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Detection of Erythrocyte Membrane Structural Abnormalities in Lecithin: Cholesterol Acyltransferase Deficiency Using a Spin Label Approach

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The membrane fluidity of erythrocytes from patients with Lecithin: cholesterol acyltransferase (LCAT) deficiency was studied by means of electron spin resonance. The temperature dependence of the separation of the outer extrema of the spectra of 2-(3-carboxy-propyl)-4,4-dimethyl, 2-tridecyl-3-oxazolidinyloxyl spin probe was monitored for normal, presumed carrier and clinically affected subjects. The temperature profile of controls was significantly different from that of the presumed carriers and the clinically affected individuals. The results show that the compositional abnormalities previously noted in erythrocyte membranes from patients with LCAT deficiency are associated with alterations in the physicochemical state of the membrane. An investigation of the spectral lineshapes below 10°C allowed a distinction to be made at the membrane level between clinically affected subjects and clinically normal heterozygous carriers. Alterations in the temperature dependence of electron spin resonance parameters may provide a sensitive index of red cell membrane alterations in pathological states of generalized membrane involvement.

Key words: erythrocyte membranes, LCAT deficiency, electron spin resonance, spin label

Lecithin: cholesterol acyltransferase (LCAT) deficiency is a rare familial disorder in which plasma LCAT activity is very low or absent [1]. The impairment of cholesterol esterification results in increased plasma levels of free cholesterol and lysolecithin [1]. Abnormal plasma lipoproteins are formed [2,3] and the pathological manifestations of the disease, which include corneal opacity, anemia, renal impairment and premature atherosclerosis, appear to involve abnormal deposition of lipids in affected tissues.

Erythrocyte membrane lipid alterations have been demonstrated in LCAT deficiency [4–6]. Gjone et al [4,6] and Godin et al [5] have shown that LCAT deficiency is accompanied by an increase in erythrocyte membrane phosphatidylcholine (PC) and a decrease

Abbreviations used in this paper: LCAT, Lecithin: cholesterol acyltransferase; ESR, electron spin resonance; 5-doxyl stearic acid, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolindinyl-1-oxyl; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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in membrane phosphatidylethanolamine (PE). The electron spin resonance technique is well established as a valuable tool for exploring structural aspects of biological membranes [8]. In the present study we have employed a spin labeled fatty acid probe [2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolindinyl-1-oxyl or trivially known as 5-doxyl stearic acid] incorporated into hemoglobin-free erythrocyte membranes from two recently identified patients with LCAT deficiency and their relatives [5]. The temperature dependent spectral characteristics of 5-doxyl stearic acid show that the physiochemical state of membrane components in clinically affected individuals is abnormal and that similar but distinguishable alterations are present in the membranes of clinically unaffected relatives who are presumed carriers of this genetic disorder.

METHODS AND MATERIALS

The blood samples were obtained from apparently healthy volunteers and a Canadian family of Italian and Swedish origin, in which two clinically affected siblings and a number of clinically normal family members (including the parents, another sibling, and the maternal grandparents) were investigated. Erythrocytes were separated from plasma by washing twice with isotonic saline and then subjected to a stepwise hemolytic procedure for the preparation of hemoglobin-free membranes [7].

The 5-doxyl stearic acid spin probe was introduced into the erythrocyte membrane by incubating on ice a suspension of membranes in Tris-HCl buffer (10 mM, pH 7.4) with 1.2 $\mu\ell$ of probe (5×10^{-3}) per mg of membrane protein. This procedure ensured that the ratio of spin probe to phospholipid is approximately 1:100. Following incubation, membranes were centrifuged at 40,000 × g for 30 min and the resulting pellet drawn into a 20 $\mu\ell$ micropipette which, in turn, was inserted into the resonant cavity of the ESR spectrometer.

Electron spin resonance (ESR) spectra were obtained with an X-band (9.0 GHz) homodyne spectrometer employing a Varian 12" magnet equipped with a Mark II FIELDIAL control. Phase sensitive detection at 100 GHz was achieved with an Ithaco Dynatrac 391A lock-in amplifier. Field calibration was carried out using a proton magnetometer whose resonant frequency was monitored by a Hewlett-Packard 5246L frequency counter. The same frequency counted, using a 5256 plug-in, served to measure the microwave frequency. The microwave power level on the sample was never greater than 10 mW and the amplitude of the 100 GHz modulation was 0.5.G. The temperature of the sample in the cavity was controlled using a Varian temperature controller and was measured using a copper-constantan thermocouple inserted into the cavity adjacent to the sample. The maximum relative error in temperature is estimated to be ±0.1°C. The spectra at each temperature were recorded with either a 5 min or a 10 min scan over 0.1T (100G) using a time constant of 0.4s. The difference between the measured spectral features for the two scan times was well within the error of ±0.3G for measuring a peak maximum. The rate of change of temperature was maintained at 8°C per hr, usually in 2°C every 15 min. Thermal equilibration was carried out with the microwave bridge tuned. The temperature range covered was 2°C to 48°C and no evidence of hystersis was ever detected.

