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ERYTHROCYTE MEMBRANES — COMPRESSION OF LIPID PHASES BY INCREASED CHOLESTEROL CONTENT

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SUMMARY

Lipid and protein interactions were studied in guinea pig erythrocytes containing a normal or a two-fold increased amount of cholesterol. The electron spin resonance (ESR) spectra of cholesterol-loaded cells labeled with fatty-acid probes showed an increase in the local viscosity of the membrane as compared with control cells. This increase reflects changes in the interior of the lipid matrix of the membrane because the probes resisted destruction by ascorbate, were unaffected by the action of pronase, and gave spectra similar to those of liposomes. No differences were observed between control and cholesterol-loaded cells in the conformation of the membrane proteins by either the infrared spectra or the ESR spectra of cells labeled with maleimide probes.

INTRODUCTION

The cholesterol content of erythrocytes from guinea pigs fed diets supplemented with cholesterol is nearly double that of the controls[1,2]. No associated changes occur in the content of phospholipids or fatty acids in these cholesterol-loaded cells until after the eventual development of a hemolytic anemia and an associated reticulocytosis[3]. Permeability of the cholesterol-loaded cells to Na^+ diffusion and transport and to several hydrophobic and hydrophilic non-electrolytes is decreased[2]. One possible explanation for this apparently general reduction in permeability may be an increase in the local viscosity of the membrane matrix. Accordingly, we investigated the effects of increased cholesterol content on lipid and protein interactions in the erythrocytes membrane by means of electron spin resonance (ESR) and infrared spectroscopy. We found that the added membrane cholesterol increases the local viscosity of the lipid phases of the membrane without any detectable changes in the protein interactions.

EXPERIMENTAL

Erythrocytes were loaded with cholesterol *in vivo* by addition of 1 % cholesterol to the diet of male guinea pigs for 5–8 weeks[1]. Their mean lipid values are shown in

Abbreviation: ESR, electron spin resonance.

TABLE I

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF ERYTHROCYTES AND ERYTHROCYTE GHOSTS FROM CONTROL AND CHOLESTEROL-FED GUINEA PIGS

Data are means ± S.E. from 4 animals per group; control and 1% cholesterol-containing diets had been fed 6–8 weeks. Erythrocytes were isolated from heparinized blood and washed 3 times in buffered saline[2]. Ghosts were prepared according to the method of Doge *et al.*[4] modified by the addition of 1 mM $MgCl_2$ to the wash solution. Protein was determined by the method of Lowry *et al.*[5] with bovine serum albumin as standard. Lipids were extracted with isopropanol–chloroform (1:0.6, by vol.) followed by chloroform–methanol (1:1, by vol.) according to a modification of the method of Rose and Oklander[6]. Phospholipid and cholesterol in the lipid extracts were determined as previously described[1].

Source of erythrocyte	*Erythrocyte*			*Ghost*		
	Cholesterol content (pg)	*Phospholipid content (pg)*	*Cholesterol/ phospholipid (moles)*	*Cholesterol content (pg)*	*Phospholipid content (pg)*	*Cholesterol/ phospholip (moles)*
Control guinea pigs	0.093 (±0.003)	0.180 (±0.006)	1.04 (±0.03)	0.105 (±0.001)	0.214 (±0.007)	0.97 (±0.03)
Cholesterol-fed guinea pigs	0.184* (±0.012)	0.231 (±0.021)	1.63[1] (±0.16)	0.200[1] (±0.014)	0.221 (±0.009)	1.79* (±0.11)

* Control *vs.* cholesterol-fed significantly different at $P < 0.01$.

