free sulphydryl groups in the membrane (table 1). Polyacrylamide gel electrophoresis (fig.1) of these membranes showed that high M_r membrane polypeptide aggregates were formed

Table 1
The erythrocyte G-6-PD levels, membrane microviscosity, free sulphydryl contents, cholesterol:phospholipid ratio and lipid peroxidation levels of normal erythrocytes and those of patients with the common Mediterranean- and A-types of G-6-PD deficiency (standard deviations are shown)

	Control erythrocytes	G-6-PD-deficient erythrocytes
G-6-PD levels (mU/10^9 red cells)	133 ± 12 (5)	32 ± 26 (6)
Peroxidation level (A_{532}/mg protein)	0.095 ± 0.002 (4)	0.156 ± 0.03 (6)
Sulphydryl level (nmol/mg protein)	82 ± 2 (4)	71 ± 4 (6)
Relative microviscosity parameter at 25°C $[(r_o-1)/r]^{-1}$	2.43 ± 0.1 (4)	1.91 ± 0.2 (6)
Cholesterol:phospholipid ratio	0.89 ± 0.07 (5)	0.66 ± 0.05 (5)
ANS Binding constant (μM)	17.0 ± 1 (3)	17.0 ± 1 (3)
Number of ANS molecules bound (nmol/g)	90.0 ± 5.0 (3)	220 ± 20 (3)

The figures in brackets represent the number of samples

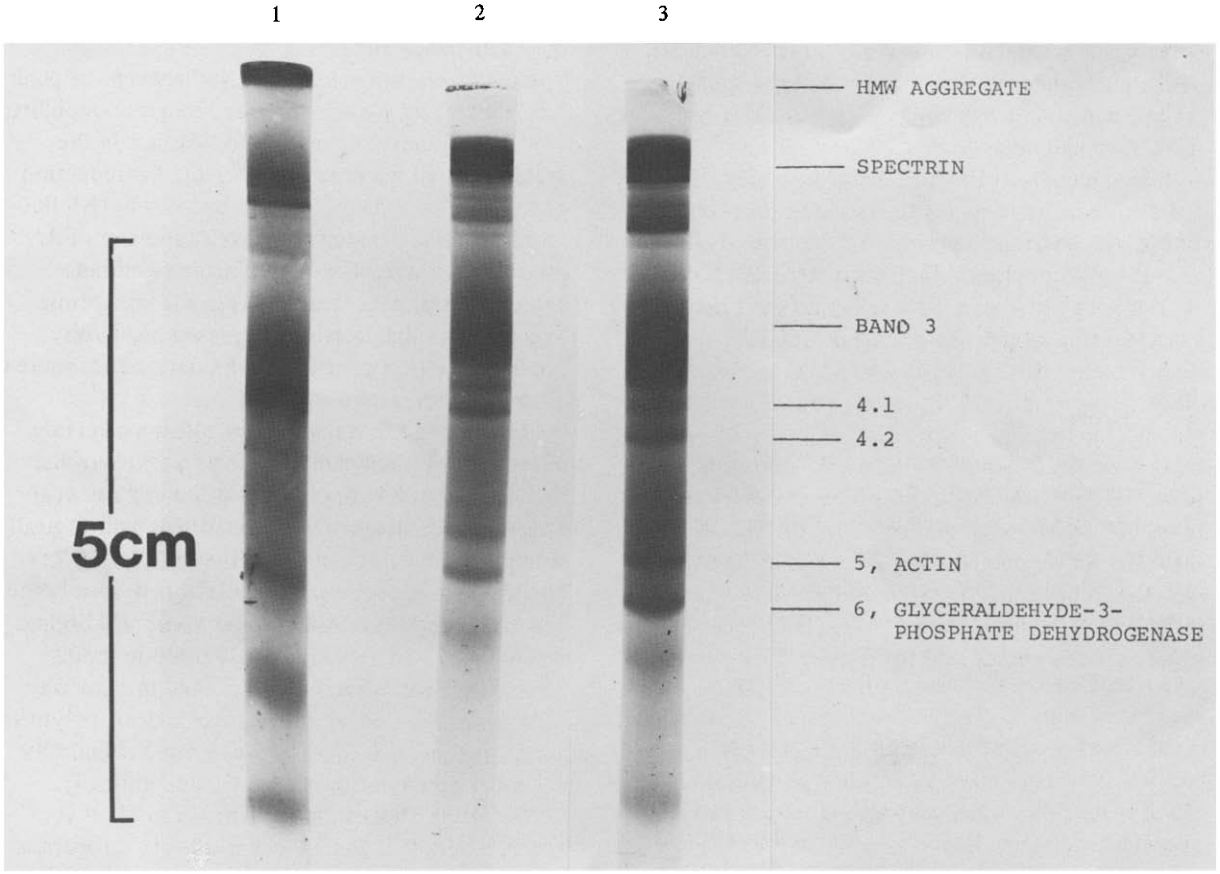

Fig.1. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate of red cell membranes from patients with G-6-PD deficiency of the common type (1); similar conditions to lane 1 except the samples were treated with dithiothreitol prior to electrophoresis (2); normal red cell membranes (3).

appearing at the top of the gel (lane 1) with a decrease in the intensity of the spectrin bands 1 and 2 and an obscuring of bands 4.1 and 4.2. The high M_r aggregates disappeared when the samples were treated with dithiothreitol prior to electrophoresis (lane 2) and the protein patterns appeared more normal. This showed that the aggregates originated from cross-linked sulphydryl groups on the membrane proteins.

