Evidence for an Altered Physical State of Membrane Proteins in Erythrocytes in Friedreich's Ataxia

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SUMMARY: Electron spin resonance, scanning electron microscopic, and SDS-polyacrylamide gel electrophoretic studies of erythrocytes in Friedreich's ataxia have been performed. No alteration in the physical state of membrane lipids, in morphology, or in the staining profile of erythrocytes in Friedreich's ataxia could be demonstrated. An altered conformation and/or organization of proteins in erythrocyte membranes in this disorder was suggested by spin labeling studies (P<0.025), favoring the possibility of a generalized membrane abnormality in Friedreich's ataxia. These findings are discussed in relation to other inherited neurological diseases where similar studies have been performed.

INTRODUCTION

Friedreich's ataxia (FA) inherited in an autosomal recessive manner is characterized clinically by ataxia of gait, dysarthria, muscle weakness, impaired proprioception and/or vibratory sense, and areflexia. In addition, scoliosis, pes cavus, cardiomyopathy, and an extensor plantar response are observed as the disease progresses. Other accessory symptoms and signs are observed in some FA patients (Barbeau, 1976). The pathological changes in FA include degeneration of posterior nerve roots and ganglia, posterior columns, Clarke's columns, and the spinocerebellar and pyramidal tracts (Barbeau, 1976).

Huang and co-workers (1978) found major differences in the high density lipoprotein (HDL) fraction of plasma lipoprotein in FA. In FA patients the relative proportion of cholesterol and triglycerides was increased, while the relative protein content was decreased. The cholesterol content of membranes can be exchanged with the plasma lipoproteins. Consequently, an abnormality in HDL in FA could result in a modified structure or fluidity of erythrocyte membranes. Moreover, taurine was found to have increased renal clearance rates in FA, suggesting a specific membrane transport defect (Lemieux et al, 1976). Taurine in the heart has been shown to increase calcium ion retention, the extra Ca\(^2+\) is known to have rigidizing effects on membrane lipids (Ohnishi and Ito, 1974). These possibilities of altered conformation and/or organization of proteins or fluidity of lipids of red cell membranes led to our investigation of erythrocyte membranes in FA by electron spin resonance, scanning...
electron microscopy, and SDS \times polyacrylamide gel electrophoresis.

MATERIAL AND METHODS
The spin labels employed in the current studies, 2,2,6,6-tetramethylpiperidin-1-oxyl-4 maleimide (MAL-6), and 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolindinylolyl (5-nitroxyde stearate of 5-NS) were obtained from Syva. All other chemicals and reagents were of the highest purity commercially obtainable.

The studies were carried out on twelve patients with Friedreich's ataxia as defined by the criteria of the Quebec Co-operative Study of Friedreich's Ataxia (Barbeau, 1976) and on an equivalent number of age and sex matched normal controls. All but one patient were ambulatory and only three were on medication. One subject was given 1200 mg lecithin, six times per day, another was given Beminal® (500 mg), 3 times per day, and the third was given diazepam, 5 mg per day. Blood was obtained in heparinized tubes by venipuncture and erythrocyte membranes (ghosts) were prepared as previously described (Butterfield, 1977b).

Electron spin resonance (ESR) spectra were recorded on a Varian E-109 electron spin resonance spectrometer employing an E-238 quartz aqueous sample cell. Modulation and power broadening of the ESR spectral lines were avoided by use of low microwave powers (16 milliwatts) incident on the E-238 rectangular cavity and by employing a modulation amplitude of 0.2 G. Membrane ghosts were spin labeled with MAL-6 or 5-NS as previously described (Butterfield, 1977a).

Control and FA unheparinized blood were drawn from seven FA patients and six controls by venipuncture through a short catheter infusion set and were processed for scanning electron microscopy (SEM) after the method of Miller et al (1976). Two or three drops of blood were dripped directly from the infusion set into 1% glutaraldehyde in phosphate buffer, pH 7.4. The unmanipulated erythrocytes were gently inverted and allowed to settle for 2 hours. One drop of cells was then pipetted to collagen-coated coverslips in a moist petri dish. Cells were then dehydrated through ascending ethanol solutions, processed through critical point drying in liquid C02, and coated with gold palladium. Samples were studied in a Cambridge Steroscan MKII-A scanning electron microscope at 20kV. Between 200 and 600 cells were counted for each subject.

SDS — polyacrylamide gel electrophoresis (Fairbanks et al, 1971) and estimation of membrane protein content (Lowry et al, 1951) were performed as previously described.

RESULTS
No alteration in the resulting protein profile of FA erythrocyte membranes, which had been subjected to SDS — polyacrylamide gel electrophoresis, compared to that of corresponding control volunteers could be demonstrated. Also, no altered morphology of FA cells from the seven subjects studied compared to control samples was observed by SEM.

The two spin labels used in these studies report on different environments within the erythrocyte membrane (Butterfield, 1977a,b). 5-NS is thought to orient in the lipid bilayer part of the membrane with its long alkyl chain on the average parallel to the alkyl chains of the membrane lipids, and its polar head group near the polar head groups of the lipid molecules (Hubbell and McConnell, 1971). A typical spectrum of 5-NS in erythrocyte membranes is shown in Figure 1. Several authors have discussed the interpretation of spectra like that in Figure 1 and the use of labels like 5-NS to deduce information about membrane fluidity via the order parameter S (Berlinier, 1976; McConnell and McFarland, 1970). The larger the value of S, the more rigid is the local microenvironment reported by the paramagnetic center of the spin label (Berlinier, 1976; McConnell and McFarland, 1970) which, in the case of 5-NS, is about 5 carbon atoms into the lipid bilayer from the membrane surface.