In order to assess the difference in the physicochemical state of the erythrocyte membrane of the experimental subjects or normal controls we have chosen to measure the splitting ($2T'_{11}$) between the outer extrema of the ESR spectrum of 5-doxyl stearic acid incorporated into the membranes. This parameter ($2T'_{11}$) is linearly related to the order parameter S according to the expression

$$2T'_{11} = 2A_O + (4S/3)\,[T_{ZZ} - (1/2)(T_{XX}+T_{yy})]$$

where A_O, T_{XX}, T_{yy}, T_{ZZ} are the hyperfine coupling constants [see ref 8, p 476]. The interpretation of S and hence $2T'_{11}$ is not straightforward. Strictly speaking S is a function of both spatial and motional order. However in the case of a system of randomly oriented membrane fragments, S (and hence $2T'_{11}$) can be viewed as a measure of motional order [8, p 454]. The change of $2T'_{11}$ with temperature can be used to evaluate the variation of membrane fluidity with temperature and the comparative characteristics of different membrane samples. Thus, for each membrane sample we have measured the temperature profile of $2T'_{11}$.

RESULTS

The temperature dependence of $2T'_{11}$ between 2°C and 48°C for the control (normal) samples is shown in Figure 1. The results shown in Figure 1 represent more than 100 spectra obtained from erythrocyte ghosts of seven different apparently normal individuals. The solid line in Figure 1 is a least-squares regression for all of the available data; the regression parameters are given in the legend. The temperature profile for the control membranes shows a discernible discontinuity at 31.5±0.5°C. The correlation coefficient for the 2° to 31.5°C region is 0.995 and that for the higher temperature region is 0.986, indicating a highly reliable linear correlation between $2T'_{11}$ and temperature over the two ranges.

The experimental values of $2T'_{11}$ obtained from five family members under investigation (parents, two clinically affected siblings and one clinically normal child) are shown in Figure 2, which also depicts the regression lines for the control samples. It is evident that the temperature profile of the family is different from that of the control samples. The initial portion of the graph, up to about 21.5°C, has the same slope as the control samples but is lower by approximately one gauss, thus indicating a more fluid membrane. At 21.5°C there is an inflection in the temperature profile and the membranes of the experimental subjects approach the same fluidity as the control samples at about 31°C. Above 34°C the

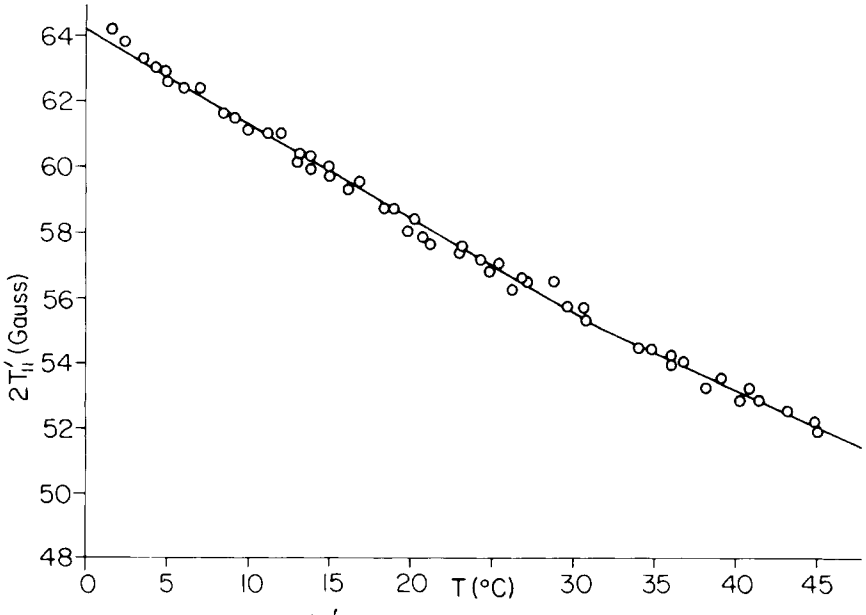

Fig. 1. The temperature variation of $2T'_{11}$ for normal control samples. The solid line is the least squares regression line. The least squares parameters for the line from 0°C to 31°C; a = 0.295 ± 0.005, b = 64.3 ± 0.1 and r = 0.992. The least squares parameters for the line from 32° to 48° are a = −0.22 ± 0.01, b = 62.0 ± 0.4 and r = 0.08.

