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A Generalized Membrane Defect in Heritable Myotonia: Studies of Erythrocytes in an Animal Model and Patients (40725)

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There is substantial evidence from electrophysiological studies that the prolonged contraction with repetitive action potentials of skeletal muscle characteristic of myotonia is associated with a reduced chloride conductance of the surface membranes (1, 2). However, few studies have approached the pathophysiology of myotonia by examining the composition and molecular structure of the muscle cytomembranes. Our previous studies using purified fragmented sarcoplasmic reticulum (FSR) from goats with hereditary myotonia demonstrated increased amounts of saturated fatty acids, increased sialic acid, increased bound calcium, increased calcium sequestration, and increased permeability to calcium when compared to normal (3, 4). These findings were interpreted as being compatible with less fluid, more permeable FSR incapable of maintaining appropriate calcium homeostasis.

In order to explore the concept that hereditary myotonia may be manifest as a generalized membrane defect, we have extended our studies to erythrocytes from the myotonic goat and from patients with myotonic muscular dystrophy. Additional data are included for hemogram studies in the goat.

Materials and methods. Experimental animal model and blood collection. The experimental animal model used in these studies was the myotonic goat. These animals came from a herd which has been line-bred over the past 20 years. They exhibited the characteristic manifestations of myotonia which were confirmed by electromyographic measurements (4). Normal goats of a different genetic background were purchased from a local breeder and had no evidence of myotonia. All goats were allowed to graze in the same pasture and were free of other diseases. Normal and myotonic goats were studied in pairs which were age- and sex-matched, and the data from all experiments were analyzed by Student's *t* test.

Blood was collected by venipuncture from the jugular vein into either sodium heparin (Venoject vacuum blood collection tubes, Kimble-Terumo, Inc., Elkton, MD.) or, for some studies, into tubes using no anticoagulant. When no anticoagulant was used, the blood was drawn into glass syringes that had been siliconized and was immediately centrifuged at 2000 *g* for 6 min in siliconized glass tubes. The plasma and buffy coat were removed and the erythrocytes were washed three times in a Ringer's–glucose buffer (0.154 *M* NaCl, 5.6 m*M* KCl, 6.0 m*M* $NaHCO_3$, 0.68 m*M* $CaCl_2$, 3.9 m*M* dextrose, pH 7.4) and once in 0.154 *M* NaCL–10 m*M* HEPES, pH 7.4

Hematological methods. Standard methods were used for determinations of red cell counts, packed cell volumes, and hemoglobin concentrations on heparinized blood (5).

Osmotic fragility assays were carried out on defibrinated blood by the method of Shen *et al.* (6) within 3 hr of collection. Data from the hemolysis curves were analyzed and a value for the mean erythrocyte fragility, X_{50}, was calculated. This value is the sodium chloride concentration at which 50% hemolysis occurs and represents the fragility of the greatest number of cells (7).

Erythrocyte membrane isolation. Erythrocyte membranes were prepared by a modification of the method of Hanahan and

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Ekholm (8). Erythrocytes which were isolated from blood collected with no anticoagulant were washed four times as described above, resuspended to a 50% packed cell volume in the buffer, and then hemolyzed with 20 m*M* NaCl–10 m*M* HEPES, pH 7.4. The membranes were pelleted by centrifuging at 20,000 *g* for 60 min, washed four times in 10 m*M* NaCl–1 m*M* HEPES, pH 7.4, and resuspended in buffer by homogenization with a Potter–Elvehjem tissue grinder (Wheaton Scientific, Millville, N.J.) and stored at −70°C. The membrane pellets were white or cream colored, indicating minimal hemoglobin contamination. When examined by electron microscopy the samples consisted of sealed membrane vesicles with no traces of particulate material. Protein was estimated by the method of Lowry *et al.* (9) using bovine serum albumin as standard.

