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Influence of Increased Membrane Cholesterol on Membrane Fluidity and Cell Function in Human Red Blood Cells

Richard A. Cooper

Hematology-Oncology Section, Department of Medicine, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania 19104

Cholesterol and phospholipid are the two major lipids of the red cell membrane. Cholesterol is insoluble in water but is solubilized by phospholipids both in membranes and in plasma lipoproteins. Morever, cholesterol exchanges between membranes and lipoproteins. An equilibrium partition is established based on the amount of cholesterol relative to phospholipid (C/PL) in these two compartments.

Increases in the C/PL of red cell membranes have been studied under three conditions: First, spontaneous increases in vivo have been observed in the spur red cells of patients with severe liver disease; second , similar red cell changes in vivo have been induced by the administration of cholesterol-enriched diets to rodents and dogs; third, increases in membrane cholesterol have been induced in vitro by enriching the C/PL of the lipoprotein environment with cholesterol-phospholipid dispersions (liposomes) having a C/PL of >1.0. In each case, there is a close relationship between the C/PL of the plasma environment and the C/PL of the red cell membrane. In vivo, the C/PL mole ratio of red cell membranes ranges from a normal value of 0.9–1.0 to values which approach but do not reach 2.0. In vitro, this ratio approaches 3.0.

Cholesterol enrichment of red cell membranes directly influences membrane lipid fluidity, as assessed by the rotational diffusion of hydrophobic fluorescent probes such as diphenyl hexatriene (DPH). A close correlation exists between increases in red cell membrane C/PL and decreases in membrane fluidity over the range of membrane C/PL from 1.0 to 2.0; however, little further change in fluidity occurs when membrane C/PL is increased to 2.0–3.0.

Cholesterol enrichment of red cell membranes is associated with the transformation of cell contour to one which is redundant and folded, and this is associated with a decrease in red cell filterability in vitro. Circulation in vivo in the presence of the spleen further modifies cell shape to a spiny, irregular (spur) form, and the survival of cholesterol-rich red cells is decreased in the presence of the spleen. Although active Na-K transport is not influenced by cholesterol enrichment of human red cells, several carrier-mediated transport pathways are inhibited. We have demonstrated this effect for the cotransport of Na + K

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and similar results have been obtained by others in studies of organic acid transport and the transport of small neutral molecules such as erythritol and glycerol.

Thus, red cell membrane C/PL is sensitive to the C/PL of the plasma environment. Increasing membrane C/PL causes a decrease in membrane fluidity, and these changes are associated with a reduction in membrane permeability, a distortion of cell contour and filterability and a shortening of the survival of red cells in vivo.

Key words: cholesterol, red blood cells, cell membrane, membrane fluidity, fluorescence polarization

Lipids account for approximately 50% of the weight of red cell membranes. Cholesterol and phospholipid are the major lipids (Table I). Small amounts of glycolipids are also present in human red cells, and larger amounts are found in the red cells of other mammalian species. Both glycolipids and phospholipids are polar, whereas cholesterol is a neutral lipid.

A constant feature of mammalian red cell membranes is that the mole ratio of neutral lipid (cholesterol) to polar lipid (phospholipid + glycolipid) is always approximately 0.9–1.0 [1]. However, the relative amounts of glycolipid and phospholipid vary considerably, as do the relative amounts of the various phospholipid classes. Four phospholipids predominate in human red cell membranes: lecithin (phosphatidylcholine), sphingomyelin, phosphatidylserine, and phosphatidylethanolamine. Small amounts of free fatty acids are also present in human red cells, but neither triglycerides nor cholesterol esters are constituents of red cell membranes.

CHOLESTEROL–PHOSPHOLIPID INTERACTIONS

The importance of the association between polar lipids and sterols is emphasized by the fact that the membranes of all higher organisms, including plants and animals, contain both. In those few instances where sterols are known not to occur in prokaryotes and lower eukaryotes, molecules are present which appear to mimic the sterol structure [2]. For example, caratenols are found in certain mycoplasmas and tetrahymanol occurs in tetrahymena piriformis. Cholesterol is the predominant sterol in all cells within the animal kingdom, whereas sterols with an alkylated side chain predominate in plants. The structural requirements for sterols in cell membranes are quite specific. They must possess a β-OH on the third carbon, a $\Delta 5$ double bond, and alternating transantistereochemistry, creating a planar ring structure and an uncyclized side chain at C17 [2, 3].

