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Spin Label Studies of Erythrocytes With Abnormal Lipid Composition: Comparison of Red Cells in a Hereditary Hemolytic Syndrome and Lecithin: Cholesterol Acyltransferase Deficiency

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Erythrocytes from patients with familial lecithin:cholesterol acyltransferase (LCAT) deficiency have been shown to exhibit an increase in membrane fluidity which is surprisingly small in view of the extensive alterations both in membrane lipid composition (namely, an elevation in cholesterol and phosphatidylcholine contents as well as a decrease in phosphatidylethanolamine) and in the functional properties of these cells. In the hope of deriving some information concerning the interrelationship between the structural and functional abnormalities, we have used the spin probe 5-doxyl stearic acid to investigate the temperature-dependent fluidity properties of red cells from two patients with a hereditary hemolytic syndrome (HHS) whose red cells are also characterized by qualitatively similar alterations in phosphatidylcholine and phosphatidylethanolamine but, unlike those in LCAT deficiency, have relatively normal levels of membrane cholesterol. A small increase in membrane fluidity of HHS erythrocytes equivalent to that previously observed in LCAT deficiency was found, indicating that membrane cholesterol level does not exert an important modulatory influence on membrane fluidity in these cells. It is concluded that while the distinct patterns of structural and functional erythrocyte alterations in these two disorders cannot be explained on the basis of differences in bulk membrane fluidity, the marginally increased fluidity may underlie the abnormalities in osmotic fragility and membrane p-nitrophenylphosphatase activity which are shared in common by both types of modified red cells.

Key words: erythrocyte membranes, electron spin resonance, LCAT deficiency, phosphatidylcholine elevation, cholesterol

Erythrocyte membrane alterations have been described in a variety of pathological states of hematological [1] and nonhematological [2–5] origin. Detailed studies of such structurally modified erythrocytes may provide valuable information concerning interrelationships between membrane compositional and functional properties. We have recently had the opportunity to investigate erythrocyte membrane abnormalities in two hereditary disorders, namely, familial lecithin:cholesterol acyltransferase (LCAT) deficiency [6] and a hereditary hemolytic syndrome (HHS) characterized by increased levels of membrane

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TABLE I. Lipid Compositional Properties of Erythrocytes From Patients With Familial Lecithin: Cholesterol Acyltransferase (LCAT) Deficiency or a Hereditary Hemolytic Syndrome (HHS) Compared With Normal Controls

Subjects	Phospholipid[a] (μg P/μg total lipid P)				Cholesterol[a] (μmole/mg protein)
	PE	PC	Sph	PS	
Controls (n = 12)	0.352 (±0.033)	0.296 (±0.015)	0.156 (±0.031)	0.166 (±0.014)	0.598 (±0.058)
HHS (n = 2)	0.304 (±0.023)	0.363 (±0.011)	0.126 (±0.002)	0.181 (±0.008)	0.532 (±0.022)
LCAT-deficient (n = 2)	0.183 (±0.009)	0.560 (±0.011)	0.155 (±0.014)	0.068 (±0.011)	0.665 (±0.021)

Values are given as mean ± SD.

[a]Erythrocyte membrane cholesterol/phospholipid ratios (mean ± SD) in the three groups of subjects were: 0.81 ± 0.06 (controls), 0.78 ± 0.03 (HHS), and 0.86 ± 0.01 (LCAT-deficient).

TABLE II. Alterations in Osmotic Fragility (in 0.05 M NaCl) and Mg^{++}-Stimulated Membrane p-Nitrophenylphosphatase (NPPase) Activity in Erythrocytes From Patients With LCAT Deficiency or HHS

Subjects	Hemolysis in 0.05 M NaCl	NPPase activity[a]
Controls (n = 12)	91 (±4)	0.101 (±0.022)
HHS (n = 2)	31 (±11)	0.188 (±0.011)
LCAT-deficient (n = 2)	43 (±9)	0.186 (±0.027)

Values are given as mean ± SD.

