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NUCLEAR MAGNETIC RESONANCE EVIDENCE FOR ABNORMAL WATER TRANSPORT IN DUCHENNE MUSCULAR DYSTROPHY ERYTHROCYTES

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SUMMARY: The mean residence times, τ_a, of water within erythrocytes of ten Duchenne muscular dystrophy patients and nine controls have been determined by pulse nuclear magnetic resonance methods. Eight of the DMD results were found to be clearly higher than those of the controls. Since the values of τ_a are shown to be reciprocal measures of membrane water permeability, they reflect a new membrane abnormality associated with DMD. The results are discussed in relation to the general problem of membrane abnormalities in DMD. Finally, additional NMR experiments are suggested as a basis for further investigation of DMD membrane properties.

INTRODUCTION

Duchenne muscular dystrophy is a degenerative muscle disease inherited as an X-linked recessive trait (1). Previous studies suggest that the disease is not confined to just the muscle but is also expressed as abnormalities in the membranes of various cellular systems, including the erythrocyte. Recent reviews have surveyed these results and pointed out the rather widespread disagreement in many of the findings (2,3).

In an effort to better characterize these membrane abnormalities in DMD, we have carried out a pulsed nuclear magnetic resonance study of water movement across the erythrocyte membrane on 10 DMD patients and 9 male controls within the same age range. The NMR technique, which has been specially designed for erythrocyte studies (4,5), produces a quantity τ_a, the mean residence time of water within the cell. Under the given experimental conditions, τ_a is a recip-

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Abbreviations: DMD, Duchenne muscular dystrophy; NMR, Nuclear magnetic resonance; τ_a, mean residence time; E_a, Arrhenius activation energy.

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rocal measure of the membrane permeability to water (6). Determination of τ_a over a range of temperatures (4°-37°C) also provides an activation energy, E_a, which serves to further characterize the membrane.

MATERIALS AND METHODS

Blood was obtained from patients diagnosed with Duchenne muscular dystrophy and young male controls who showed no signs of hematological or neurological disorder by venipuncture into Vacutainers with heparin used as anticoagulant. The final heparin concentration was 10 USP units/ml. The dystrophic patients were diagnosed by the conventional methods and were regularly examined outpatients. All samples were used within 6 hours of collection and kept constantly mixed to prevent settling.

The procedure for the measurement of the mean residence time of water within the erythrocyte and the activation energy of water transport across the red blood cell membrane using pulsed nuclear magnetic resonance spectroscopy has been described in detail previously (4). The mean residence time is determined by signal averaging of the output from a Spin-Lock CPS-2 pulsed spectrometer (Spin-Lock Ltd., Port Credit, Ontario, Canada) employing the Carr-Purcell-Meiboom-Gill pulse sequence (7,8), with only the tops of the peaks sampled by a home-built accessory. This magnetization decay is fit by non-linear regression analysis to the two-site exchange equations given by Hazelwood et al. (9). The Arrhenius activation energy is determined by measurement of mean residence time over the temperature range 4°-37°C and linear regression analysis of the plot of $\ln(1/\tau_a)$ vs. 1/temperature.

Temperature was measured by a Duo-wrap 36 gauge Cu-constantan thermocouple (Thermo Electric Co., Inc., Saddle Brook, N. J.) calibrated with a Keithley 1608 digital multimeter (Keithley Instruments, Inc., Cleveland, Oh.) against a Mettler FP-5 temperature controller (Mettler Instrument Corp., Princeton, N. J.). The error was determined to be within $\pm$ 0.2°C. The thermocouple was inserted into a melting point capillary tube containing heat-sink compound for good conduction and this assembly inserted directly into the NMR sample tube. The sample temperature was controlled to within $\pm$ 0.2°C by a YSI Model 72 Proportional Temperature Controller (Yellow Springs Instrument Co., Yellow Springs, Wy.) employing thermally regulated nitrogen gas.

RESULTS AND DISCUSSION

The results of τ_a measurements at 25°C and 37°C for DMD patients and controls are presented in Table 1 and Figure 1. To summarize the 25°C results briefly: all controls fell in the 19-22 msec. range; of the DMD cases, 8 are in a high range of 21-25 msec., while two have low values of 18-19 msec. Four DMD subjects, including one of the two low cases, were remeasured after 1-2 months and agreement was within 2.3% in all cases. The differences between the elevated set and controls, as well as that between the two DMD groups, are statistically highly significant ($p < 0.0002$ and 0.00005, respectively). The two lower

Table 1. Mean residence time values

	No. of Samples	τ_a(msecs.) at 25°C	p	τ_a(msecs.) at 37°C	p
High Duchenne patients	8	22.6 ± .9		17.3 ± .9	
			<.0002		<.00005
Controls	9	20.6 ± .7		15.0 ± .6	
			<.006		<.08
Low Duchenne patients	2	18.6 ± .2		14.1 ± .6	

Values given are average ± standard deviation. p values are from Student's t-test.

