Quebec Cooperative Study of Friedreich's Ataxia

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

D. A. BUTTERFIELD, P. K. LEUNG, W. R. MARKESBERY AND A. BARBEAU

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.*

RÉSUMÉ: *Nous avons étudié des érythrocytes provenant de patients avec ataxie de Friedreich grâce à des techniques de résonance paramagnétique électronique, de microscopie à balayage électronique et d'électrophorèse sur gel de polyacrylamide SDS. Nous n'avons pas pu démontrer des modifications dans la morphologie, dans le profil de coloration ou dans l'état physique des lipides membranaires des ataxiques. Cependant une possibilité ($p<0.025$) de conformation ou d'organisation altérée des protéines de ces mêmes membranes est suggérée par les études de résonance paramagnétique électronique. Ceci soulève la possibilité d'une anomalie membranaire généralisée dans l'ataxie de Friedreich. Ces résultats sont comparés à ceux obtenus avec des méthodes semblables dans plusieurs autres maladies neurologiques héréditaires.*

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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

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electron microscopy, and SDS × polyacrylamide gel electrophoresis.

MATERIAL AND METHODS

The spin labels employed in the current studies, 2,2,6,6-tetramethyl-piperidin-1-oxyl-4 maleimide (MAL-6), and 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolindinylolyl (5-nitroxide 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 Freidreich'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 $C0_2$, 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 (Berliner, 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 (Berliner, 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 sulfhydryl (SH) groups, although a small amount of amino group binding may also occur (Chapman et al, 1969). The low field

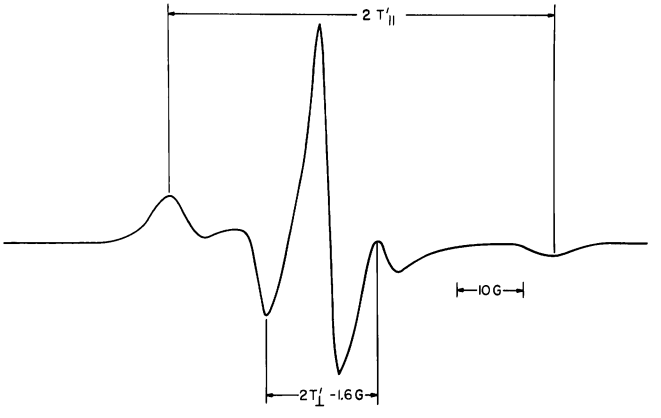

Figure 1 — Typical ESR spectrum of 5-NS in normal erythrocytes. The T-tensor parameters are indicated.

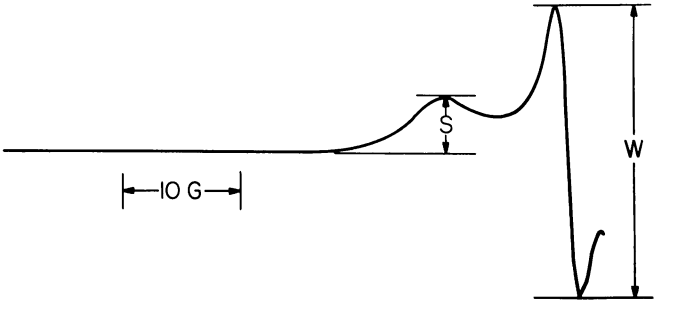

Figure 2 — Typical ESR spectrum of human erythrocyte membranes labeled with MAL-6. Only the $M_I = +1$ weakly and strongly immobilized lines (of amplitudes W and S, respectively) are presented.

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($M_I = +1$) lines of a typical spectrum of erythrocyte ghosts labeled with MAL-6 are shown in Figure 2. Considerable discussion of the interpretation and use of MAL-6 in the study of erythrocyte membrane proteins in several disease states has been presented in previous papers from our laboratory (Butterfield, 1977a,b; Butterfield et al, 1977). The ratio of the ESR spectral amplitude of MAL-6 attached to weakly-immobilized SH groups (W) to that of MAL-6 attached to strongly immobilized SH group (S) is a sensitive and convenient monitor of conformational and/or organizational differences of membrane proteins in erythrocytes (Butterfield 1977a,b; Butterfield et al, 1977).

The W/S ratio of MAL-6 attached to membrane proteins is increased in FA erythrocytes compared to normal controls ($P<0.025$, Table 1), suggesting that there is an alteration in the physical state of membrane proteins in FA. The order parameter of 5-NS is unchanged in FA erythrocytes compared to control ($P<0.25$, Table 1). The half-width at half maximum amplitude of the low-field ESR line of 5-NS has been suggested as being a more sensitive monitor of membrane fluidity than the order parameter (Mason et al, 1977). Use of this approach resulted in a trend indicative of a more rigid lipid environment in FA erythrocytes but the level of significance was borderline ($0.05<P<0.1$) (data not shown)

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 Ca^{+2} might lead to the results of the current study. This is a possibility since even a small change in Ca^{+2} 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 myotonic dystrophy was reduced (Roses and Appel, 1975) and 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,

TABLE 1

Electron Spin Resonance Parameters of MAL-6 and 5-NS in Erythrocyte Membranes from Friedreich's Ataxia Patients and Normal Controls+

MAL-6		5-NS	
$^{(W/S)}$ Control	$^{(W/S)}$ FA	$^{**(S)}$ Control	$^{(S)}$ FA
4.89±0.07	5.14±0.07	0.682±0.004	0.690±0.003
P***<0.025		P<0.25	
N 12	12	7	7

* Means±S.E.M. are presented.