[a]μmoles p-nitrophenol/hr/mg membrane protein.

phosphatidylcholine (PC) [7]. Red cells in these two relatively uncommon disease states have previously been shown [6, 7] to share a number of abnormalities in common, including an elevation in membrane PC accompanied by a decrease in phosphatidylethanolamine (PE) (Table I), a decrease in osmotic fragility, and an increase in the activity of membrane Mg^{++}-dependent p-nitrophenylphosphatase (Table II). Despite these similarities, a number of differences between these two types of cells were shown to exist. These included alterations in membrane cholesterol (Table I) and sulfhydryl group latency in LCAT deficiency [6] and abnormalities in the Na^+, K^+-ATPase activity and 2,3-diphosphoglycerate content in erythrocytes of patients with HHS [7].

In seeking to understand the molecular basis of the qualitative differences between these two types of cells, it seemed unlikely that an elevation in membrane PC/PE ratio, which was greater in LCAT deficiency than in HHS (Table I), could be the sole determinant of the characteristic patterns of abnormalities seen in both disorders. One possible modifying factor to be considered was membrane fluidity, which may be modulated by a number of factors, including the PC/Sphingomyelin (Sph) ratio, an increase in which would be associated with enhanced fluidity [8, 9] and cholesterol, whose effects on fluidity are dependent upon its concentration in the membrane, and on temperature [10–12]. The level of membrane cholesterol is marginally decreased in HHS and significantly elevated in

LCAT deficiency, while the ratio of PC to Sph is increased from a value of 2 in control cells to approximately 3 in both HHS and LCAT deficiency (Table I). Earlier studies from our laboratory have shown that membrane fluidity is increased relative to normal in homozygotes for LCAT deficiency [13]. The present report describes an analogous study examining the temperature-dependent electron spin resonance (ESR) parameters of the fatty acid spin probe 5-doxyl stearic acid [2-(3-carboxylpropyl)-4,4-dimethyl-2-tridecyl-3-oxazolindinyl-1-oxyl] incorporated into hemoglobin-free erythrocyte membranes from two members of a family with HHS. The availability of pathologically altered erythrocytes from patients with these two rare hereditary disorders has provided us with a unique opportunity to investigate the role of membrane lipids in determining structural and functional properties of the erythrocyte membrane.

EXPERIMENTAL METHODS

Blood samples were obtained from patients and normal controls by venipuncture, using EDTA as anticoagulant. Erythrocytes were separated from plasma by centrifugation and washed twice with isotonic saline. Hemoglobin-free erythrocyte membranes were prepared by the step-wise hemolytic procedure described previously, including a final wash in 10 mM Tris, pH 7.4 [14]. The results presented in Tables I and II are taken from [6] and [7], where experimental procedures are presented in detail.

The 5-doxyl stearic acid was introduced into the erythrocyte membrane as described in our earlier work on LCAT deficiency [13]. As before, in order to detect possible differences in the physicochemical state of erythrocyte membranes from experimental and control subjects, we have chosen to measure the splitting $2T'_{\prime\prime}$ between the outer extrema of the ESR spectrum of 5-doxyl stearic acid incorporated into the membranes. The larger the magnitude of $2T'_{\prime\prime}$, the greater the degree of motional order in the sample. As pointed out in our previous studies, it is highly desirable that $2T'_{\prime\prime}$ be monitored as a function of temperature so as to maximize the amount of experimental information derivable from the system and allow the detection of subtle membrane alterations in the clinical samples. We have, therefore, examined the temperature profile of $2T'_{\prime\prime}$ over the temperature range 2° to 45°C and the details of the experimental arrangement for measuring the ESR spectra have been described previously [13].

RESULTS AND DISCUSSION

The variation with temperature of $2T'_{\prime\prime}$ for 5-doxyl stearic acid incorporated into the erythrocyte membranes of the two HHS patients, J.M. and her father C.M. is shown by the solid lines in Figures 1, and 2, respectively.

The dashed lines in the figures represent the temperature profile for normal controls (see [13]). It is worthwhile to note, at this point, that the reproducibility of this line from control sample to control sample is extremely high. So far, we have investigated some 20 normal samples with a variation about the line always being within the experimental error of ±0.3 G.