Protein analysis. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (10). Erythrocyte membranes were dissolved in 4 *M* urea, 1% SDS, and 2% 2-mercaptoethanol at 37°C for 1 hr, then heated at 100°C for 5 min. Eighty micrograms of protein were applied to each gel which contained 6% acrylamide, 0.1 *M* sodium phosphate, pH 7.0, and 1% SDS. Electrophoresis was performed in a phosphate buffer (0.1 *M* sodium phosphate, 1% SDS, 0.38 *M* glycine, pH 7.0) at 3 mA per gel and the gels were stained in 5% methanol, 7% acetic acid, and 0.1% Coomassie blue. Molecular weights were calibrated with known protein standards.

Lipid analysis. Lipids were extracted from erythrocyte membranes (11) and separated using thin-layer chromatography (tlc) techniques. The lipids (4–8 mg total lipid) were streaked on a tlc plate (precoated silica gel 60, EM Laboratories, Elmsford, NY) using a Chromatocharger (Camag Chemie-Erzengnisse, und Adsortionstechnik A. G., Muttenz, Switzerland). The plates were developed in petroleum ether: ethyl ether: acetic acid (80:20:1), stained with Rhodamine 6G, and the individual lipids were identified by comparison to a standard lipid mixture (Non-Polar Lipid Mix A, Supelco, Inc., Bellefonte, Pa.). Free cholesterol was eluted from the silica gel with 4:1 chloroform–methanol and analyzed (12).

The phospholipid, cholesterol ester, triacylglycerol, and free fatty acid fractions were quantitated by gas chromatography techniques. Fatty acids were methylated (13), and the methyl esters were analyzed with a Varian 2100 gas chromatograph (Varian Instruments, Palo Alto, Calif.) at 175–225°C using a column packed with SP-2340 on 100/200 mesh Supelcoport (Supelco, Inc.) and flame ionization detection. The composition of the fatty acid mixtures was estimated from the relative

TABLE I. ERYTHROCYTE VALUES FOR NORMAL AND MYOTONIC GOATS[a]

	Normal	Myotonic	Male[b]	Female[b]
Red blood cells ($\times 10^6$/mm^3)	17.5 ± 3.6	15.6 ± 2.3	16.6 ± 2.6	17.1 ± 3.9
Packed cell volume (%)	34.5 ± 4.1	33.7 ± 6.6	32.3 ± 4.6	35.9 ± 5.7
Hemoglobin (g/dl)	12.1 ± 2.0	11.6 ± 0.5	11.7 ± 0.6	12.0 ± 2.0
Mean corpuscular volume (μ^3)	20.2 ± 3.0	20.5 ± 3.6	20.1 ± 2.4	20.5 ± 3.9
Mean corpuscular hemoglobin (pg)	6.6 ± 0.9	6.6 ± 1.0	6.9 ± 0.8	6.4 ± 1.0
Mean corpuscular hemoglobin concentration (g/dl)	32.4 ± 2.6	29.4 ± 2.9	31.8 ± 3.5	30.4 ± 2.8
mg protein/cell ($\times 10^{-12}$)[c]	5.3 ± 2.6	5.7 ± 2.5	—	—

[a] Values were determined by standard hematologic techniques (5) and represent mean ± standard deviation of eight normal and eight myotonic goats.
[b] Normal and myotonic goat (n = 8).
[c] Values represent mean ± standard deviation of five normal and five myotonic goats.

peak areas computed on a Varian Integrator, Model 485, using internal standards of eicosenoic acid, trieicosenoin, and cholesteryl-11-eicosenoate (Nu-Chek-Prep, Inc., Elysian, Minn.) and ditetradecanoyl-L-α-lecithin (Supelco, Inc.) as reference.

Sialic acid and calcium assays. Sialic acids were assayed by the thiobarbituric acid method after the membranes were hydrolyzed in 0.1 *N* H_2SO_4 at 80°C for 1 hr (14). Absorption at 549 and 532 nm was measured in order to correct for the minor contribution of the interfering chromophore at 532 nm, and *N*-acetylneuraminic acid was used as control for the assay.