The molecular interactions which occur between sterols and polar lipids in membranes are not completely understood. Huang has recently proposed that hydrogen bonding occurs between the carbonyl oxygen of the phospholipid acyl side-chains and the 3β-OH of the sterol [4]. This particular model permits an efficient alignment between the acyl chains of phospholipids and the planar sterol nucleus. In most biologic membranes, one of the phospholipid acyl chains is saturated and the other has at least one unsaturated double bond, a cis double bond at C9-10. This asymmetry of acyl chain structure accommodates the asymmetry of the sterol molecule, caused by the protrusion of two angular methyl groups (C18 and C19) from one face of the sterol nucleus. Models of sterol-phospholipid interaction suggest that phospholipids are capable of accomodating up to 2 moles of cholesterol per mole of phospholipid. For amounts of sterol in excess of a C/P ratio of 2.0, some other molecular configuration is required. This concept is supported by an increasing body of experimental data [5]. It should be noted that, although cholesterol is the usual sterol present in red cell membranes, other sterols which have the structural requirements described above may gain access to the membrane. This has been observed

TABLE I. Lipids of the Normal Human Red Cell Membrane

	μmole/10^{11} cells
Cholesterol	36.1
Phospholipid	38.0
Glycolipid	1.0
Free fatty acid	2.6
	% of total phospholipids
Sphingomyelin	26.0
Lecithin	30.5
Phosphatidylserine (+ phosphatidylinositol)	13.2
Lysolecithin	1.3
Other (polyglycerol phosphatide, phosphatidic acid)	1.7
	Mole/mole
Cholesterol/phospholipid	0.95

in patients who accumulate cholestanol due to an inherited metabolic derangement [6] and in patients who absorb inappropriate quantities of dietary plant sterols, such as β-sitosterol [7].

The interactions between sterols and phospholipids have a number of important consequences for membrane structure. For example, sterols increase the efficiency of packing of phospholipids in artificial membranes [8]. The close interpositioning of sterols with phospholipids causes a degree of immobility to be imposed upon the ten acyl carbon atoms nearest the membrane's surface, while increasing the freedom of motion deep within the hydrophobic core of the membrane [9], thus creating what Chapman has called an "intermediate fluid state" [10]. One manifestation of this effect, which is seen when sterols are added to pure phospholipids, is a dimunition and finally eradication of the endothermic gel to liquid-crystal phase transition which is normally seen upon cooling. Another manifestation is the effect which sterols have on the fluidity of lipid bilayers.

MEMBRANE LIPID FLUIDITY

Under physiologic conditions the lipids of biologic membranes are in a liquid crystalline state. Although the hydrophobic interactions and the hydrogen bonding which occur between lipids maintain the integrity of the lipid bilayer, they also permit appreciable amounts of molecular motion within that bilayer. This motion appears to be greatest within the hydrophobic core of the membrane and least near the hydrocarbon-water interface. It is the process of molecular motion within the membrane that is referred to as "fluidity" and it is this process which imparts viscous properties to the hydrophobic portion of the membrane. This property of membrane lipids is most readily assessed in terms of the motion of small, hydrophobic probes that insert into the membrane and that are detectable by means of nuclear magnetic resonance, electron spin resonance, or fluorescence. The random motion of these probes within the membrane is referred to as rotational diffusion, and this motion is influenced by the viscosity of the microenvironment in which the probe resides. Therefore, measurements of the movement of hydrophobic probes is a means of assessing the viscosity of membrane lipids.

Concepts of membrane fluidity must not be confused with recent studies which have begun to define the membrane's viscoelastic properties. Whereas lipids have viscous properties, they do not have significant elastic properties. The surface viscoelasticity of red cells results from the character of certain membrane proteins, spectrin being the most important, and not from the fluidity of membrane lipids [11].