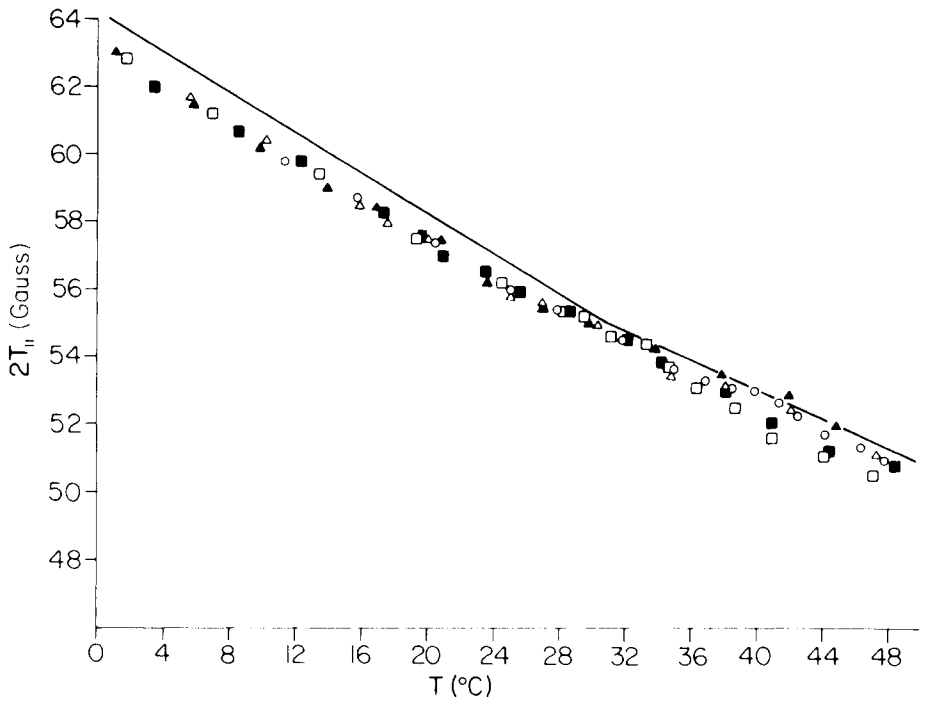

Fig. 2. The temperature variation of $2T'_{11}$ for the father (□), mother (■), son (heterozygote, ○), daughter (homozygote, Δ) and son (homozygote, ▲). Solid line corresponds to the normal control samples.

five different samples diverge and are either on the solid line of the normal samples or below it. Thus, it would appear that the variation in $2T'_{11}$ with temperature in the patients who are likely homozygotes for the abnormal genetic trait [5,9] and in their parents (who are presumed heterozygous carriers for the disorder [5,9]) serves to clearly distinguish these individuals from the normal population. The fact that the clinically unaffected sibling also exhibits the abnormal profile suggests that he too is a carrier.

It is apparent, however, that the 1 gauss difference between the normal profile and the experimental subjects is quite small, in view of the error (±0.3G) in estimating the value of $2T'_{11}$, so that the question of statistical significance arises. In order to test this we have employed a general least-squares analysis of variance program which is supported by the computing centre at the University of British Columbia. The program takes all of the data (both from control and experimental subjects) and assesses the number of homogeneous groups using a multiple range test based on a linear hypothesis (ie $2T'_{11} = aT + b$) using the Scheffe test [14]. Such an analysis of the experimental data, based on the intercept of the linear regression, revealed that at the 99% confidence level ($P = 0.01$) two homogenous groups exist; the control samples and the experimental subjects (parents and children). Thus, although the difference in the measured parameter ($2T'_{11}$) is quite small we feel that this conservative [14] statistical test supports our contention that the temperature profile of $2T'_{11}$ can be used to detect physicochemical differences between the membranes of apparently healthy controls and heterozygous and homozygous carriers of LCAT deficiency.