Membrane bound calcium was measured with a Perkin–Elmer 290 Atomic Absorption Spectrophotometer after digestion of the membranes in a $LaCl_3$–HNO_3 solution.

Results. There were no significant differences between normal versus myotonic goats for the red cell counts, packed cell volumes, or hemoglobin concentrations (Table I). These values were found to be comparable to those previously reported for goats (15). Erythrocytes from normal and myotonic goats had similar cell volumes with no statistically significant differences in the amount of protein per cell.

Osmotic fragility assays were used to compare normal and myotonic goat erythrocytes, since we had observed that plasma from myotonic goats exhibited less hemoglobin contamination than normal. Hemolysis curves confirmed the observation that myotonic cells were less susceptible to osmotic lysis than normal. The fragility curves were analyzed by a least squares formulation and the mean erythrocyte fragility, X_{50}, was calculated. This value represents the concentration of sodium chloride at which 50% hemolysis occurs (7). The X_{50} values for normal and myotonic goat erythrocytes, shown in Table II, were significantly different. These results indicated that myotonic erythrocytes were more resistant to hemolysis than normal cells (0.1383 ± 0.0035 *M* NaCl for normal and 0.1315 ± 0.0026 *M* NaCl for myotonic, $P < 0.03$). These data also verified the remarkable sensitivity which goat erythrocytes have to hemolysis as compared to those from humans, which have X_{50} values at 0.067 *M* NaCl (7).

The protein composition of the goat erythrocyte membrane was found to be

TABLE II. MEAN OSMOTIC FRAGILITY OF NORMAL AND MYOTONIC GOAT ERYTHROCYTES[a]

Animal pair	Normal	Myotonic
1	0.134	0.128
2	0.137	0.131
3	0.142	0.134
4	0.140	0.133
Mean	0.1383	0.1315[b]
Standard deviation	0.0035	0.0026

[a] Values are expressed as X_{50} (*M* NaCl), which represents the NaCl concentration at which 50% hemolysis occurs.

[b] Data are significant at $P < 0.03$ as analyzed by Student's *t* test.

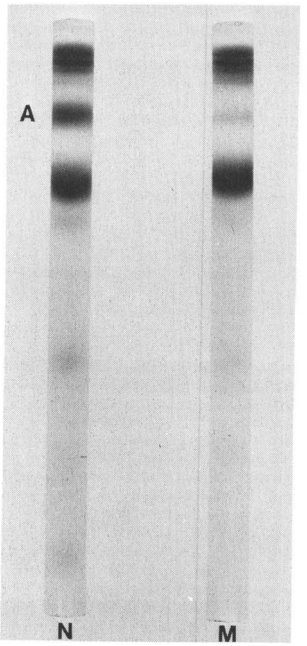

FIG. 1. Sodium dodecyl sulfate–polyacrylamide gels of erythrocyte membranes from normal (N) and myotonic (M) goats. Band A corresponds to a 140,000 molecular weight protein which represented 18.2% of the total protein in normal and 3.7% in myotonic membranes as determined by densitometric scanning of the Coomassie blue-stained gels. Electrophoresis was performed using 6% polyacrylamide in a phosphate buffer (0.1 *M* sodium phosphate, 1% SDS, 0.38 *M* glycine, pH 7.0).

comprised of seven major bands by SDS–polyacrylamide gel electrophoresis (Fig. 1). The molecular weights of six of these corresponded closely to the six major proteins of human erythrocyte membranes (16). However, an additional protein was found in the goat membrane which has not been reported for human red cell membranes. This protein, labeled Band A in Fig. 1, had a molecular weight of 140,000 and comprised 18.2% of the total protein in normal membranes as determined by densitometric scanning of the gels. Furthermore, this protein was markedly reduced in erythrocyte membranes from myotonic goats and comprised only 3.7% of the total protein content. Its functional significance remains unknown, although a protein similar in molecular weight was noted in sheep erythroycte membranes (17).

