Quebec Cooperative Study
of Friedreich's Ataxia

Erythrocyte Membrane Lipids in Friedreich's Ataxia

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SUMMARY: *In a study of the lipid composition of erythrocyte membranes in Friedreich's ataxia, the concentration of the major membrane components (phospholipids, cholesterol and protein) in ataxic patients, family members, and control subjects were found to be the same. The total fatty acid distribution was also normal. However, an altered distribution of phospholipid classes in erythrocytes was noted (an increase of PI + PS and a decrease of PE in Friedreich's ataxia patients).*

RÉSUMÉ: *Nous avons étudié la composition lipidique des membranes érythrocytaires, ainsi que la concentration des principales composantes de la membrane (phospholipides, cholesterol et protéines) chez des sujets atteints d'ataxie de Friedreich, des membres de leurs familles et des sujets témoins. Aucune différence majeure ne fut trouvée. La distribution des acides gras totaux était également normale, cependant nous avons noté une altération dans la distribution des classes de phospholipides dans les érythrocytes (une augmentation de PI + PS et une diminution de PE dans l'ataxie).*

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INTRODUCTION

The possibility of a generalized membrane defect in Friedreich's ataxia has been suggested. This was based in part on the preliminary observation of a difference in the stability of erythrocyte membranes in Friedreich's ataxia patients, as determined by SDS polyacrylamide gel electrophoresis (Shapcott et al., unpublished).

Lipids are also of fundamental importance in membrane structure, and the possibility of an abnormality in lipid composition was suggested by the greatly increased cholesterol/protein ratio in the plasma high density lipoprotein (HDL) fraction in Friedreich's ataxia (Huang et al., 1978). As part of the study of membrane function in Friedreich's ataxia, we measured the major lipid components of erythrocyte membranes in Friedreich's ataxia patients, in unaffected family members, and in control subjects.

SUBJECTS AND METHODS

A. Subjects

Typical Friedreich's ataxia (Group Ia) patients and non-affected family members (obligatory heterozygotes and siblings*) were studied. Control subjects were apparently healthy adult volunteers from laboratory and secretarial personnel.

B. Preparation of Ghosts

Fasting blood samples were collected in heparinized tubes and the plasma removed. The red cells were washed once with cold 5mM sodium phosphate (pH 8) — 0.15 M NaCl and the ghosts prepared according to the method of Fairbanks et al. (1971). The ghosts were divided into aliquots for further analysis and were then lyophilized and the weight of each fraction determined.

*Siblings: are either heterozygotes or normal.

Inorganic phosphorus was determined by direct addition of the ammonium molydate — p — semidine reagents to an acidified solution of ghosts (Wybenga, 1974). From this, the weight of phosphate buffer remaining in the ghosts was calculated, and the net weight of lyophilized ghosts was determined by subtraction of the amount of remaining buffer from the lyophilized ghost weight.

Total proteins

Lyophilized ghosts were dissolved by incubation in 0.1 M NaOH overnight at 37°. The method of Lowry (1951) was then used to measure protein in the solution.

Lipids

About 6-8 mg lyophilized ghosts (equivalent to 1 ml ghost preparation before lyophilization) was shaken gently with 1.0 ml chloroform-methanol (2:1) for 5 min. The mixture was centrifuged, the supernatant divided into fractions for the various lipid determinations, and the solvent was evaporated with a stream of nitrogen at room temperature.

For determination of the phospholipid classes, erythrocytes were extracted directly according to the method of Dodge and Phillips (1967).

Total phospholipid

An aliquot (0.1) of the total lipid extract was digested with sulfuric acid and the phosphorus determined according to the method of Wybenga (1974). Quantities of each reagent were reduced proportionally to accommodate the relatively small amount of phosphorus.

Phospholipids

The major phospholipid classes were separated by thin layer

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chromatography (Noel et al., 1972) and lipid phosphorus in each class measured by the method of Bartlett (1959).

Cholesterol

Total and free cholesterol were determined in aliquots of the total lipid extract as described by Dryer (1970).

Fatty acids

Phytanic acid (100μg), methanol (0.4 ml), and 10 M NaOH (60μl) were added to 0.4 of the total lipid extract, and the mixture saponified by heating 15 min at 100°. Methyl esters were prepared by heating the fatty acids in 20 μl of a methylating reagent (made from mixing 0.4 ml methanol and 0.1 ml acetyl chloride) at 120° for 10 min. Excess reagent was evaporated and the produce repeated.

The dried esters were dissolved in methanol (20μl) and 2μl injected onto a 6 ft glass column packed with 10% SP 2330 on 100/120 Chromosorb WAW (Supelco) in a Hewlett-Packard model 5750 gas chromatograph equipped with a flame ionization detector. The oven temperature was programmed from 150 to 200°C at 2°/min. Peak identifications were confirmed for one Friedreich's ataxia patient with a gas chromatograph-mass spectrometer (GC-MS) combination consisting of a Hewlett Packard model 7130-A gas chromatograph connected to an AE1-MS30 mass spectrometer. The fatty acid compositions reported here are calculated directly from relative peak areas and are not corrected for relative recoveries or responses. However, the reproducibility of the procedure was monitored by carrying out the extraction, methylation, and chromatographic steps on mixtures of known quantities of the acids with each lot of samples.