We have also been able to examine the erythrocyte membranes of the maternal grandparents of our patients, and the data presented in Figure 3 strongly suggest that the grand-

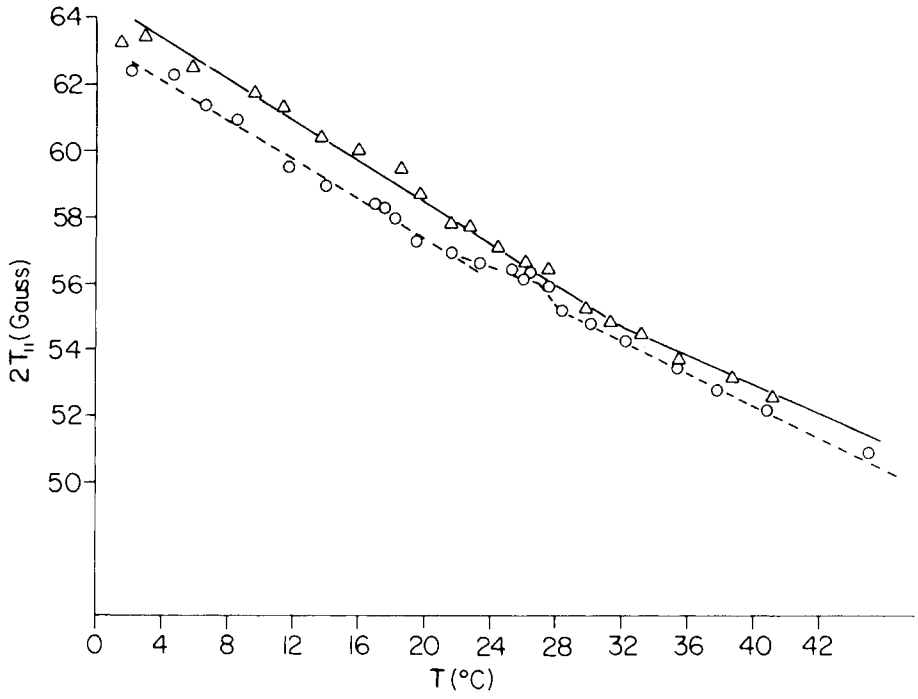

Fig. 3. The solid line depicts the least squares line for the maternal grandfather. The parameters for this line from 0° to 31°C are: a = −0.305±0.011, b = 64.5±0.2 and r = 0.991. Those from 32° to 48°C are: a = -0.22±0.01, b = 62.1±0.4 and r = 0.98. Thus the least squares parameters for the temperature profile of the grandfather are identical, to within experimental error, to those of the normal cells. (Compare caption to Figure 1).

The dashed line refers to the temperature profile of the maternal grandmother.

father is normal but the grandmother is a carrier. This conclusion has been tested statistically and it is found at the 99% confidence limit that the grandfather is homogeneous with the control samples and the grandmother with the parents and children. It seems quite clear, therefore, that significant differences exist in the $2T'_{11}$ temperature profiles of heterozygotes and homozygotes for LCAT deficiency as compared with normal controls.

More detailed spectral analyses of membranes at temperatures below 12°C revealed differences in the line shapes among patients, clinically unaffected carriers and normal controls. Figure 4 shows spectra obtained from carriers (curves C to F), a normal individual (A) and the grandfather (B) who also appears to be normal. The spectra are almost identical except for a greater separation of the outermost extrema ($2T'_{11}$) in the normal samples, reflecting their reduced fluidity compared to carriers. The spectra labeled G and H from clinically affected subjects also show a qualitative difference from the normal control. The different features (indicated by an arrow) are probably due to changes in the absorption manifold associated with transitions involving the $m_I = -1$ nuclear spin state. This qualitative change might be due to relatively slower motion about the long axis of the fatty acid spin probe in the abnormal membranes [8]. Irrespective of the microscopic interpretation of the qualitative differences between the low temperature line shape characterizing patients' membranes on the one hand and those of carriers and normal controls on the other, the spin probe approach described here seems capable of distinguishing between homozygotes possessing clinical manifestations of LCAT deficiency and clinically normal heterozygotes, who are carriers of the abnormal gene.