Membrane fluidity is strongly influenced by the composition of membrane lipids. For example, the number of saturated double bonds within the phospholipid acyl chains in membranes is an important determinant of the membrane's fluid qualities. Indeed, variation in acyl chain saturation is the most common determinant of membrane fluidity in nature. Saturated acyl chains form highly ordered membranes in which fluidity is of low magnitude. Conversely, phospholipids with unsaturated acyl chains form fluid-disordered membranes. Red cell membrane fluidity is also influenced by the amount of cholesterol relative to phospholipid [5, 12, 13] and by the relative amounts of the various phospholipids [14]. Amphiphilic molecules, such as soaps, alcohols, and detergents, partition between aqueous and lipid phases, and have a fluidizing effect on membrane lipids. Lysophosphatides are a naturally occurring amphipathic substance. Local anesthetics, such as procaine, and psychotrophic drugs, such as chlorpromazine, also exert a fluidizing effect under physiologic conditions [15]. Recent studies have suggested that the process of complement lysis is associated with the generation of amphipathic molecules which increase the fluidity of the hydrophobic core of membranes [16].

Because lipid fluidity is very sensitive to temperature, a demand is imposed upon nature to control fluidity under conditions in which temperature changes, for example, in cold-blooded animals exposed to varying ambient temperatures or warm-blooded animals during torpor or hibernation. Similar needs exist in plants which are cold-insensitive. In eukaryotes, sterols buffer the effects of temperature on fluidity by decreasing the activation energy for rotational diffusion, thus permitting less change in fluidity per degree change in temperature than would occur in the absence of sterols. The concept of homeoviscous adaptation is derived from the observation that simple organisms as diverse as bacteria and tetrahymena, are able to maintain membranes having a constant fluidity at a variety of growth temperatures [17–20]. This is achieved by varying the degree of saturation and branching of acyl fatty acids. A similar process of adaptation has been demonstrated in eukaryotic organisms, such as crustacean plankton, which grows in water of widely varying temperatures while maintaining a degree of fatty acid saturation such that the gel-to-liquid crystal phase transition of its membrane lipids is always approximately 2° lower than the ambient temperature [21]. Fish and frogs also vary the saturation/unsaturation ratio of their acyl fatty acids during cold adaptation [22, 23] and this has also been observed in the red cell and mitochondrial membranes of echidna, a primitive mammal, during periods of torpor [24]. Additional mechanisms have been observed in high mammals during hibernation. For example, ground squirrels increase the lysophosphatide content of their cardiac mitochondria [25], and hamsters decrease the cholesterol phospholipid ratio (C/PL) of their brain lipids [26]. All of these changes in membrane lipid composition serve to permit a normal fluidity under conditions of decreased temperature.

SYNTHESIS AND EXCHANGE

Mature red cells lack specific adaptative processes with which to control membrane fluidity. Instead, they depend upon the lipid composition of plasma lipoproteins. Similarities exist between membranes and lipoproteins in the sense that both contain cholesterol and phospholipid in close association with proteins. In addition, plasma lipoproteins contain cholesterol esters and triglycerides. More than two-thirds of the sterol in plasma lipoproteins is in the form of cholesterol esters. From the specific structural requirements for sterols in membranes described above, it is clear that cholesterol esters do not effectively associate with phospholipids in the membrane bilayer.

Two types of exchange diffusion are necessary to describe the movement of cholesterol between plasma lipoproteins and red cell membranes. The first is simple equilibrium exchange in which one molecule of membrane cholesterol exits into plasma in exchange for one molecule of cholesterol in plasma which enters the membrane. This process of equilibrium exchange has a half-time of 2 h, and was first described with isotopically labeled cholesterol by Gould and associates more than 25 years ago [27]. Studies with whole plasma and with isolated plasma lipoproteins in vitro and in vivo have demonstrated that the equilibrium involves the entire free cholesterol pools of both plasma lipoproteins and red cell membranes [28–32].

The second type of exchange diffusion between cholesterol in membranes and plasma lipoproteins concerns the partition of cholesterol within this exchangeable pool; that is, how much cholesterol is in lipoproteins and how much is in membranes.

This partition is best appreciated when it is recognized that cholesterol does not exist as a solute in water, nor does it by itself form micelles or liposomal membranes, as is the case for fatty acids and phospholipids. Sterols are virtually insoluble in water. They are solubilized in lipoproteins and in membranes by amphipathic lipids, such as phospholipids. Therefore, the partition of cholesterol between membranes and lipoproteins is determined by the amount of phospholipid relative to the amount of cholesterol within each compartment, ie, the cholesterol/phospholipid ratio. This has been confirmed by studies in a variety of systems in vivo and in vitro [5, 33, 34]. It is of particular interest because it underlies the pathogenesis of spur cell anemia in man [34] and it may play a role in the development of atherosclerosis in animals fed cholesterol-rich atherogenic diets [35].

