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INCREASE IN THE NEGATIVE CHARGE OF ERYTHROCYTE MEMBRANE PROTEINS IN HEREDITARY NEUROMUSCULAR DISEASES

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The muscular dystrophies are monogenic inherited human diseases which, according to most authorities [7-9], are based on a generalized defect of cell membranes. On account of this view, many attempts have been made to discover changes in those membranes most accessible for clinical analysis of the cells, namely erythrocytes [3]. Interest in erythrocytes is also due to the fact that these cells, like target cells, contain a contractile apparatus, consisting of the contractile proteins spectrin and actin [4]. A whole range of disturbances in the erythrocyte membrane have not been discovered in hereditary muscular dystrophies: changes in shape and deformability of the cells, in cation transport, and in activity of membrane-bound protein kinases [3]. Meanwhile data on phosphorylation of membrane proteins are contradictory. For instance, intensification of phosphorylation of spectrin and of band 3 protein has been found in Duchenne progressive muscular dystrophy (PMD) [10, 11], although in another publication [5] no such effect could be found.

Since an increase in phosphorylation of proteins must lead to an increase in the negative charge on their surface, this effect can be used as the basis for estimating the level of protein phosphorylation. In the investigation described below, the protein surface charge was studied by measuring the efficiency of quenching of protein fluorophores by nitrate anions.

EXPERIMENTAL METHOD

The diagnosis of various forms of hereditary muscular dystrophies was based on clinical, clinico-genealogic, and electromyographic analysis. Erythrocytes were washed twice in physiological saline and hemolyzed by the addition of 4 volumes of 0.01 M Tris-HCl (pH 7.4), incubated for 20 min at 4°C, after which the hemolysate was passed through a column (0.5 × 8.0 cm) filled with sepharose 4B, and the erythrocyte membrane fraction was collected. The protein content in the membrane was estimated spectrophotometrically by measuring absorption of the cell ghost suspension in 1% SDS at 280 and 310 nm [6]. Fluorescence was measured on a "Hitachi MPF-2" spectrofluorometer (Japan) in cylindrical microcuvettes with a capacity of 0.4 ml. Protein fluorescence was excited at 262 nm in the region of minimal absorption of nitrate, and recorded at 335 nm, in the region of emission of tryptophanyls. Allowing for the geometry of the cuvettes used, a correction for optical screening under the conditions of excitation and recording used was introduced [1], in accordance with the equation:

$$F = F_{meas} / 1 - 1.68 \cdot M^{-1} \cdot C, \quad (1)$$

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TABLE 1. Average Parameters of Quenching of Protein Fluorescence of Erythrocyte Ghosts by Nitrate (M ± m)

Subjects tested	f	K, M^{-1}
Healthy blood donors (n = 8)	1,01±0.03	17,30±1,87
Patients with Duchenne PMD (n = 5)	1,04±0,13 $p>0,05$	5,54±1,31 $p<0,001$
With Erb-Roth PMD (n = 4)	0,88±0,06 $p>0,05$	9,08±0,67 $p<0,01$
With Landouzy-Dejerine PMD (n = 2)	0,97±0,08 $p>0,05$	9,45±0,85 $p<0,01$
With Charcot-Marie neural amyotrophy (n = 2)	0,98±0,10 $p>0,05$	7,50±2,21 $p<0,01$

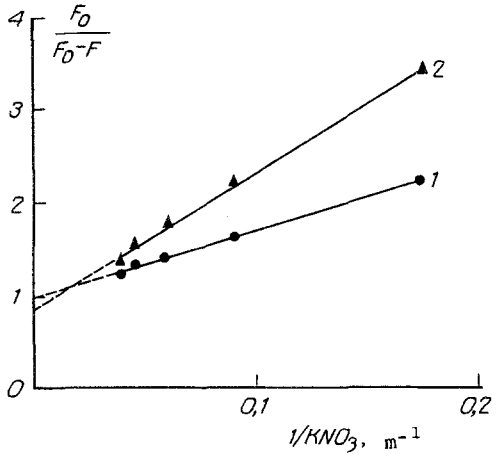

Fig. 1. Typical curves of quenching of protein fluorescence in erythrocyte ghosts from a healthy blood donor (1) and a patient with Duchenne PMD (2) by nitrate anions. Protein concentration 0.4 mg/ml; F, F_0) intensity of fluorescence with and without KNO_3 respectively.

where: F denotes the intensity of fluorescence with a correction made for optical screening by nitrate, F_{meas} is the measured intensity of fluorescence, and C the concentration of nitrate, in M.

The fraction of fluorophores accessible for the quenching agent (f) and the quenching constants (K) were estimated by a modified Stern–Volmer equation [2]:

$$\frac{F_0}{F_0-F} = \frac{1}{f \cdot K \cdot C} + \frac{1}{f}, \quad (2)$$

where: F_0 is the intensity of fluorescence in the absence of the quenching agent.

EXPERIMENTAL RESULTS

A change in the parameters of quenching of fluorescence may be due both to a change in accessibility of the fluorophores for the quenching agent and to a change in the quenching constant, which in turn depends on the charge of the microenvironment of the fluorophores.

Typical curves of quenching of protein fluorescence of a healthy donor and a patient with Suchenne PMD, illustrated in Fig. 1, are well extrapolated by a straight line which intercepts on the ordinate a segment equal to 1/f. The quenching constant (I) was determined from the slope of the quenching curves, and was equal to 1/f·K.

The averaged parameters of quenching of protein fluorescence by nitrate for healthy blood donors and patients with various forms of hereditary neuromuscular diseases are given in Table 1. They show that accessibility of the fluorophores for nitrates does not differ significantly in all the groups, and is close to 1.

The quenching constants of protein fluorescence by nitrate in all forms of PMD studied and in patients with Charcot-Marie amyotrophy were significantly lower than in healthy blood donors. It is interesting to note that the changes discovered are characteristic both of patients with a primary defect in the myofibrils (PMD) and also for patients with neurogenic amyotrophies. Considering that the quenching constant by nitrate of free tryptophan, which under physiological conditions has a neutral charge, is 25.5 M^{-1} [1], it can be concluded that the lower values of the quenching constant in healthy blood donors (K = 17.3 M^{-1}) point to a negative charge on the surface proteins of the erythrocyte membrane and its increase in myodystrophies. If this fact is compared with data in the literature [10] on increased phosphorylation of spectrin and band 3 protein (the main proteins of the erythrocyte membrane), it can be tentatively suggested that the change in the quenching constant of the membrane tryptophanyl groups is due to increased phosphorylation of the membrane proteins.

The data obtained thus confirm the hypothesis relating to the generalized character of membrane damage in this particular pathology, and specifically they are evidence of the genetically determined disturbance of function of the contractile membrane proteins, linked with a change in the level of their phosphorylation.

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