In contrast to noncovalently bound lipid-specific spin labels like 5-NS, MAL-6 is covalently bound to membrane protein sulphydryl (SH) groups, although a small amount of amino group binding may also occur (Chapman et al, 1969). The low field

Figure 1 — Typical ESR spectrum of 5-NS in normal erythrocytes. The T-tensor parameters are indicated.

Figure 2 — Typical ESR spectrum of human erythrocyte membranes labeled with MAL-6. Only the M1 = + 1 weakly and strongly immobilized lines (of amplitudes W and S, respectively) are presented.
DISCUSSION

No definitive change in lipid fluidity as reported by spin labeling methods or in the SDS — polyacrylamide gel electrophoretic staining profile of erythrocyte membranes from patients with Friedreich's ataxia was observed in these studies. In addition, no alterations in surface morphology were found in the FA samples examined by SEM. However, altered ESR parameters of a protein specific spin probe, suggesting changes in the physical state of membrane proteins in FA were observed (P<0.025, Table 1). The absolute difference of the mean values of the W/S ratio of MAL-6 in FA and control ghost membranes is not large. There could be a small change in a protein present in large amounts in the erythrocyte membrane in FA, or there could be a large change in a protein which constitutes only a small fraction of the total ghost protein. Other interpretations are possible.

It is known that at least two kinds of phospholipids exist in membranes: those that are free to move in the bilayer and those that are tightly attached to integral membrane proteins (so called boundary-lipids) (Jost et al., 1977). A small change in boundary-lipids (perhaps as a result of alterations in HDL) may lead to an altered conformation and/or organization of proteins in the red cell membrane as suggested in this study. The absence of fluidity differences in FA erythrocytes is not inconsistent with such a possible biochemical defect. The resulting spectrum is a time average of all spin label molecules in the membrane. Many more 5-NS molecules will likely be located in the free lipid portion of the bilayer since it constitutes the larger fraction of lipids in the membrane; hence, a small change in boundary lipid might not be detected in these experiments. Also, a slight increase in the amount of bound Ca²⁺ might lead to the results of the current study. This is a possibility since even a small change in Ca²⁺ concentration in the buffer causes a large change in the W/S ratio of MAL-6 attached to proteins in erythrocyte ghosts (D.A.B. unpublished results).

The absence of changes in both lipid fluidity and surface morphology in erythrocytes in FA is in contrast to such alterations observed in other inherited neurological diseases. Electron spin resonance studies of erythrocytes have shown an increased membrane fluidity in myotonic muscular dystrophy (Butterfield, 1977a), an increased membrane rigidity in Duchenne muscular dystrophy (Wilkerson et al., 1978), and no change in Huntington's disease (Butterfield et al., 1979). A nonspecific increased number of stomatocytes has been observed in SEM studies of erythrocytes in myotonic and Duchenne muscular dystrophy (Miller et al., 1976) and Huntington's disease (Markesbery and Butterfield, 1977).

Differences in the ESR parameters of MAL-6 have been observed in all these diseases (Butterfield, 1977a,b; Butterfield et al., 1977). However, different alterations in different proteins in each of these diseases could give rise to similarly altered spectra. Biochemical evidence supports the concept of different primary defects in these diseases. No alteration in the activity of membrane-bound protein kinase was observed in Huntington's disease erythrocytes (Butterfield et al., 1978a) while that of Duchenne muscular dystrophy was increased (Roses et al., 1975). Na⁺+K⁺-ATPase activity is increased in Huntington's disease erythrocytes (Butterfield et al., 1979).

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<thead>
<tr>
<th>MAL-6</th>
<th>5-NS</th>
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<tr>
<td>(W/S) Control</td>
<td>(W/S) FA</td>
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<tr>
<td>4.89±0.07</td>
<td>5.14±0.07</td>
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<tr>
<td>P&lt;0.025</td>
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<td>N</td>
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* Means±S.E.M. are presented.
** S- value is calculated by: S = T_{11} - T_{12} (TrT)
   (T_{11} T_{12} T_{13})(TrT)

where primed values are obtained experimentally (Figure 1) and unprimed crystal values are obtained from Jost et al. (1971). TrT is the trace of the nitrogen nuclear hyperfine tensor and equal to T_{11} + 2T_{12}.

*** P - value calculated by a two-way analysis of variance (Brownlee, 1960).
1978), while in myotonic muscular dystrophy it is normal (Roses and Appel, 1975), and in Duchenne muscular dystrophy it is abnormally stimulated by ouabain (Brown et al., 1967). The biophysical and biochemical studies and the current results suggest that the basic molecular defects in each of these disorders and Friedreich's ataxia are different.

In summary, our findings in the present experiments suggest an alteration in the conformation and/or organization of membrane proteins in erythrocytes in Friedreich's ataxia and support the concept that this disorder may be associated with a generalized membrane abnormality.

ACKNOWLEDGMENTS

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REFERENCES