SPUR CELL ANEMIA

The syndrome of spur cell anemia represents an abnormality of red cell membrane cholesterol content which results from a primary disorder of plasma lipoprotein metabolism [36]. It occurs in patients with severe liver disease, usually cirrhosis of the alcoholic. Red cells have bizarrely spiculated shapes, and they undergo premature destruction in vivo, primarily in the spleen. Of the two major lipids of the red cell membrane, phospholipids are present in normal amounts but cholesterol is increased by 25–65% [33]. This results in an increase in membrane C/PL from normal values of 0.95 to values as high

as 1.60. It is of interest to compare this to values which can be achieved in vitro when cholesterol is mixed with purified phospholipids in water. Under conditions of high lipid concentration and gentle agitation, multilamellar liposomes form in which the maximum C/PL is 1.0 [37]. This value has been considered to be the upper limit of C/PL, and normal membranes have C/PL values which are below 1.0. Values greater than 1.0 can be achieved in vitro when lipids are suspended at a low concentration in water and energy is added, usually in the form of sonication [5, 34, 38, 39]. Thus, although values of less than 1.0 are preferred, values of C/PL greater than 1.0 can be achieved in vitro and are found in disease states in vivo.

Clinical observation has demonstrated that the spur cell membrane phenomonon is acquired, since normal transfused blood develops the abnormality [40]. Similar observations have been made when normal red cells are incubated for 24 h in serum from patients with spur cells [36]. Since cholesterol is transferred from plasma lipoproteins to cell membranes, it was of interest to examine the role of plasma cholesterol concentration in this process. It was observed that the enrichment of red cell membranes with cholesterol did not correlate with the serum cholesterol concentration but correlated strongly with the C/PL of low-density lipoproteins [34] (Fig 1). Thus, by means of equilibrium partition, red cells acquire cholesterol from lipoproteins which have an increased C/PL.

The syndrome of spur cell anemia is not unique to man, but has been described in rodents (guinea pigs and rabbits) [41, 42] and recently in dogs [43] fed cholesterol-rich atherogenic diets. The accumulation of cholesterol is not confined to red cell membranes. In man and in dogs platelet membranes are affected as well [36, 43], and in rodents an increase in C/PL has been observed in the surface membranes of macrophages [44] and liver cells [45]. Moreover, we have recently observed an increase in the C/PL of the surface membrane of lymphoblasts growing as ascites tumors in cholesterol-fed mice. Nor are changes isolated to surface membranes. Cholesterol equilibrates between cell surface membranes and internal membranes [46], causing an increase in the C/PL of microsomal membranes in cholesterol-fed animals [47]. Thus, an increase in the C/PL of plasma lipoproteins leads to an increase in the C/PL of many cell membranes.

CHOLESTEROL ENRICHMENT IN VITRO

Studies have been carried out in vitro to examine the equilibrium partition of cholesterol between human red cells and sonicated lipid dispersions containing various quantities of cholesterol [5, 34]. The C/PL of red cell membranes is directly influenced by the C/PL of lipid dispersions containing cholesterol cosonicated with various lecithins and with sphingomyelin. In each case, red cell cholesterol is unaffected by dispersions with a C/PL of 1.0 and is directly proportional to the C/PL of the dispersions over a range of membrane C/PL from 0.4 to 2.7 (Fig 2). Moreover, the addition of cholesterol esters to these dispersions had no influence on the partition of cholesterol. Thus, studies with pure lipid dispersions confirm the conclusions reached with human and experimental spur cell anemia.