The lipid composition of normal and myotonic goat erythrocyte membranes is shown in Table III. Goat membranes contained about 40% lipid (38.3% for normal and 39.5% for myotonic). Approximately 60% of the lipid was phospholipid and 30% was free cholesterol, resulting in a free cholesterol:phospholipid molar ratio of 0.99 ± 0.11 for normal and 1.00 ± 0.09 for myotonic. No statistical differences were noted in the lipid composition of membranes from normal and myotonic goats.

The phospholipid fatty acids of erythrocyte membranes from normal and myotonic goats were similar with no significant differences (Table IV). The major fatty acids (palmitic, stearic, oleic, linoleic, and arachidonic) comprised approximately 90% of the total phospholipid fatty acids. The ratio of saturated to unsaturated fatty acids (i.e., palmitic + stearic/oleic + linoleic + arachidonic) was 0.194 ± 0.019 for normal and 0.206 ± 0.011 for myotonic.

Erythrocyte membranes as isolated from normal and myotonic goats showed statistically significant differences in the amount of membrane bound calcium (Table V). Membranes from normal animals contained 1.00 ± 0.07 μg calcium/ml cells whereas those from myotonic had 1.77 ± 0.23 μg calcium/ml cells ($P < 0.02$).

Striking differences were found in the membrane sialic acid content. Membranes from myotonic goats consistently contained increased sialic acid when compared to normal ($P < 0.005$, Table VI). The values in Table VI are presented as paired data and were analyzed by a paired Student's t test, since the animals were age- and sex-matched and the assays performed in a paired manner. Although there was some overlap among values for normal and

TABLE IV. PHOSPHOLIPID FATTY ACIDS OF ERYTHROCYTE MEMBRANES FROM NORMAL AND MYOTONIC GOATS[a]

	Normal	Myotonic
16:0	6.8 ± 0.7	6.5 ± 0.5
16:1	0.8 ± 0.2	0.7 ± 0.2
18:0	8.2 ± 0.6	9.5 ± 0.5
18:1	44.2 ± 2.4	42.1 ± 1.7
18:2	11.8 ± 1.5	11.2 ± 1.9
20:3	3.5 ± 1.4	3.7 ± 0.9
20:4	20.7 ± 2.8	23.5 ± 1.2
Unknown	4.2 ± 2.0	2.6 ± 1.2

[a] Fatty acids are denoted by chain length:number of double bonds. Values are expressed as mean ± standard deviation of percentages of total phospholipid fatty acids for five normal and five myotonic goats.

TABLE III. LIPID COMPOSITION OF ERYTHROCYTE MEMBRANES FROM NORMAL AND MYOTONIC GOATS[a]

	Normal	Myotonic
Phospholipid	63.5 ± 2.9	62.5 ± 1.9
Cholesterol free	32.1 ± 2.2	32.2 ± 2.8
Cholesterol ester	1.9 ± 1.0	1.9 ± 0.9
Free fatty acid	2.2 ± 1.0	2.1 ± 0.9
Triacylglycerol	0.3 ± 0.2	0.3 ± 0.1

[a] Values are expressed as percentage total lipid and represent the mean ± standard deviation of five normal and five myotonic preparations.

TABLE V. CALCIUM CONTENT OF ERYTHROCYTE MEMBRANES FROM NORMAL AND MYOTONIC GOATS[a]

	Normal	Myotonic
	1.05	1.81
	0.92	1.97
	1.04	1.52
Mean	1.00	1.77[b]
Standard deviation	0.07	0.23

[a] μg calcium/ml cells.
[b] Data are significant at $P < 0.02$ as analyzed by Student's t test.