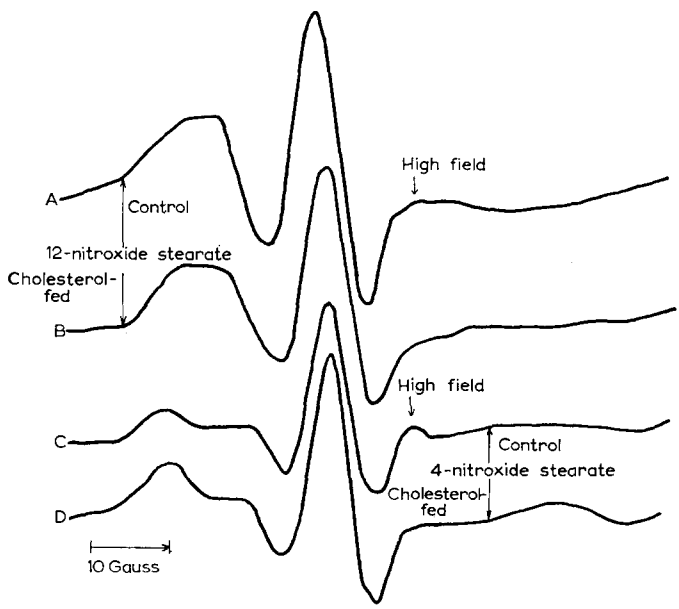

Fig. 1. ESR spectra of stearate probes in erythrocytes from control and cholesterol-fed guinea pigs. Curves A and B show 12-nitroxide stearate in control and cholesterol-loaded cells respectively. Curves C and D show 4-nitroxide stearate in control and cholesterol-loaded cells respectively. Arrows denote prominence of high-field lines in control erythrocytes. The spin-labeled fatty acids 4-nitroxide stearate and 12-nitroxide stearate, both *N*-oxyl-4′,4′-dimethyloxalidine derivatives of the appropriate keto stearates, were prepared as previously described[7]. The probes were incorporated into the cells by addition of 0.01 volumes of probes in ethanol (concentration = 0.1–0.001 M) to cell suspensions (hematocrit = 50%). ESR spectra of aqueous suspensions of cells were recorded at 22 °C in a Varian X-band spectrometer, model 4500.

Table I. A 96 % increase in the content of cholesterol was accompanied by an increase in the molar ratio of cholesterol/phospholipid from 1.04 in normal cells to 1.90 in cholesterol-loaded cells.

The effect of cholesterol-loading on the ESR spectra of membrane-bound stearate probes is shown in Fig. 1. With both probes the added cholesterol caused a broadening of the spectra and a relative decrease in the height of the high-field line. Such changes indicate decreased motion of the probes in the erythrocytes with increased cholesterol[8].

Several lines of evidence suggest that the fatty acid probes were bound in the hydrocarbon matrix of the cell membranes and therefore reflect changes in that phase of the membrane. The hyperfine coupling constant of the spectra, 14.8 gauss, indicates that the probes were in apolar regions of the membrane[9]. Destruction of the membrane-bound probes by ascorbic acid was less than 5 % as determined from the area under the ESR curves before and after 1 h incubation at 22 °C (20 mM ascorbate, Calbiochem, Los Angeles, Cal., 0.05 ml/ml suspension). Since ascorbic acid destroys spinlabels in an aqueous but not in a hydrophobic medium, this also indicates that the probes were in hydrophobic regions of the membrane[10]. Incubation of labeled red cell ghosts with a proteolytic enzyme released 55 % of the membrane protein (0.71 ± 0.043 and 0.37 ± 0.023 pg protein per ghost before and after incubation with 20 mg/ml pronase for 1 h at 37 °C, respectively) yet had no effect on the ESR spectra (identical to those shown in Fig. 1). This observation and the similarity of the spectra of labeled cells to labeled aqueous dispersions of lecithin and cholesterol previously published[11] are further evidence that the probes were bound in the lipid phase of the membranes. Finally, Hubbell and McConnell[12] have reported that these fatty acid probes bind perpendicular to the plane of the membrane. Presumably the carboxyl group of these probes is oriented toward the aqueous interface of the membrane, and the hydrocarbon end is located in the hydrocarbon zones of the membrane. Consequently, the increase in the cholesterol content of the erythrocytes results in a compression of the lipid phase of the membrane.