If the equilibrium partition of cholesterol in terms of phospholipid was the sole determinant of the C/PL of membranes and lipoproteins, then all structures involved in this exchange should have identical C/PL values. Yet red cells, which have a C/PL of 0.95, are in equilibrium with various phospholipid dispersions with a C/PL of 1.0, and also with low-density lipoprotein, which has a C/PL of 0.8, and high-density lipoprotein, with a C/PL of 0.2. Platelet membranes have a C/PL of 0.6, and the surface membranes of

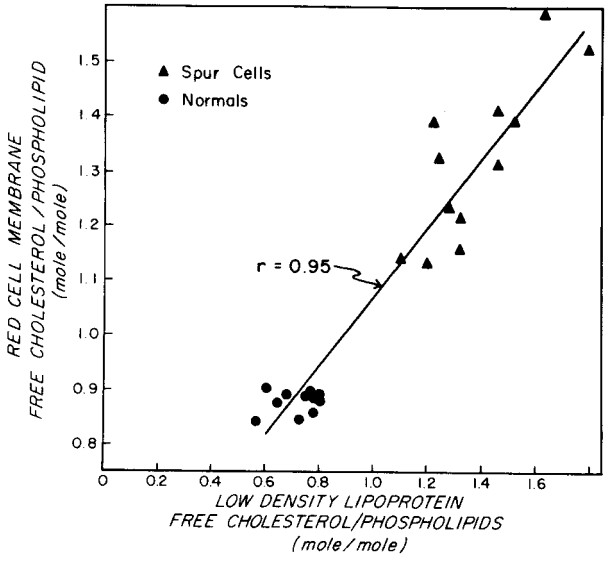

Fig 1. Relationship between the cholesterol/phospholipid (C/PL) mole ratio of red cell membranes of, and serum low-density lipoproteins from, patients with spur cells. A close correlation exists between the C/PL of membranes and lipoproteins [48].

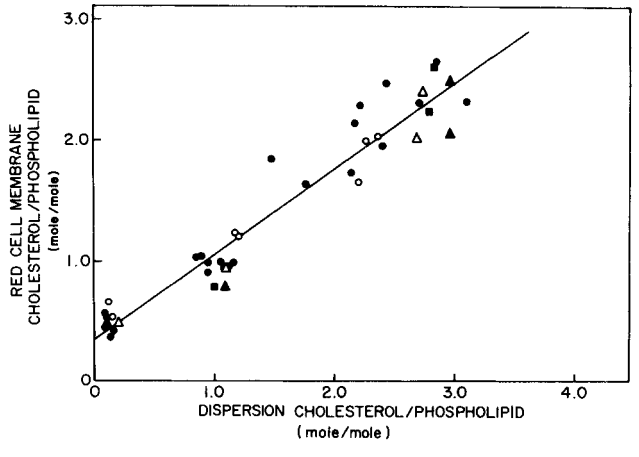

Fig 2. Effect of lipid dispersions on the cholesterol/phospholipid mole ratio of red cell membranes in vitro. Cells were incubated with dispersions for 24 h at 37°C. Phospholipids present in dispersions were ●) L-dipalmitoyl lecithin; ○) egg lecithin; ■) L-dimyristoyl lecithin; ▲) D,L-dipalmitoyl lecithin; △) bovine brain sphingomyelin. The final C/PL of red cell membranes correlated closely with that of lipid dispersions over a broad range of dispersion C/PL, irrespective of the phospholipid present in the dispersion [5].

nucleated cells have C/PL values of 0.4–0.8. Both are in equilibrium with plasma lipoproteins. When high-density lipoprotein is incubated with various phospholipid dispersions, its C/PL remains unchanged in the presence of dispersions with a C/PL of 1.0, and it increases in the presence of dispersions with C/PL values of greater than 1.0 [34]. A part of this seeming disparity results from variable quantities of glycolipids in membranes and lipoproteins. Like phospholipids, these are polar lipids capable of solubilizing cholesterol. In red cells of various mammalian species, glycolipids represent up to 15% of the total membrane lipids. Large amounts are present in the central nervous system. However, glycolipids alone do not account for the wide range of C/PL values observed. It is likely that the association between phospholipids and proteins, which are essential for certain functional and structural properties of both membranes and lipoproteins, occupy some phospholipids in a way which precludes their availability to solubilize cholesterol.