The results in Figures 1 and 2 show that the temperature profiles of $2T'_{\prime\prime}$ in the HHS patients were lower than the control curve by approximately one gauss. This alteration, which was also present in the erythrocyte membranes of individuals with LCAT deficiency [13], indicated a more fluid membrane. The profiles of the patients were parallel to the control curve up to about 30°C, at which temperature there was an inflection with the

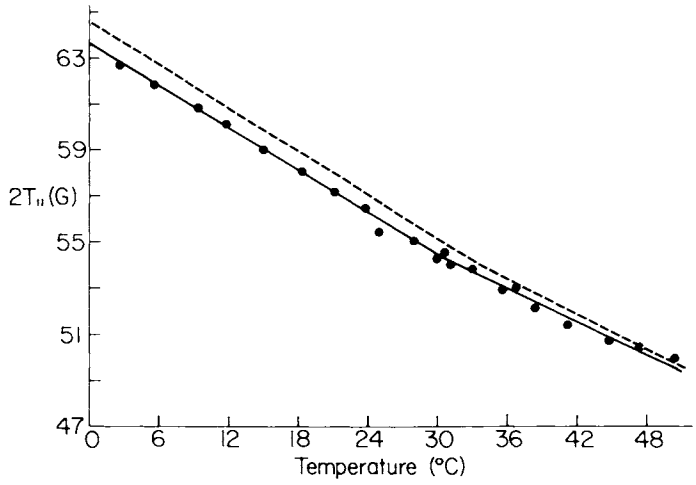

Fig. 1. The temperature variation $2T'_n$ for J.M. (solid line). The dashed line corresponds to the normal control samples (see [10]).

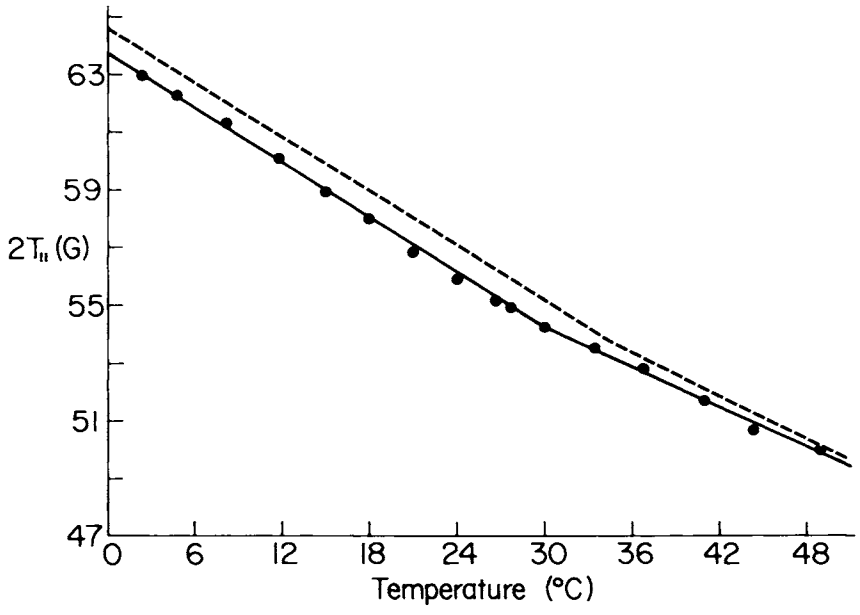

Fig. 2. The temperature variation of $2T'_n$ for the father (C.M.).

membranes of the experimental subjects approaching the same fluidity as the control samples at 34°C. A similar behavior was exhibited by the temperature variation of $2T'_{\prime\prime}$ for erythrocyte membranes from patients with LCAT deficiency, except that the inflection occurred at a lower temperature of 21.5°C [13].

As in our previous studies, we have carried out a statistical analysis of the data in order to ascertain the confidence level for the distinction between the HHS subjects and the normal controls. The analysis assesses the number of homogeneous groups using a multiple-range test based on a linear hypothesis employing the Scheffe test [13]. On the basis of the linear regression intercept, it was apparent that at the 99% confidence level ($P = 0.01$), two distinct homogeneous groups exist – the control samples and the HHS patients. Thus, as was the case in LCAT deficiency, although the difference between the control and experimental profiles is small (1 gauss, as compared with an error of ±0.3 gauss), the statistical test confirms the contention that the differences are significant.

