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Erythrocyte membrane abnormalities in Duchenne muscular dystrophy monitored by saturation transfer electron paramagnetic resonance spectroscopy

(fatty acid spin label/spin exchange/Becker syndrome/differential diagnosis)

L. S. WILKERSON*, RAY C. PERKINS, JR.†, ROBERT ROELOFS‡, LARRY SWIFT§, L. R. DALTON†, AND JANE HARTING PARK*

* Departments of Physiology, † Chemistry, ‡ Neurology, and § Pathology, Vanderbilt University, Nashville, Tennessee 37232

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ABSTRACT **Saturation transfer electron paramagnetic resonance and the spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl were used to study erythrocytes from patients with Duchenne muscular dystrophy or Becker syndrome and from age-matched normal boys. There were significant differences in the spectral intensities of erythrocytes from Duchenne patients when compared to controls. Spectral intensities increased with time in the former; no such change was observed in the latter. Saturation transfer electron paramagnetic resonance spectra of erythrocytes from patients with Becker syndrome were significantly different from those from Duchenne patients but were not significantly different from normals. These observations suggest the possible usefulness of these techniques in the differential diagnosis of Duchenne muscular dystrophy. Spin label concentration spectral studies suggest that the observed spectral differences between Duchenne patients and controls were due to differential spin exchange phenomena.**

"Muscular dystrophy" is a term that describes a group of diseases characterized by an inherited progressive muscle weakness and degeneration. Duchenne muscular dystrophy (DMD) is inherited in males as a recessive sex-linked characteristic and is manifested by dystrophy of the proximal muscles, often appearing in the first 5 years of life and progressing to complete immobilization by the early to middle teens. Numerous biochemical abnormalities have been observed in DMD—for example, alterations in muscle creatine phosphokinase (1), glycolytic enzymes (2, 3), calcium transport (4, 5), and activation of adenylate cyclase by epinephrine and sodium fluoride (6). In spite of these observations, as well as many others, the primary biochemical defect remains unknown.

Recently, the hypothesis was advanced that DMD might be caused by a generalized genetic defect in surface membranes, which could be expressed in membranes not pathologically involved in DMD. This hypothesis was supported by a considerable amount of evidence of abnormalities in erythrocytes from patients with DMD. It was observed that the Na^+,K^+-ATPase of erythrocyte ghosts, normally inhibited by ouabain, was stimulated *by ouabain* when tested in erythrocytes from patients with DMD (7). DMD erythrocytes were abnormal in several other respects: appearance by scanning electron microscopy (8), sodium and potassium fluxes (9, 10), membrane protein kinase (11), and the response of adenylate cyclase to epinephrine (12). Finally, and more directly related to the present experiments, it has been postulated that any functional abnormality in the erythrocyte membrane might be related to an altered environment within the membrane. This hypothesis has been investigated by spin labeling and electron paramagnetic resonance (EPR). On the basis of conventional EPR and the spin label 4-maleimido-2,2,6,6-piperidinooxyl, it was suggested (13) that there were alterations in the membrane protein conformation or organization in DMD. However, no alterations were found when the fatty acid spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl methyl ester or 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-NS) was used (14).

In the present study, the effects of fatty acid spin label 5-NS were reexamined by the more sensitive techniques of saturation transfer electron paramagnetic resonance (ST-EPR). Theory and experiment have confirmed that passage saturation transfer electron paramagnetic resonance exhibits sensitivity to ranges of system dynamics not observable with ordinary EPR (15–17). This increased sensitivity has been extensively documented for the case of nitroxide-labeled molecules undergoing rotational diffusion in solution. It was shown for such systems that , by using ST-EPR, rotational times as slow as 10^{-3} sec could be studied, whereas the lower limit of sensitivity for EPR is 10^{-7} sec (15, 17). Therefore, it was not unreasonable to speculate that differences between DMD and normal erythrocyte membranes might exist that could be monitored by ST-EPR but not by conventional EPR. That this was the case is demonstrated by the 5-NS labeling experiments presented in this paper.

MATERIALS AND METHODS

Erythrocytes were obtained from heparinized blood from patients with DMD or Becker syndrome and from age-matched normal boys (17 patients with DMD, ages 3–14 years; 3 patients with Becker syndrome, ages 7–16 years; 12 controls, ages 6–15 years). Intact erythrocytes were prepared by centrifuging the blood at 1500 × *g* for 15 min in the cold, removing the serum, and washing the cells twice by suspension and centrifugation in 0.15 M NaCl/5 mM sodium phosphate buffer, pH 7.5. After the final wash, the packed erythrocytes were resuspended 1:1 (vol/vol) in the same buffer and stored at 4° until used. The period of storage was never more than 16 hr and in most cases the erythrocytes were used immediately. Control experiments demonstrated that storage at 4° for these periods had little, if any, effect on the experimental variables measured.