Fluidity studies at 25°C on the apolar lipid region of the membrane, measured by incorporation of 1,6-diphenyl-1,3,5-hexatriene, showed small increases in the average bulk lipid fluidity of the G-6-PD-deficient membranes as indicated by the decrease in the relative microviscosity parameter (table 1). The anionic amphipathic fluorescent probe ANS was used to seek information on changes in charge at the membrane surface. The fluorescence intensity, which is dependent on the polarity of the environment of the probe and the environmental constraints, was decreased in the G-6-PD-deficient membranes. Titrations of varying concentrations of normal and G-6-PD-deficient membranes at a fixed concentration (10 μM) of ANS were performed and the double reciprocal plot extrapolated to estimate the limiting fluorescence enhancement when all the probe is bound to the membrane. Bound ANS in G-6-PD-deficient membranes is less fluorescent than in normal membranes, indicating different characteristics in the binding sites. There was, however, no change in the wavelength of maximum emission. To determine the number of binding sites for ANS and the binding constants, a Scatchard analysis was performed on the data as outlined in [22]. The results indicate that the binding of ANS to the membranes of G-6-PD-defi-

cient red cells is markedly increased in terms of the number of probe molecules bound (table 1) but the affinity of the membrane for the probe is unchanged at a dissociation constant of 17.0 ± 1.0 μM.

The cholesterol:phospholipid ratio in the G-6-PD-deficient membranes was decreased by 26%. The phospholipid content was not significantly altered but it was the cholesterol content that was lowered in this condition. Red cell ATP and DPG levels were normal suggesting the normal operation of the glycolytic pathway in this disorder.

4. Discussion

Normal red cells are capable of withstanding oxidative stress by increasing the activity of glucose-6-phosphate dehydrogenase (G-6-PD). The defective operation of the pentose phosphate pathway in red cells deficient in G-6-PD leads to the failure to maintain glutathione in its reduced state; its role is to protect the sulphydryl groups of the enzymes, haemoglobin and membrane proteins from oxidation.

The resulting production of activated oxygen radicals and the increased oxidative stress manifests itself in an increase in the lipid breakdown product [23,24], malonyldialdehyde, resulting from the peroxidation of the lipid fatty acid chains with 4 or more conjugated double bonds, thereby increasing the ratio of saturated to unsaturated lipid fatty acyl chains. In the G-6-PD-deficient erythrocytes the increased bulk lipid fluidity is presumably a consequence of the decreased cholesterol:phospholipid ratio. This increase is consistent with a decreased lipid packing density [25]. It is not clear whether it is oxidation of cholesterol or decreased synthesis of cholesterol at the reticulocyte stage which is responsible for the changes in microviscosity in the G-6-PD-deficient mutants, since NADPH, whose level is affected in this disorder, is required for cholesterol biosynthesis. Furthermore, since normally the unsaturated lipids are protected from free radical attack by the presence of cholesterol in biomembranes [26], this decreased cholesterol level may enhance their susceptibility to the reactivity of free radicals and therefore peroxidation. A similar relationship between lipid peroxidation and lipid fluidity has been shown in bovine red cell membranes during the onset of Heinz body anaemia which accompanies intensive brassica feeding [27]. Using electron spin resonance [28] an increase in the fluidity of the membrane in G-6-PD Helsinki cells was shown and a defect in the lipid region of the membranes suggested.

Changes in the packing of ANS between the polar headgroups and changes in polar headgroup mobility express themselves through modifications in the accessibility of water to the ANS and the reduction of the quantum yield [29]. The decreased ANS fluorescence intensity and the increased number of ANS binding sites in the G-6-PD-deficient membranes suggests a less-ordered structure in the head-group region due to the decreased lipid packing density, possibly resulting from lipid peroxidation and lowered cholesterol levels.

The drug-induced haemolysis which occurs in chronic G-6-PD-deficiency is a consequence of the oxidation of sulphydryl groups in the red cell membrane; membrane aggregate formation is pathologically related to decreased red cell deformability [30,31]. Our studies suggest the presence of high M_r membrane protein aggregates crosslinked via disulphide bridges in G-6-PD mutants of the non-haemolytic variety. Their formation is presumably related in some way to the peroxidation level, as similar high M_r polymers are formed in fresh erythrocytes treated chemically to induce peroxidation. (C. R. E., unpublished).

This work implies that a further important contributing factor to the altered rheological properites of G-6-PD mutants is the restricted capacity of the cell to break down oxidising radicals, e.g., superoxide anions $O_2^{\cdot-}$, which might participate in lipid peroxidation. Lipid peroxidation has also been implicated in the free radical reactions and membrane alterations associated with ageing cells and tissues [32,33] by initiating changes in their mechanical properties leading to their entrapment in the fine circulation of the spleen [34,35].

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