RESULTS

Table 1 summarizes the distribution of the major components (phospholipids, cholesterol, protein) of erythrocyte membranes from Friedreich's ataxia patients, unaffected family members, and external controls. No significant differences among the groups is seen for these components except for a somewhat lower total cholesterol/total protein ratio for the obligatory heterozygotes ($p<002$ compared with external controls and $p<0.05$ compared with Friedreich's ataxia). However, it must be noted that these results are based on a small number (N=4) of heterozygotes. Determination of free cholesterol (by precipitation with digitonin) and gas chromatographic analysis of cholesterol esters showed that most of the cholesterol is in the free form with very low concentrations of cholesterol esters.

In Table 2, the distribution of the major phospholipid classes in erythrocytes of Friedreich's ataxia patients and control subjects is given. There is a significant decrease in phosphatidylethanolamine (PE, $p<0.001$) and an increase in phosphatidylinositol + phosphatidylserine (PI + PS, $p<0.01$) in Friedreich's ataxia patients is noted.

Table 3 shows the distribution of membrane fatty acids in Friedreich's ataxia patients and unaffected family members while in Table 4 the sum of the fatty acid concentrations are expressed as a percentage of the ghost weight and also relative to total ghost protein. The only difference in the distribution and total quantity of fatty acids among the groups is a tendency toward a lower fatty acid/protein ratio for the obligatory heterozygotes.

DISCUSSION

The concept of a generalized membrane defect in several inherited disorders, particularly the neuromuscular diseases, is becoming widely

TABLE 1

Composition of Erythrocyte Membranes (Mean± S.D.)

	Friedreich's Ataxia (N=16)	Obligatory Heterozygotes (N=4)	Siblings (N=10)	External Controls (N=15)
Total Phospholipid (% Dry Ghost Weight)	26.0±2.8	26.8±1.7	26.1±3.3	25.2±4.2
Total Cholesterol (% Dry Ghost Weight)	10.23±1.99	8.93±0.82	9.79±1.32	10.46±2.48
Total Protein (% Dry Ghost Weight)	40.63±2.61	42.63±2.00	40.81±2.14	39.69±6.22
Total Phospholipid/ Total Protein	0.65±0.09	0.63±0.04	0.65±0.07	0.66±0.19
Total Cholesterol/ Total Protein	0.249±0.048	0.210±0.025	0.240±0.031	0.269±0.074

TABLE 2

Percentage Composition of Erythrocyte Phospholipids (Mean ± S.D.)

	Friedreich's Ataxia (N=12)	Controls (N=9)
Total Phospholipid (mg/ml)	3.21±0.35	2.97±0.72
Lysophosphatidylcholine	Trace	Trace
Spingomyelin (S)	28.49±4.63	26.86±2.90
Phosphatidylcholine (PC)	29.22±3.96	32.00±1.87
Phosphatidylinositol (PI) +Phosphatidylserine (PS)	19.84±2.68	14.03±2.99*
Phosphatidylethanolamine (PE)	22.45±3.47	27.10±3.24**

* $p < 0.001$
** $p < 0.01$

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TABLE 3

Percentage Composition of Membrane Fatty Acids (Mean ± S.D.)

	Friedreich's Ataxia (N=13)	Obligatory Heterozygotes (N=4)	Siblings (N=10)	External Controls (N=14)
C14:0	1.57±0.63	1.31±0.99	1.98±1.06	2.27±1.35
C16:0(+C17:0)	27.27±3.68	25.29±3.41	27.09±3.40	26.85±4.11
C16:1	1.17±0.34	1.42±0.48	1.10±0.48	0.93±0.35
C18:0	18.71±2.49	17.54±2.92	17.25±2.70	17.48±2.60
C18:1	21.49±2.74	23.51±2.17	20.22±2.39	21.61±2.20
C18:2	13.16±2.00	13.57±1.00	14.43±2.15	13.70±3.31
C20:3	1.59±1.80	0.61±0.59	0.97±0.55	0.71±0.55
C20:4 +C22:0	15.16±5.44	16.73±2.70	17.13±2.37	16.62±3.58

TABLE 4

Total Fatty Acids ($C_{14:0}$ - $C_{20:4}$) in Erythrocyte Ghosts (Mean ± S.D.)