The experiments described in this paper were done with the spin label 5-NS obtained from Syva, Palo Alto, CA. The spin label was dissolved in methanol to a concentration of 1 mM and was stored in the dark and cold until used. For the concentration studies, the stock solution was appropriately diluted with methanol. An aliquot containing 0.25 ml of the appropriate

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Abbreviations: DMD, Duchenne muscular dystrophy; EPR, electron paramagnetic resonance; 5-NS, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl; ST-EPR, saturation transfer electron paramagnetic resonance.

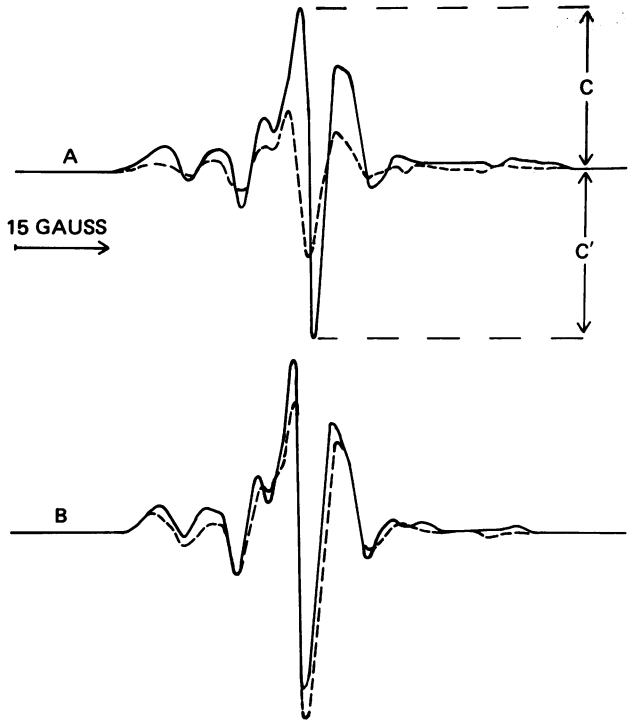

FIG. 1. Tracings of representative ST-EPR spectra for erythrocytes from a DMD patient (- - -) and a control (—). (*A*) Early time point. (*B*) Late time point.

concentration of spin label was measured into an aluminum foil-shielded test tube. The methanol was evaporated under a stream of dry nitrogen gas, leaving a thin film of spin label. One milliliter of the erythrocyte suspension was added to the test tube and the tubes were incubated for 10–30 min (early time point) or 20–24 hr (late time point) at 37° in the dark.

After appropirate incubation times (i.e., early or late), an aliquot of the spin-labeled erythrocytes was transferred to a quartz flat cell (S-812; James F. Scanlon Co., Solvang, CA) and the EPR and ST-EPR spectra were measured at 37° in a Varian E-109 Century Series EPR spectrometer. The same flat cell was used in all experiments and the microwave cavity temperature was maintained at 37° ± 0.5° with a Varian E-257 temperature controller. The instrument was operated at 10 mW microwave power, 1.0 G modulation amplitude (peak–peak), and 100 kHz modulation frequency for the EPR measurement. ST-EPR spectra were recorded at 63 mW microwave power and 5.0 G modulation amplitude, and the absorption signal was detected out of phase with the second harmonic of the 50-kHz modulation. The cavity used was a Varian E-231 rectangular TE_{102} microwave cavity.

RESULTS

Our results with conventional EPR techniques and the spin label 5-NS confirmed those presented by Appel and co-workers (14) in that we could detect no differences in the fluidity profile of erythrocytes from DMD patients and from normal boys. However, by using the more sensitive technique of ST-EPR, differences were observed (Fig. 1). At the early time point, the intensity of the signal for the DMD erythrocytes was 50%–60% of the age-matched normal control value (intensity was measured as the sum of the absolute values of the heights of the C and C′ peaks). However, upon incubation for 20–24 hr, the signal intensity for the DMD erythrocytes had increased to a value close to that of the control. The signal intensity of the control did not change. A demonstration of the nature of the