DMD results fall below those of the controls, although this difference is not statistically significant at 37°C.

This partition of DMD results into two groups does not appear to be entirely arbitrary. The two patients in the low group were noticeably less incapacitated by the disease than others of comparable age. Many previous studies have shown that not all DMD patients exhibit the same experimentally-determined characteristics (10-12). It is, thus, conceivable that DMD progresses at different rates or is manifested as different disease states in some patients, and this variation may be correlated with differences in water transport rates. The present findings are also compatible with the possibility of genetic heterogeneity in DMD, as has been suggested elsewhere (13,14). Measurements of τ_a in DMD patients over a longer time period, as the disease progresses, may help clarify some of these possibilities as well as establish the extent of variability.

Under conditions of constant volume and surface area, τ_a is inversely proportional to the permeability of the membrane (6). Tillmann et al. (15) have shown that DMD red blood cells do not show an abnormal cell shape or volume when suspended in plasma. Because the cell shape is determined by the ratio of surface area to volume, normal cell shape and volume imply that the surface area is also normal. Therefore, the increased mean residence time of water in DMD erythrocytes suggests that the red blood cell membranes exhibit a diminished water permeability.

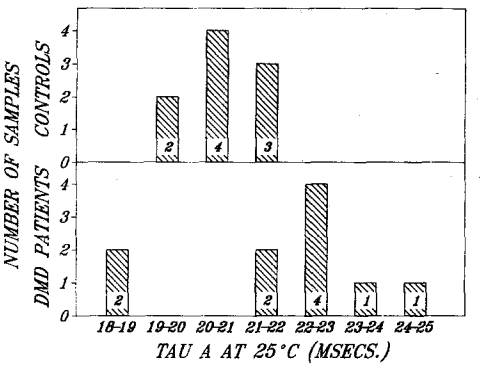

Figure 1. Distribution of mean residence time measurements at 25°C of erythrocytes from patients with Duchenne muscular dystrophy and normal controls.

The activation energy, E_a, was found to be the same for both DMD groups and controls, which suggests a corresponding similarity in that portion of the membrane responsible for water transport. The principle pathway is generally considered to be through integral Band III protein (16), although transport through lipids also occurs to a lesser extent (17). It is interesting, therefore, that freeze fracture studies of DMD erythrocyte membranes indicate a decrease in the number of intramembranous particles, which consist of integral membrane Band III protein and associated lipids (11). The present results, therefore, may not necessarily reflect a change in the nature of the integral Band III protein, but, rather, a decrease in the amount present in the membrane, and hence, a reduction in the number of water transport channels. This, in turn, could reflect a smaller amount of Band III protein available to the membrane or a less favorable lipid environment for its incorporation. These suggestions are offered only tentatively and as a possible basis for further inquiry.

To our knowledge, the only previously reported NMR measurement of τ_a in DMD erythrocytes consists of a single value each for one patient and two carriers (18). No significant difference was reported, but the method employed, especially in its use of high concentrations of manganese, has been shown to lead to substantial systematic errors (4). In the version used in this laboratory, however, the NMR method provides a degree of reproducibility ($\sim$2%) which is

relatively high for biological systems and which has enabled the differences reported here to be established.

It is unlikely that alterations in so general a process as membrane water transport should be specific to DMD. However, the present NMR method permits numerous variations potentially useful in elaborating these initial findings. As an example, detailed NMR studies on normal erythrocytes have been carried out here in the presence of sulfhydryl blocking agents, permitting the protein and lipid transport paths to be separately examined (19). ESR studies have suggested that the membrane sulfhydryl groups may be altered in Duchenne dystrophy (20). Also, we can now measure τ_a as the intracellular calcium level is varied (21). An alteration in the response to calcium of DMD erythrocytes has already been reported (22). These and other related approaches will in the near future be applied to DMD subjects and controls. Preliminary studies in our laboratory also indicate that the carrier state may manifest an altered water permeability. These results are being examined as a possible basis for a carrier detection test

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