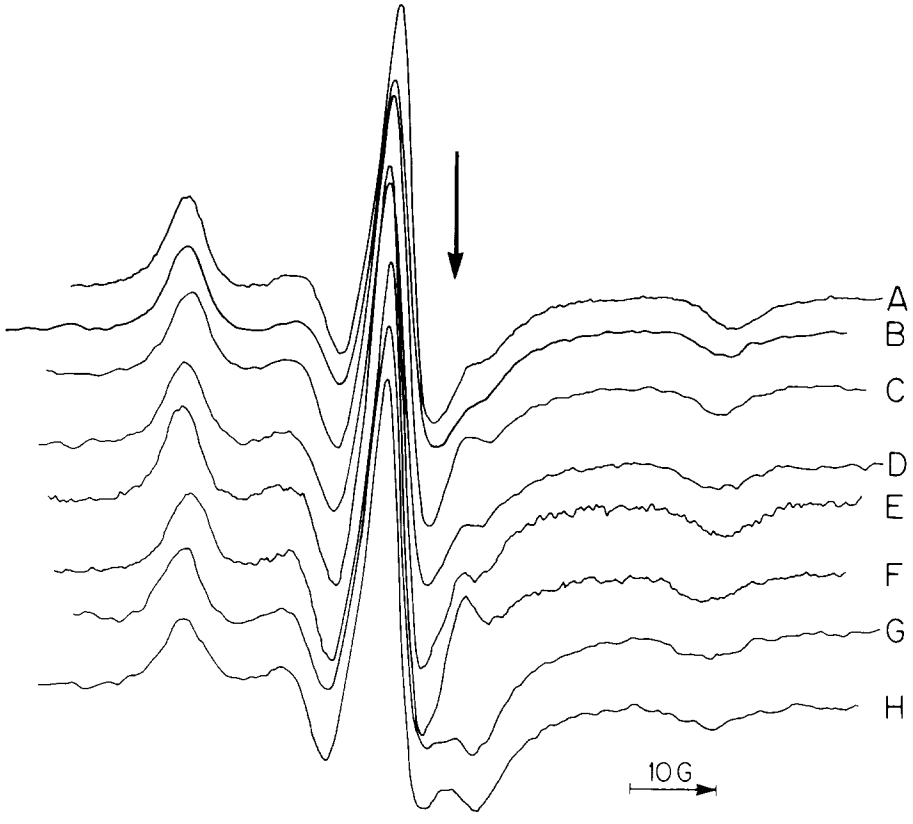

Fig. 4. The spectra of a normal sample, grandfather, grandmother, father, mother, son (heterozygote), daughter (homozygote), and son (homozygote) are labeled A, B, C, D, E, F, G, and H, respectively. All spectra were recorded between 0°C and 10°C.

DISCUSSION

Erythrocyte membrane lipid alterations have been demonstrated in LCAT deficiency [5,6]. The major changes observed were an increase in membrane cholesterol content, a reduction in the phosphatidylethanolamine and a corresponding increase in the phosphatidylcholine. In addition, a decreased latency of membrane protein sulfhydryl groups was noted both in homozygotes and in clinically unaffected heterozygotes for the deficiency, suggesting the presence in both groups of alterations in native membrane structure, possibly related to the lipid compositional abnormalities. Results in the literature [8,13] indicate that lipid arrays containing phosphatidylethanolamine have a higher gel-to-liquid crystalline phase transition temperature than systems composed of phosphatidylcholine. Thus the decrease in the percentage of phosphatidylethanolamine in the erythrocyte membrane would be expected to cause an increase in the fluidity of the membrane, with a corresponding decrease in the value of $2T'_{11}$ at a given temperature. The results obtained for both heterozygotes and homozygotes are consistent with this suggestion. It is interesting, in this regard, that an interrelationship between erythrocyte membrane fluidity, as modulated by cholesterol, for example, and the exposure of membrane protein sulfhydryls has recently been demonstrated [15]. The changes in $2T'_{11}$ are, however, relatively small and one might have expected a greater change in $2T'_{11}$. A general explanation for this is the

suggestion that the fatty acid probe is preferentially sequestered in the more fluid portions of the membrane [13] so that changes in a more rigid component may well be masked. A more specific explanation in the case of the homozygotes is that the marked increase in membrane cholesterol observed in this case will tend to offset the decrease in PE producing a more rigid membrane than expected. Nonetheless, the results that we have obtained demonstrate that significant differences in the value of $2T'_{11}$ can be detected in homozygotes and heterozygotes for LCAT deficiency.

Alterations in physicochemical properties of erythrocyte membranes have been recently described in a number of other disease states, including myotonic muscular dystrophy [10] and Huntington chorea [11], suggesting that these represent diseases of generalized membrane involvement. In contrast, erythrocytes from patients with Duchenne muscular dystrophy were reported to have normal fluidity characteristics, as assessed using a spin labeled stearic acid [12] derivative. These studies [12], however, were performed at a single temperature only. Our present findings emphasize the importance of examining the temperature-dependent properties of ESR spectra in attempting to detect physicochemical membrane alterations associated with pathological states.

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