The effect of red cell membrane C/PL on the effective viscosity ($\bar{\eta}$) of red cell membrane ghosts was examined by measuring the rotational diffusion of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene [5] (Fig 3). A close relation between C/PL and $\bar{\eta}$ was seen through the range of red cell C/PL values extending from 0.5 to 2.0. However, further increases in membrane C/PL to values between 2.0 and 2.7 did not cause a further increase in $\bar{\eta}$. Values for membrane $\bar{\eta}$ ranged from 2.0 P at a membrane C/PL of 0.5 to approximately 6.0 P at C/PL values of 2.0–2.7. No phase transition was apparent over the temperature range 10–40° with red cell membranes at any C/PL studied (Fig 4). The activation energy for rotational diffusion, ΔE, was decreased from 8.3 kcal/mole in normal membranes to 5.8 kcal/mole in cholesterol-rich membranes (C/PL, 2.54), indicative of a high degree of order in the lipid structures of cholesterol-enriched membranes. Conversely, cholesterol depletion increased ΔE to 11.8 kcal/mole at a membrane C/PL of 0.35.

Although a linear relationship exists between the C/PL of dispersions and the C/PL of membranes throughout the broad range of membrane C/PL observed (Fig 2), the influence of C/PL on membrane fluidity was continuous only to a membrane C/PL value of 2.0 (Fig 3). This upper limit of 2.0 in terms of membrane C/PL represents a 1:1 interaction of cholesterol molecules with the acyl chains of phospholipids. It is possible that membranes with C/PL values in excess of 2.0 have domains which include cholesterol in a form that fails to influence membrane fluidity. Alternatively, such domains may exclude DPH, and therefore the dynamics within these domains may not be represented by the rotational diffusion of this probe.

CHOLESTEROL EFFECTS ON CELL CONTOUR

Striking changes in cell contour occur in association with increases in membrane cholesterol. These are characterized by a folding and scalloping of the cell margins (Fig 5). Similar changes occur when normal red cells are enriched with cholesterol by incubation with cholesterol-rich lecithin dispersions or with serum from pateints with spur cells [34, 36]. Moreover, a similar morphology was observed in a patient with spur cells who had undergone splenectomy [48]. The addition of cholesterol to red cells under these circumstances also increases membrane surface area in proportion to the contribution that cholesterol makes to the surface area of normal red cells. The magnitude of this increase in surface area is 0.20–0.25% for each 1.0% increase in membrane cholesterol [32]. Thus, the effect of added cholesterol is to increase surface area and alter cell contour.

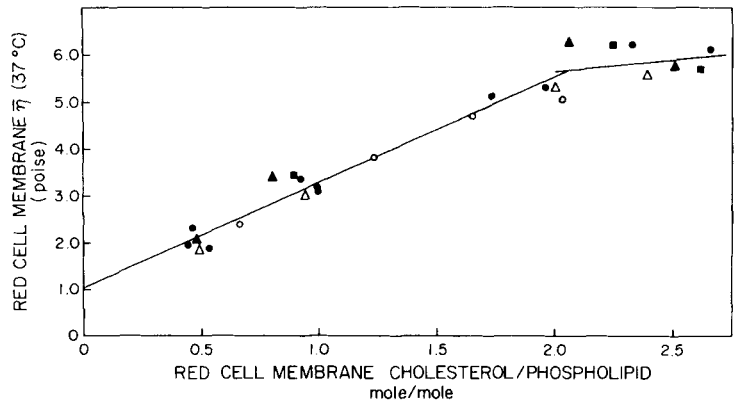

Fig 3. Relationship between the cholesterol/phospholipid mole ratio of red cell membranes and membrane fluidity after incubation of red cells as described in Figure 2. Fluidity was assessed as the rotational diffusion of diphenylhexatriene (DPH) and expressed as the effective microviscosity ($\bar{\eta}$) in poise. A close relationship existed between membrane C/PL and $\bar{\eta}$ to a maximum at a C/PL of 2.0. Thereafter, additional cholesterol did not influence $\bar{\eta}$, as determined with DPH [5].

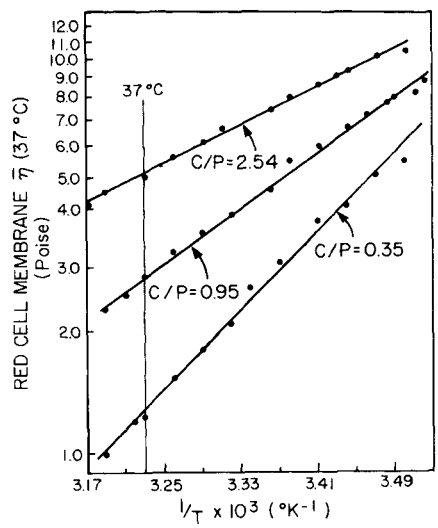

Fig 4. Fluidity of red cell membranes as a function of temperature. Red cells were enriched with or depleted of cholesterol in vitro, as in Figure 3. Effective microviscosity ($\bar{\eta}$) was expressed relative to the reciprocal of the absolute temperature. Cholesterol-enriched membranes had a greater $\bar{\eta}$ at all temperatures. No phase transition was observed [5].