	Total Fatty Acid (% Dry Ghost Weight)	Total Fatty Acid/ Total Protein
Friedreich's Ataxia (N=13)	13.9±3.6	0.34±0.09
Oblitatory Heterozygotes (N=4)	12.8±2.1	0.30±0.06
Siblings	16.1±4.0	0.39±0.09
External Controls (N=14)	13.4±2.6	0.34±0.07

accepted. Alterations in the composition of the two major membrane components, lipids and proteins, would affect the physical state of the membrane with far reaching consequences in membrane function being likely. Based on the hypothesis that the defect is manifested in membranes of many different tissue cells, and because of its availability and relative ease of isolation, the erythrocyte membrane is a widely used model. The possibility of a difference in erythrocyte and fibroblast membrane protein composition in Friedreich's ataxia will be discussed in detail in other papers in this series.

Defects in lipid metabolism have been associated with a number of inherited diseases, a relevant example being the large increase in plasma phytanic acid in Refsum's disease, a hereditary form of ataxia (Steinberg, 1978).

Erythrocyte lipids have also been measured in a number of disease states and an abnormal composition has been found in patients with familial lecithin: cholesterol acyltransferase (LCAT) deficiency (Gjone et al., 1968). Much work has been described on erythrocyte lipids in Duchenne Muscular Dystrophy with abnormal (Kunze et al., 1973; Kalofoutis et al., 1977) and normal (Kobayashi et al., 1978; Roses and Appel, 1978) phospholipid composition being reported. It has been suggested that oxidation of polyunsaturated fatty acid components during their extraction and analysis may contribute to anomalous results, and differences in lipid content and fatty acid composition of erythrocyte phospholipids between adult and child controls has been reported (Kobayashi et al., 1978). These examples show that comparison of membrane lipid determinations must be based on identical experimental methods, and that variables such as age or the effects of unrelated disease states must also be considered.

In an alternative approach to the elucidation of membrane structure, Butterfield et al. (1974, and this issue) have described the results of ESR experiments which demonstrate different lipid environments in erythrocyte membranes of myotonic muscular dystrophy and Friedreich's ataxia patients. It has been suggested that very subtle differences in chemical composition or altered protein-lipid organization could account for the greater membrane fluidity in myotonic muscular dystrophy, and that these differences would probably not be measurable by the usual chromatographic techniques.

Interpretation of the differences in erythrocyte phospholipids in Friedreich's ataxia is difficult at this stage of our investigation, especially in view of the normal distribution in plasma (Huang et al., 1978) and in platelets (Filla et al., this issue), and also in view of the conflicting results described for the muscular dystrophies. Although our results are consistent with our hypothesis of a generalized membrane defect, they may simply reflect two extremes of the normal range, resulting from differences in diet or other environmental factors. Further studies of the fatty acid composition of the erythrocyte phospholipid classes along with lipid determinations in fibroblasts and biopsy material are planned in order to determine the extent of these differences. The normal fatty acid distribution in erythrocyte membranes of Friedreich's ataxia patients and unaffected family members is in contrast with the anomalies of fatty acid distribution in plasma lipoproteins, where a decrease of linoleic acid ($C_{18.2}$) has been observed in Friedreich's ataxia patients (Davignon et al., this issue). In this respect, dietary fatty acids are incorporated into circulating red cells by exchange as well as by synthesis of new cells. However, it has been suggested that the erythrocyte can maintain its lipid balance even when the plasma levels are altered as long as the enzyme system controlling exchange between the cell and its environment is functioning normally (Nelson, 1972).

The possibility of an error in the identification of a fatty acid or of missing an abnormal acid which elutes with a normal acid peak in a gas chromatographic analysis should

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always be considered. Therefore, in this study, the membrane fatty acids listed in Table 3 from one Friedreich's ataxia patient were determined by mass spectrometry. Branched chain fatty acids, such as phytanic acid, give characteristic fragmentation patterns in their spectra and, thus, it should be possible to identify such compounds even in the presence of normal straight-chain acids. However, no abnormal acids were found in the patient examined and therefore the presence of large amounts of abnormal acids in erythrocyte membranes of Friedreich's ataxia patients seems unlikely. There remains the slight possibility that a subtle difference in acid structure (for example, a difference in the ratio of cis-trans isomers) not readily detectable by mass spectrometry may occur in Friedreich's ataxia.

In our fatty acid determinations, we used an internal standard so that the total fatty acid concentration as well as their distribution could be measured. Phytanic acid was chosen as the standard since it elutes in a peak free area of the chromatogram, and it was found not to be present in significant amounts in erythrocyte ghosts of Friedreich's ataxia or control subjects.

The results of our study of erythrocyte membrane in Friedreich's ataxia suggest a normal concentration of the major components, proteins, phospholipids and cholesterol. Similarly, the total fatty acid concentration and distribution is normal; however, an increased percentage of PI + PS and a decrease of PE in erythrocytes of Friedreich's ataxia patients was found. Although no gross abnormalities in lipid composition were uncovered, the results along with the abnormalities observed by E.S.R. suggest that further work on membrane lipids be done, particularly the study of the fatty acid composition of individual phospholipids.

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