Measurement of erythrocyte membrane elasticity as a diagnostic aid in Duchenne muscular dystrophy

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SUMMARY Using a micropipette aspiration technique, erythrocyte membrane shear elastic modulus was determined for 23 patients with Duchenne muscular dystrophy, for 14 female carriers, and for three control groups (age matched boys and adult males and females). On average the elastic modulus was slightly greater than normal for both affected males and female carriers. However, there was overlap in the values from all groups. The decreased deformability of dystrophic membranes demonstrated by the test is not great enough to be used for carrier detection nor to make the possibility of prenatal diagnosis likely.

Duchenne muscular dystrophy (DMD) is an inherited X linked recessive trait causing progressive degeneration of skeletal muscle. At present, measurements of serum creatine kinase (SCK) levels, which are characteristically raised in affected males, are used to aid detection of asymptomatic female carriers. It has also been suggested that quantification of this enzyme might enable prenatal diagnosis. However, as the method is not certain for either purpose, considerable interest exists in finding an alternative diagnostic technique.

It has been suggested that DMD is caused by a defect in the muscle plasma membrane and that abnormalities exist in the red blood cell membrane. However, in the erythrocyte studies, there has been considerable variation and disagreement between results obtained by different workers. The claim that abnormal red cell shapes occur in dystrophic blood has not been confirmed in all studies. Moreover, although anomalies in the membrane enzyme and lipid content have been described, these results have been contradicted. Variable results have also been obtained in studies of the filterability of suspensions of dystrophic erythrocytes. However, micropipette aspiration measurements of red cell deformability and membrane elasticity have shown that both are lower than normal in DMD. In addition, abnormalities have been detected in the structure of dystrophic membranes using electron spin resonance spin labelling techniques.

Despite these variable results, interest has been maintained in the possible use of red cell measurements for diagnostic purposes in DMD. Thus, Missirilis et al. used a micropipette aspiration method to test the rigidity of the cell membrane. They found that boys suffering from DMD had much larger values for the membrane shear elastic modulus than normal. Previously, Percy and Miller, using a different micropipette technique, had claimed that the overall deformability of erythrocytes was significantly decreased for both female carriers and affected donors. Thus, it seemed possible that measurement of membrane elasticity might be useful as a diagnostic aid in DMD.

To test this hypothesis we obtained blood from female carriers and boys affected by DMD and also from appropriate control groups. A micropipette method similar to that of Missirilis et al. was used to measure red cell membrane shear elastic modulus. This reflects the ease with which the membrane can be deformed at constant area. As only a small quantity of blood is required, it might be possible to apply the method to prenatal diagnosis. However, before studying fetal blood it was necessary to ensure that the test could discriminate between dystrophic and normal blood.

Subjects

Blood samples from 23 patients with DMD and from 14 female carriers were obtained with the aid of the staff of the Jerry Lewis Muscle Research Centre at the Hammersmith Hospital, London. The boys had been diagnosed using clinical, histochemical, electronmicroscopical, serum enzyme, and electromyographic criteria. The carriers were
classified as definite (having either two affected children or raised SCK plus an affected son or male relative) or possible (having one affected son or male relative but normal SCK). Age matched boy controls were taken from among patients in a paediatric neurological ward at Guy’s Hospital where blood was in any case being taken for routine clinical purposes. They were not suffering from muscular disorders but some were being treated with anticonvulsant drugs (see Results). Normal adult male and female donors were volunteers from the staff of this medical school.

Methods

Blood was obtained by venepuncture, anticoagulated with heparin, and transferred to cold storage as soon as possible after sampling (within 0 to 1 hours for normal blood from Guy’s Hospital and 0 to 2 hours for dystrophic blood from Hammersmith Hospital). Normal samples were stored 1 to 2 hours and dystrophic samples 2 to 3 hours in the cold before a small aliquot was diluted a thousandfold in isotonic buffer (NaCl 120 mmol/l, NaHCO₃ 25 mmol/l, CaCl₂ 2-5 mmol/l, KCl 2-6 mmol/l, MgSO₄ 1-2 mmol/l, KH₂PO₄ 1-2 mmol/l, pH 7-4) containing glucose (2 mg/ml) and human serum albumin (2 mg/ml) (Fraction V, Sigma Chemicals, Poole, England). For some adult male donors (see Results) blood was obtained by finger prick and the 10 μl sample was immediately diluted as above, without anticoagulant. In all cases, micropipette measurements were carried out straight after dilution.