** S- value is calculated by: $S = \frac{T'_{11} - T'_{\perp}}{(T_{11} \quad T_{\perp})_{XL}} \quad \frac{TrT}{TrT'}$

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 $T_{11} + 2T_{\perp}$.

*** 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 oubain (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.

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REFERENCES

BARBEAU, A. (1976). Friedreich's ataxia 1976—an overview. Can. J. Neurol. Sci. 3, 389-397.

BERLINER, L. J. (ed.) (1976). Spin Labeling — Theory and Applications, Academic Press, New York.

BROWN, H. D., CHATTPADHYAY, S. K., and PATEL, A. B. (1967). Erythrocyte abnormality in human myopathy. Science 157, 1577-1578.

BROWNLEE, K. A. (1960). Statistical Theory and Methodology in Science and Engineering, John Wiley, New York.

BUTTERFIELD, D. A. (1977a). Electron spin resonance studies of erythrocyte membranes in muscular dystrophy. Accounts Chem. Res. 10, 111-116.

BUTTERFIELD, D. A. (1977b). Electron spin resonance investigations of membrane proteins in erythrocytes in muscle diseases: Duchenne and myotonic muscular dystrophy and congenital myotonia. Biochim. Biophys. Acta 470, 1-7.

BUTTERFIELD, D. A., OESWEIN, J. Q., and MARKESBERY, W. R. (1977). Electron spin resonance study of membrane protein alterations in erythrocytes in Huntington's disease. Nature 267, 453-455.

BUTTERFIELD, D. A. OESWEIN, J. Q., PRUNTY, M. E., HISLE, K. C., and MARKESBERY, W. R. (1978). Increased sodium plus potassium adenosine triphosphatase activity in erythrocyte membranes in Huntington's disease. Ann. Neurol. 4, 60-62.

BUTTERFIELD, D. A., PURDY, M. J., and MARKESBERY, W. R. (1979). Electron spin resonance, hematological, and deformability studies of erythrocytes from patients with Huntington's disease. Biochim. Biophys. Acta, in press.

CHAPMAN, D., BARRATT, M. D., and KAMAT, V. B. (1969). A spin label study of erythrocyte membranes. Biochim. Biophys. Acta 173, 154-157.

FAIRBANKS, G., STECK, T. L. and WALLACH, D. F. H. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10, 2606-2617.

HUANG, Y. S., NESTRUCK, A. C., BARBEAU, A., BOUCHARD, J. P., and DAVIGNON, J. (1978). Plasma lipids and lipoproteins in Friedreich's ataxia and familial spastic ataxia — evidence for an abnormal composition of high density lipoproteins. Can. J. Neurol. Sci. 5, 149-156.

HUBBELL, W. L., and McCONNELL (1971). Molecular motion in spin labeled phospholipids and membranes. J. Amer. Chem. Soc. 93, 314-326.

HUXTABLE, R. (1976). Metabolism and function of taurine in the heart. In: Taurine, Raven Press, (Huxtable, R. and Barbeau, A., eds.), pp 99-119.

JOST, P. C., LIBERTINI, L. J., HERBERT, V. C., and GRIFFITH, O. H. (1971). Lipid spin labels in lecithin multilayers: a study of motion along fatty acid chains. J. Mol. Biol. 59, 77-98.

JOST, P. C., NADAKAVUKAREN, K. K., and GRIFFITH, O. H. (1977). Phosphatidyl-choline exchange between the boundary lipid and bilayer domains in cytochrome oxidase containing membranes.

LEMIEUX, B., BARBEAU, A., BERONIADE, V., SHAPCOTT, D., BRETON, G., GEOFFROY, G., and MELANCON, S. (1976). Amino acid metabolism in Friedreich's ataxia. Can. J. Neurol. Sci. 3, 373-378.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. C., and RANDALL, R. J. (1951). Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265-275.

MARKESBERY, W. R., and BUTTERFIELD, D. A. (1977). Scanning electron microscopy studies of erythrocytes in Huntington's disease. Biochem. Biophys. Res. Commun. 78, 560-564.

McCONNELL, H. M., and McFARLAND, B. G., (1970). The chemistry and physics of spin labels. Quart. Rev. Biophys. 3, 91-136.

MASON, R. P., GIAVEDONI, E. B., and DALMASSO, A. P. (1977). Complement-induced decrease in membrane mobility: introducing a more sensitive index of spin-label motion. Biochemistry 16, 1196-1201.

MILLER, S. E., ROSES, A. D., and APPEL, S. H. (1976). Scanning electron microscopy studies of erythrocytes in muscular dystrophy. Arch Neurol. 33, 172-174.

OHNISHI, S., and ITO, T. (1974). Calcium induced phase separations in phosphatidylserine-phosphatidylcholine membranes. Biochemistry 73, 881-887.

ROSES, A. D., and APPEL, S. H. (1975). Phosphorylation of component a of the human erythrocyte membrane in myotonic muscular dystrophy. J. Membr. Biol. 20, 51-58.

ROSES, A. D., HERBSTREITH, M. H., and APPEL, S. H. (1975). Membrane protein kinase alteration in Duchenne muscular dystrophy. Nature 254, 350-351.

WILKERSON, L. S., PERKINS, R. C., ROELOFS, R., SWIFT, L., DALTON, L. R., and PARK, J. H. (1978). Erythrocyte membrane abnormalities in Duchenne muscular dystrophy monitored by saturation transfer electron paramagnetic resonance spectroscopy. Proc. Nat. Acad. Sci. U.S.A. 75, 838-841.