A compression of the lipid phases in the erythrocyte membrane in response to an increase in cholesterol content is in agreement with previous reports that the addition of cholesterol to aqueous dispersions of phospholipid decreased the fluidity of the hydrocarbon chains[13–15]. It is interesting to note, that the molar ratio of cholesterol to phospholipids in the erythrocytes from cholesterol-fed guinea pigs is well above 1, the highest possible in stable, aqueous dispersions[16]. The mechanism by which more cholesterol can be accommodated in membranes is not known. However, high cholesterol/phospholipid ratios have been reported for solid phase cholesterol:phospholipid systems[17] and for human erythrocytes and plasma lipoproteins in certain pathological cases[18–20].

We also investigated possible alterations in the protein phases of the cholesterol-loaded membranes by means of infrared and ESR spectroscopy of cells labeled with a protein sulfhydryl reagent (*N*-1-oxyl-2,2,5,5-tetramethyl-pyrrolidinyl maleimide, Synvar, Palo Alto, California). In both cases the methods used have been previously described[21,22]. The infrared and ESR spectra of both normal and cholesterol-loaded cells were identical to each other and were similar to those reported by others[11,22]. In particular, there was no change in the amide I and II bands of the infrared spectra which are sensitive to protein conformation. Also, the ESR spectra of the membrane-

bound maleimide probes did not differ in either the narrow-line (freely tumbling) or broad-line (slowly tumbling) components. Thus, the cholesterol-loading of erythrocytes led to an increase in the local viscosity of the lipid phases of the membranes without apparent effects on the protein conformation in the membranes. The decreased permeability of these membranes to several permeants and to Na^+ (ref. 2) would then seem to be a logical consequence of their increased viscosity.

REFERENCES

1 R. Ostwald and A. Shannon, *Biochem. J.*, 91 (1964) 146.
2 J. Kroes and R. Ostwald, *Biochim. Biophys. Acta*, 249 (1971) 647.
3 R. Ostwald, W. Yamanaka, M. Light and J. Kroes, *8th Int. Congr. Nutr. Prague, 1969*, Abstr. 0–14.
4 J. Dodge, C. Mitchell and D. Hanahan, *Arch. Biochem. Biophys.*, 100 (1963) 119.
5 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
6 H. G. Rose and M. Oklander, *J. Lipid Res.*, 6 (1965) 428.
7 J. F. Keana, J. B. Keana and D. Beetham, *J. Am. Chem. Soc.*, 89 (1967) 3055.
8 J. Williams, R. Melhorn and A. Keith, *Chem. Phys. Lipids*, 7 (1971) 207.
9 C. Hamilton and H. McConnell, in A. Rich and N. Davidson, *Structural Chemistry and Molecular Biology*, W. H. Freeman and Co., San Francisco, 1968, p. 115.
10 W. Hubbell and H. McConnell, *Proc. Natl. Acad. Sci.*, 61 (1968) 12.
11 J. Seelig, *J. Am. Chem. Soc.*, 92 (1970) 3881.
12 W. Hubbell and H. McConnell, *Proc. Natl. Acad. Sci.*, 64 (1969) 20.
13 B. Ladbrooke, R. M. Williams and D. Chapman, *Biochim. Biophys. Acta*, 150 (1968) 333.
14 J. Hsia, H. Schneider and I. Smith, *Chem. Phys. Lipids*, 4 (1970) 238.
15 D. Chapman and S. Penkett, *Nature*, 211 (1966) 1304.
16 D. Small, *J. Am. Oil Chemists' Soc.*, 45 (1968) 108.
17 J. Zull, S. Greanoff and H. Adams, *Biochemistry*, 7 (1968) 4172.
18 R. Cooper, *J. Clin. Invest.*, 48 (1969) 1820.
19 R. Cooper and J. Jandl, *J. Clin. Invest.*, 47 (1968) 809.
20 K. Norum, J. Glomset, A. Nichols and T. Forte, *J. Clin. Invest.*, 50 (1971) 1131.
21 D. Wallach and P. Zahler, *Biochim. Biophys. Acta*, 150 (1968) 186.
22 D. Chapman, M. Barratt and V. Kamat, *Biochim. Biophys. Acta*, 173 (1969) 154.