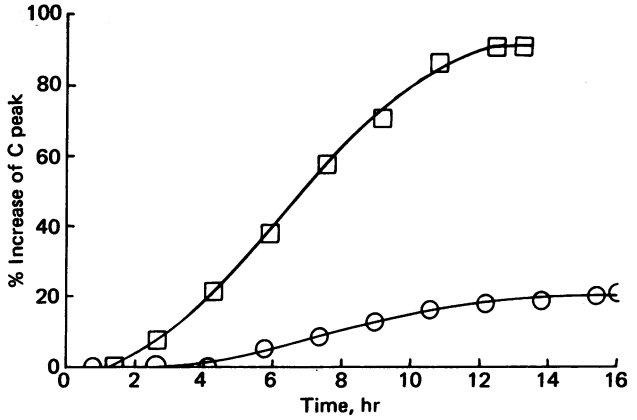

FIG. 2. Relative change in the ST-EPR C peak (see Fig. 1) as a function of time after labeling. ○, Control; □, DMD.

observed intensity behavior as a function of time is shown in Fig. 2. The spectral behavior was without discontinuities and the late time point observation represents a final kinetic data point. The C peak intensity for this DMD sample doubled (100% increase), whereas there was only a 20% increase for the normal.

The means and standard deviations of early signal intensities and late/early signal intensity ratios are shown in Table 1. The standard deviation for DMD erythrocytes did not depend on whether each determination was calculated as a statistical point or the averages for each patient were analyzed. The subgroup of normals, those <11 years old, was included because spectral changes occurred in erythrocytes from those near the onset of puberty. Thus, differences were more significant between normal boys <11 years old and boys with DMD than between normal boys of all ages and DMD patients. Furthermore, normal boys <11 years old formed a more homogeneous group as evidenced by a smaller standard deviation of the mean of the early intensity compared to that for all normal boys. The reason or reasons for the changes near puberty are unclear and will not be further considered here.

The mean early intensities for patients with DMD were considerably lower than the mean early intensities for normal control subjects. The mean late/early intensity ratios for DMD patients were considerably higher than for normal boys. The fact that the late/early intensity ratio was 1.0 for normal boys indicated that no spectral intensity changes occurred with time, whereas ratios of 1.5 and above for DMD patients were indicative of large increases in spectral intensities. Erythrocytes from patients with Becker syndrome behaved much like those from normal boys, thus distinguishing them from DMD erythrocytes.

The mean values for early intensities and for the late/early intensity ratios were compared for statistical significance (Table

Table 1. Analysis of ST-EPR spectral intensities of 5-NS labeled erythrocytes from patients and controls

Erythrocyte source	n*	Early intensity Mean	Early intensity SD	Late/early intensity ratio Mean	Late/early intensity ratio SD
DMD	43	2.33	0.68	1.55	0.48
Becker syndrome	6	2.94	0.70	0.96	0.15
Controls, <11 yr old	7	3.26	0.29	1.00	0.23
All controls	12	3.05	0.55	0.92	0.21

* Number of measurements. Numbers of subjects were: DMD, 17; Becker syndrome, 3; controls, 12.

Table 2. Statistical comparison* of ST-EPR spectral parameters of 5-NS labeled erythrocytes from patients and controls

Group comparison	*P* value: Early intensity	*P* value: Late/early intensity ratio
DMD vs. normals	0.0016	0.0008
DMD vs. normals <11 yr old	0.0012	0.0015
Becker syndrome vs. DMD	0.042	0.0263
Becker syndrome vs. normals	NS	NS
Becker syndrome vs. normals <11 yr old	NS	NS

* By *t* test of significance between two sample means for unpaired variables. NS, not significant.

2). DMD patients were highly significantly different from normals when early intensities or late/early intensity ratios were compared. Patients with Becker syndrome were significantly different from DMD patients on the basis of early intensities or late/early intensity ratios but were not significantly different from normal subjects.

The results of an experiment comparing the effect of spin label concentration on erythrocytes obtained from one DMD patient and an age-matched normal boy are shown in Table 3. For both the control and DMD erythrocytes, the spectral parameter C′/C (see Fig. 1) increased with increasing spin label concentration.

Control experiments on fatty acid spin label in buffer in the absence of erythrocytes gave a "fast motion" EPR spectrum consisting of three narrow lines and indicating that no aggregation of spin label had occurred. In the presence of erythrocytes, a small percentage of spin label (0%–10%) was free (not associated with the membrane) and gave the typical three-line response.