TABLE VI. SIALIC ACID CONTENT OF ERYTHROCYTE MEMBRANES FROM NORMAL AND MYOTONIC GOATS[a]

Animal pair	Normal	Myotonic
1	29.4	37.2
2	36.8	38.5
3	30.7	36.5
4	34.3	37.5
5	32.9	36.2
6	29.0	32.5
7	30.8	41.8
8	29.8	42.8
Mean	31.7	37.9[b]
Standard deviation	2.7	3.3

[a] μg sialic acid/mg protein.
[b] Data are significant at $P < 0.005$ as analyzed by a paired Student's t test.

myotonic, the differences were still significant when analyzed as unpaired data.

We extended these studies to myotonia in humans and found that erythrocyte membranes from patients with myotonic muscular dystrophy also had increased sialic acid. Membranes from age- and sex-matched controls had 34.2 ± 3.7 μg sialic acid/mg protein whereas those from patients with myotonic dystrophy contained 42.4 ± 5.9 μg sialic acid/mg protein ($P < 0.03$) (Table VII). These experiments are being continued as samples from patients become available.

Discussion. Previously we have reported abnormalities in the fragmented sarcoplasmic reticulum (FSR) isolated from skeletal muscle of myotonic goats (3, 4). When compared to that of normals, FSR from myotonic goats had (i) increased levels of membrane bound calcium; (ii) increased rates of calcium uptake and efflux; (iii) increased sialic acid content; and (iv) increased content of saturated fatty acids. In addition, myotonic FSR consistently contained increased proportions of free cholesterol. These findings supported the theory that myotonia is an expression of a primary membrane defect.

TABLE VII. SIALIC ACID CONTENT OF ERYTHROCYTE MEMBRANES FROM PATIENTS WITH MYOTONIC MUSCULAR DYSTROPHY AND AGE- AND SEX-MATCHED CONTROLS[a]

	Control	Myotonic
	33.3	45.1
	37.7	48.9
	36.3	40.2
	29.3	35.3
Mean	34.2	42.4[b]
Standard deviation	3.7	5.9

[a] μg sialic acid/mg protein.
[b] Data are significant at $P < 0.03$ as analyzed by Student's t test.

Several studies have suggested that myotonia may represent a generalized membrane defect. Electron paramagnetic resonance (EPR) studies on the red blood cells from patients with myotonic muscular dystrophy, myotonia congenita, and from animals with drug-induced myotonia have suggested that increased membrane fluidity may be the underlying abnormality in myotonia (18, 19). However, using conventional EPR and saturation transfer EPR with fatty acid spin labels we were unable to detect any differences between normal and myotonic goat erythrocytes (20). To further explore the concept that hereditary myotonia may be manifest as a generalized membrane defect, the current studies with erythrocytes were undertaken.

We had observed empirically that blood drawn from myotonic goats hemolyzed less than that from normal goats. These observations were verified by osmotic fragility assays which indicated that myotonic erythrocytes were less fragile than normal cells and could more effectively resist changes from an isotonic state. There were no differences in mean corpuscular volumes, however, and the lipid composition of erythrocyte membranes from normal and myotonic goats was similar. The membrane phospholipids of myotonic animals did contain a higher ratio of saturated/unsaturated fatty acids than normal goat phospholipids. Although these proportions were similar to those found in the FSR, the differences were not statistically significant.

Consistent differences were observed in the protein composition of erythrocyte membranes from normal and myotonic goats. The goat erythrocyte membrane contains seven major proteins as determined by SDS–polyacrylamide gel electrophoresis. One of the proteins, a 140,000 molecular weight component, was

markedly reduced in the myotonic membranes. This protein is not present in human erythrocytes. The relative abundance of the other higher molecular weight proteins corresponded to that reported for human membranes (16), although the lower molecular weight bands (i.e., less than 100,000 daltons) were not as prominent. This may be due to polymerization or loss of these proteins during preparation of the membranes. However, the proteins were electrophoresed under reducing conditions, and careful removal of contaminating leukocytes during membrane preparation as well as the conditions chosen for solubilization and electrophoresis elminated the possibility of proteolytic degradation (21). There were no consistent qualitative differences observed between controls and the four myotonic patients with regard to erythrocyte membrane protein composition, but these studies are being continued as samples become available. The significance of a membrane alteration between normal and myotonic goats with regard to the 140,000 molecular weight protein remains unknown and warrants further study.