The micropipette method used for measurement of membrane shear elastic modulus was similar to that of Missirlis et al.¹⁸ and has been described in a recent publication.²² Briefly, while a red cell was viewed microscopically, a tongue of membrane was aspirated from its flattened region into a narrow pipette (internal diameter 1-0 to 1-5 μm). The length of this tongue was photographed and recorded as the suction pressure was increased in steps. The measurements of length vs pressure were analysed using the theory of Evans,²³ ²⁴ modified slightly as previously described.²² On average, four measurements were made for each cell and a value for its membrane elastic modulus was calculated. Samples of between six and ten cells were measured for each donor. All measurements were made at room temperature (23 ± 1°C).

Results

The values for the membrane shear elastic modulus are summarised in the table. There was no significant difference between values for any of the control groups. A group of normal adult males, whose blood had been collected by finger prick and

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*Blood from these donors was obtained by finger puncture and was not anticoagulated.
immediately tested, was compared to a group who had been bled by venepuncture with subsequent heparinisation of the sample. The two procedures yielded nearly identical results, indicating that anticoagulation and a limited period of storage had no effect on membrane elasticity. Five of the non-dystrophic boys were receiving anticonvulsant treatment. No significant difference was found when data from these were compared to values for boys not receiving drugs. There was no correlation in any group between membrane elasticity and the age of the donors.

The average modulus was slightly higher for boys affected by DMD and for female carriers than for controls (table). This means that their red cell membranes are less easily deformed. The distributions of values obtained for dystrophic boys and definite carriers are compared to those for non-dystrophic boys and normal women in the figure. There is overlap in the values for all groups. The data from boys with and without DMD were compared using the $t$ test. The probability of the difference in mean values occurring by chance was found to lie in the range $0.01 < p < 0.025$. The same confidence limits were found when definite carriers and normal females were compared. For each of these groups the coefficients of variation of the measurements from individual donors were similar, averaging between 12 and 16%. Values for possible carriers were not significantly different from controls.

Discussion

Recently, the possible existence of membrane abnormalities in red cells from patients with Duchenne muscular dystrophy has attracted attention.\textsuperscript{5–20} This has led to the suggestion that measurements of erythrocyte deformability\textsuperscript{17} or membrane elasticity\textsuperscript{18} might form the basis for carrier detection or prenatal diagnosis or both. In particular, Missirilis et al\textsuperscript{18} found that the membrane shear elastic modulus for a group of seven dystrophic boys was more than double that for controls. The difference between the groups was statistically significant even though only two to four cells were tested per sample. We have carried out a similar study, extended to include asymptomatic female carriers, in which on average seven cells were measured for each donor.

The data which we obtained for the elastic modulus for normal donors were in fair agreement with results from other studies.\textsuperscript{18, 25} For dystrophic boys and definite carriers we found that the modulus was slightly greater than for controls. When appropriate groups were compared the differences were found to be of marginal statistical significance ($p < 0.025$). The values in all groups overlapped. Increasing the number of cells tested in each sample would decrease the standard error for each donor but would not be expected to alter the overlap between the groups. As the differences between normal and dystrophic blood are slight, it is important to avoid artefacts resulting from differences in the treatment of samples. There were slight differences in storage periods for the samples from Guy's Hospital and from the Hammersmith Hospital (see Methods).

However, we found that for two groups of ten normal male adults there was no difference in results, regardless of whether the blood was used immediately after sampling by finger prick or whether it was anticoagulated and stored for approximately 0 to 2 hours. Moreover, Meiselman et al\textsuperscript{28} have shown that storage of blood for up to 24 hours at $37^\circ$C does not significantly alter membrane elasticity, when determined using a micropipette technique similar to that of the present study. Thus we conclude that the short periods of storage used here did not affect our results.

Our data for boys with DMD do not agree with the original work of Missirilis et al\textsuperscript{18} in which much greater differences were found. It is not clear why this disagreement should occur as very similar methods were used in both studies. In the present series a larger group of dystrophic boys was tested and more cells were measured for each donor. Nor do our results from female carriers uncover any gross abnormality. It may be significant that values for definite carriers were generally greater than for possible carriers, some of whom might be expected to be normal. However, we conclude that any abnormality associated with DMD is not great enough to provide carrier detection or to make the possibility of prenatal diagnosis likely.

Previous studies of red cell abnormalities associated with DMD have tended to yield contradictory results (see introductory remarks). Here we present evidence that the membranes of erythrocytes from dystrophic donors and female carriers are slightly less flexible than normal. However, this effect might be secondary, depending, for example, on a serum factor. Thus, it is not certain that a generalised inherited membrane defect is involved in Duchenne muscular dystrophy.

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References

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