DISCUSSION

Previous experiments using the EPR technique and various spin labels have provided information about the erythrocyte membranes of patients with DMD, myotonic muscular dystrophy, and myotonia congenita (14, 13, 19, 20). By use of the spin label 4-maleimido-2,2,6,6-piperdinooxyl, evidence for altered protein conformation or organization was demonstrated in both myotonic muscular dystrophy and DMD but not in myotonia congenita (13). With the spin label 5-NS, an increased membrane fluidity was demonstrated in myotonic muscular dystrophy whereas no such demonstration was made for DMD (14).

Although previous investigators did not demonstrate differences between DMD and normal erythrocytes by using fatty acid and fatty acid ester spin labels (14), it was possible that conventional EPR techniques were not sensitive in the appropriate system dynamics region. Our results with the fatty acid spin label 5-NS and more advanced ST-EPR techniques clearly demonstrate at least three highly significant and reproducible spectral differences between erythrocytes from patients with DMD and erythrocytes from controls. First, there was a highly significant decrease of the mean early signal intensities of DMD erythrocytes compared to controls. Second, there was a highly significant and reproducible increase in signal intensity for DMD erythrocytes when they were incubated at 37° for 20 hr, with the late signal intensity approaching the control value; the signal intensity of control erythrocytes did not change with time. Third, there were differences in the effect of 5-NS concentration on the ST-EPR spectra of DMD and control erythrocytes.

Table 3. Effect of concentration of 5-NS on the ST-EPR spectra of erythrocytes from a DMD patient and a control

Erythrocyte source	Label, molecules/cell $\times 10^{-10}$	C′/C: Early	C′/C: Late	Intensity: Early	Intensity: Late
DMD	1.39	0.82	0.48	0.90	1.34
	2.09	0.92	0.71	2.00	2.25
	2.79	1.56	0.67	1.97	2.69
	4.15	1.32	0.82	2.61	3.92
	5.57	1.71	0.85	2.34	4.58
Normal	1.39	0.60	0.59	1.97	1.94
	2.09	0.75	0.70	2.51	2.53
	2.79	0.80	0.78	2.89	3.51
	4.15	0.85	1.34	3.10	3.13
	5.57	0.93	0.89	2.86	3.60

The sensitivity of the ST-EPR technique can best be understood if the general electronic magnetic resonance problem is considered. The unpaired electron spins precess about the external Zeeman field at a rate determined by the magnitude of the Zeeman field and by the molecular magnetic configuration of the unpaired electron. The spin system absorbs energy from the incident microwave field when its frequency matches that of the precessing electron. The external Zeeman field is modulated in time by a relatively small amplitude cosine modulation. The purpose of this Zeeman modulation is to improve the signal-to-noise ratio by discriminating against the low-frequency component of the $1/f$ noise of the crystal detector. The traditional absorption EPR experiment consists of detecting that portion of the time-dependent spin response that is out of phase with the first harmonic of the incident microwave and in phase with the first harmonic of the Zeeman modulation frequency. Furthermore, the power of the incident microwave is selected to ensure operation in the linear regime—i.e., the EPR signal intensity is linear with respect to the microwave amplitude. ST-EPR, however, is performed by choosing a higher microwave amplitude setting in the nonlinear regime and by detection of responses out of phase with the applied Zeeman modulation field. Choice of a nonlinear microwave amplitude increases the dependence of the spin response on those molecular processes that modulate energy transfer between the electron spin and the molecular lattice ($1/T_1$ processes) and between different portions of the resonant spectrum (rotational motion, spin exchange, etc.). Detection of responses out of phase with the applied Zeeman modulation gives reasonable assurance that the observed signal depends significantly on those saturation transfer processes. This does not imply that all out-of-phase responses exhibit ST-EPR sensitivity and that no in-phase signals do, because the distribution of information among the various possible signal responses is complex. Thus, EPR may be used to monitor events occurring at a rate competitive with the line-width parameter, T_2, whereas ST-EPR monitors events competitive with T_1. The spin–spin relaxation rate, $1/T_2$, is more than an order of magnitude faster than $1/T_1$ for most nitroxide radicals.

For the biological scientist, the experimental significance of ST-EPR is that this technique can monitor slow system dynamics of molecules much larger, for example, than hemoglobin with a molecular weight of 64,000. By contrast, the sensitivity of ordinary EPR to molecular reorientation (i.e., molecular tumbling or rotational diffusion) is restricted to reorientation times faster than 10^{-7} sec for most nitroxide-labeled molecules. This precludes the study, for instance, of proteins larger than 50 Å in diameter. ST-EPR extends the measurement

sensitivity to molecular reorientation as long as 10^{-3} sec and correspondingly allows the study of macromolecules.