Erythrocyte membranes from myotonic goats contained an increased content of sialic acid and calcium when compared to normal. Sialic acid has been shown to bind calcium (22, 23). Long and Mouat have also found that there is a correlation between the binding of calcium and the sialic acid content of erythrocytes from various species and human erythrocytes that have been partially depleted of sialic acid by neuraminidase treatment (24). However, after complete removal of sialic acid, human erythrocytes still retained the ability to bind significant amounts of calcium. These authors further determined that there were binding sites for calcium on the internal suface of the membrane. We have not characterized the location of the increased calcium in the myotonic membranes. However, excess calcium in the myotonic membrane exceeds the capacity of sialic acid to bind, suggesting increased calcium binding to additional sites not involving sialic acid. It would be reasonable to propose, then, that increased calcium binding in association with an increased sialic acid content on myotonic erythrocytes results in diffusion of calcium into the cell with subsequent localization to the internal surface of the membrane.

An alteration of the erythrocyte membrane resulting in the binding of excess calcium, including binding to the internal surface of the membrane, may explain the increased resistance of myotonic cells to hemolysis. Erythrocytes loaded with calcium using the calcium ionophore A23187 have demonstrated increased resistance to osmotic lysis, presumably due to increased membrane rigidity (25). Such rigidity could be related to a calcium induced crosslinking of proteins which provides mechanical support to the cell wall (26). However, *in vitro* osmotic fragility is dependent on several elements intrinsic to the erythrocyte, including the ratio of cell volume to membrane surface area, the total number of intracellular osmotically active components, the degree of metabolic depletion (i.e., ATP content) and qualitative as well as quantitative factors related to membrane protein and lipid composition (27). Although we observed no differences in size or volume between normal and myotonic erythrocytes, other contributing factors should not be ruled out.

Our findings of an increased sialic acid and calcium content in FSR and erythrocyte membranes from myotonic goats support the concept of a generalized membrane defect in myotonia. A relationship of these data to myotonia in humans is suggested by increased erythrocyte membrane sialic acid in four patients with myotonic muscular dystrophy. Bosmann *et al.* noted an increased electrophoretic mobility of erythrocytes from patients with myotonic dystrophy and suggested that this could be due to increased sialic acid (28). As noted earlier, there is a specific correlation between bound calcium and sialic acid in erythrocytes (24), and recent studies have demonstrated increased calcium permeability in erythrocytes from patients with myotonic dystrophy (29). Our findings of increased membrane sialic acid in patients with myotonic dystrophy are consistent with these results.

The present study has extended the previously reported cytomembrane abnormality found in the sarcoplasmic reticulum of

an animal model of hereditary myotonia congenita (3, 4) to the erythrocyte membrane of these animals and to erythrocytes from humans with myotonic muscular dystrophy. Our findings support the concept of a generalized membrane defect in myotonia characterized by increased sialic acid associated with an impaired homeostasis of the calcium ion. The application of these results to detection and diagnosis of diseases associated with myotonia is being explored.

Summary. Studies were conducted on erythrocytes from myotonic goats, an animal model of heritable myotonia, and from patients with myotonic muscular dystrophy. When compared to those of normal goats, erythrocyte membranes from myotonic goats had (i) increased levels of calcium; (ii) an increased sialic acid content, and (iii) an altered protein composition with reduction of a 140,000 molecular weight protein. Erythrocytes from myotonic goats were also less susceptible to hemolysis than those from normal. Membranes of erythrocytes from patients with myotonic dystrophy had an increased content of sialic acid as compared to controls. The differences in calcium and sialic acid are consistent with previously reported abnormalities in the sarcoplasmic reticulum from the myotonic goat, and these findings support the concept of a generalized membrane defect in myotonia.

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