Our studies have indicated, therefore, that a small but significant increase in membrane fluidity is present in the erythrocytes of patients with HHS and is equivalent to that previously described in LCAT deficiency [13]. Since HHS, unlike LCAT deficiency, is characterized by relatively normal levels of membrane cholesterol (Table I), the qualitatively similar alterations in membrane phospholipid composition would seem to play a major role in determining these changes. Other investigations have shown that phosphatidylcholine (PC) tends to favor an increase in the fluidity of phospholipid arrays while Sphingomyelin (Sph) causes an increased ordering of such systems [8, 15]. This is well exemplified by erythrocytes in abetalipoproteinemia where an elevation in the Sph/PC ratio is associated with an appreciable decrease in membrane fluidity [9]. Thus, an increased membrane fluidity in both HHS and LCAT deficiency may be explained on the basis of the increased membrane PC/Sph ratio in both these disorders. The magnitude of these fluidity changes, however, is somewhat less than might have been expected from the phospholipid abnormalities. In the case of LCAT deficiency [13], the increased levels of membrane cholesterol would likely exert a rigidifying influence [9, 11] and tend to minimize the increase in fluidity produced by the marked elevation in membrane PC and possibly the decrease in phosphatidylethanolamine (Table I).

In HHS, on the other hand, a comparable marginal increase in membrane fluidity is apparent, despite the fact that the qualitatively similar phospholipid abnormalities in these extensively functionally modified cells [7] are not accompanied by an elevation in membrane cholesterol content (Table I). Recent studies examining the condensing effect of cholesterol on egg lecithin bilayers have suggested that the spectral features of 5-doxyl stearic acid spin probe are relatively insensitive to changes in cholesterol content [16]. However, experiments by Suda et al [12] using human erythrocyte membranes have shown that increased cholesterol content was paralleled by a corresponding increase in the order parameter of 5-doxyl stearic acid incorporated into these membranes. We cannot, of course, exclude the possibility that large but highly localized abnormalities in fluidity may exist or that the 5-doxyl stearic acid probe, by virtue of its partitioning characteristics in the abnormal membranes, is excluded from regions of the lipoprotein matrix where changes in structural organization may exist. In view of the differing profiles of membrane enzymatic abnormalities in the two disorders, it is significant that the experiments of Suda et al [12] have demonstrated that alterations in membrane protein structure, induced in their studies by glutaraldehyde treatment, are able to modify the influence of cholesterol on the properties of membrane phospholipids. It should also be pointed out that the process of hemolysis employed in the preparation of hemoglobin-free membranes from intact erythro-

cytes may tend to abolish or minimize subtle differences in membrane lipoprotein organization originally present in the intact cell [17]. It does not seem likely, however, that the distinct patterns of membrane molecular modification in erythrocytes of patients with HHS and LCAT deficiency can be explained on the basis of changes in bulk membrane fluidity.

The observed increase in red cell membrane fluidity both in HHS and in LCAT deficiency may have some bearing on the fact that some erythrocyte functional abnormalities (Table II) are shared in common by both pathological states. The enhancement of membrane p-nitrophenylphosphatase activity may indicate the involvement of plasma lipoproteins [18] in the observed membrane modifications, possibly as the result of their fusion with the membrane, as appears to be the case in the production of cholesterol- and PC-enriched target cell erythrocytes in patients with obstructive jaundice [19]. Interestingly, these abnormal erythrocytes also exhibit a decrease in osmotic fragility [20], as is the case in HHS and LCAT deficiency (Table II). On the other hand, Borochov et al [8], who have demonstrated a direct correlation between membrane fluidity and PC/Sph ratio in sheep erythrocytes find, in contrast to our observations, that an elevation in this ratio is associated with an increase in osmotic fragility. It is clear, then, that further studies with other pathologically altered red cells, such as target cells and spur cells [18], which exhibit different abnormalities in membrane cholesterol and phospholipid composition, will be required in order to better define the complex interrelationships between erythrocyte structural and functional properties.

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