Our results showing a decrease in early signal intensity with DMD erythrocytes could be interpreted as an increase in polarity at the spin label insertion site for the DMD erythrocyte. Increased polarity at the insertion site could not only shift the magnetic tensor values (i.e., shift the peak positions of the resonant spectrum) but also facilitate the ease with which the unpaired electron could exchange energy with its surrounding environment. This increased exchange (relaxation rate) would give rise to a decrease in signal intensity, especially in the ST-EPR response, because, as stated above, the ST-EPR response is more dependent on lattice relaxation (T_1) than is EPR.

An alternative physical explanation for the observed spectral differences between control and DMD erythrocytes is spin exchange. This arises when the spin concentration is sufficiently high to allow interactions between the unpaired electrons. This interaction can alter spectral shapes (broaden and eventually destroy characteristic structuring) or decrease the intensity of the observed spectral response. Should an exchange of this nature occur with a frequency removed from the EPR sensitivity ($1/T_2$), but compete with $1/T_1$ processes, then ST-EPR would respond strongly to this phenomenon just as ST-EPR shows sensitivity to slow molecular reorientation. This model requires initial insertion of the spin label into local membrane sites followed by a diffusion of the spin label from that site with time or that initially all cells are not labeled and that with time the label is distributed among all cells, or both.

Our results can best be explained on the basis of spin exchange. According to this hypothesis, the lower intensity of the early signal in DMD erythrocytes could be due to localized high concentrations of spin label at specific sites in the membrane, allowing spin exchange to take place and thus decreasing the signal intensity. With time, diffusion of the spin label away from these local areas of high concentration minimizes spin exchange and thus leads to an increase in spectral intensity with time for the DMD erythrocytes. Alternatively or in coordination, the spin label could accumulate disproportionately in the erythrocyte population, leading to a highly labeled subpopulation and giving rise to low-intensity spectra via spin exchange. In time the label would distribute homogeneously and the spectra would attain its characteristic equilibrium intensity. Normal erythrocytes either would not accumulate the spin label in local high concentrations or the kinetics of redistribution in them are too rapid to be observed under our experimental conditions. Therefore, no spectral intensity changes were observed with time in control erythrocytes.

The results of the spin-label concentration experiments (Table 3) support the spin-exchange model. An increase in C′/C is indicative of an apparent increase in the rotational diffusion rate of the spin probe. The characteristic increase in C′/C as a function of concentration can be attributed to a more fluid environment. Thus, it may be concluded that the spin probe is itself partly responsible for the rotational diffusion characteristic as monitored by the ST-EPR technique for both normal and DMD erythrocytes. Such an observation is consistent with the model of accumulation of the spin probe in focal membrane sites. Sufficiently high accumulation of the spin probe may further be manifested spectrally through spin exchange. This phenomenon would lead to a lower spectral intensity such as that noted for the early DMD ST-EPR spectra.

That fatty acid spin labels, and 5-NS in particular, may accumulate at focal points in normal erythrocytes and that all cells in the population may not be equally labeled were demonstrated in an extensive study on the effect of varying concentrations of 5-NS on erythrocyte morphology (21). Erythrocytes were treated with 5-NS and examined in the scanning electron microscope, and changes in morphology were correlated with the concentration of 5-NS present. At low concentrations of 5-NS (6.02×10^6 5-NS molecules per erythrocyte), focal protrusions began to appear on the cells. The number of focal protrusions per cell as well as the number of cells bearing these protrusions increased with increasing concentrations of 5-NS. Higher concentrations of 5-NS (3.01×10^{10} 5-NS molecules per erythrocyte) caused the disappearance of biconcave cells (the cells becoming discoid) and the development of multiple surface protrusions or "knobs." Further increase in 5-NS concentration caused all cells to assume a typical echinocyte appearance. All the observed morphological changes up to the point at which cells began to lyse were fully reversible. Most of our experiments were done at cell and spin label concentrations (5.57×10^{10} 5-NS molecules per erythrocyte) at which all cells would be modified.

In summary, we have demonstrated differences in early intensity and late/early intensity ratio of the ST-EPR spectra of 5-NS labeled erythrocytes from patients with DMD compared to normal age-matched boys. The spectral differences may be the result of the phenomenon of spin exchange and suggest that the erythrocytes obtained from a DMD patient cannot distribute the fatty acid label to final equilibrium as rapidly as normal.

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