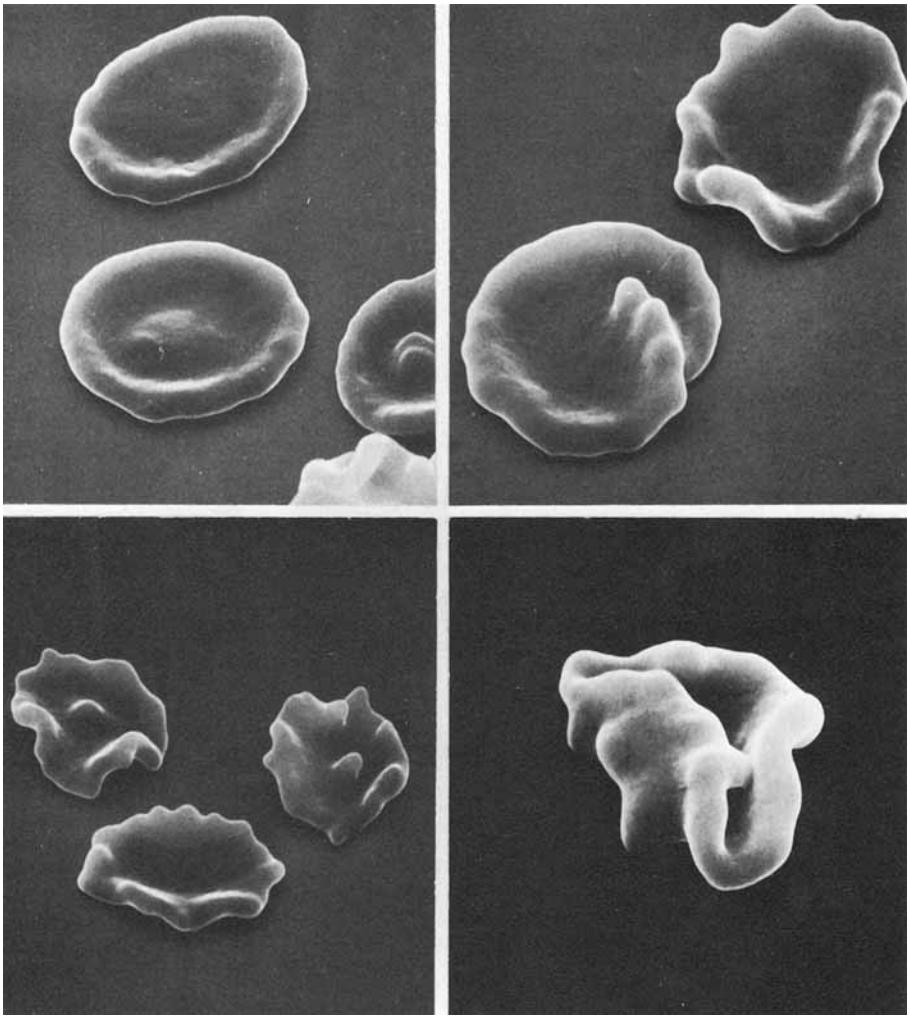

Fig 5. Scanning electron micrographs of red cells enriched with cholesterol in vitro. All photographs are from the same preparation (kindly prepared by Dr R. Weinstein) [34].

Whether due to these gross physical changes in the membrane or whether due to the more subtle changes in fluidity which are also caused by added cholesterol, the flow properties of cholesterol-rich red cells are impaired. This is seen in vitro by a decrease in the red cell's ability to traverse filters of small pore size [34, 36]. In vivo, spur cells are retarded in their circulation through the spleen, where they lose a portion of their surface membrane (Fig 6) and undergo changes in cell contour [36, 48]. This results in a thorny-appearing red cell (the "spur cell"), for which this disorder is named. Membrane remodeling in spur cell anemia is analogous to the loss of membrane that hereditary spherocytes undergo under similar conditions and has been called splenic conditioning [49].

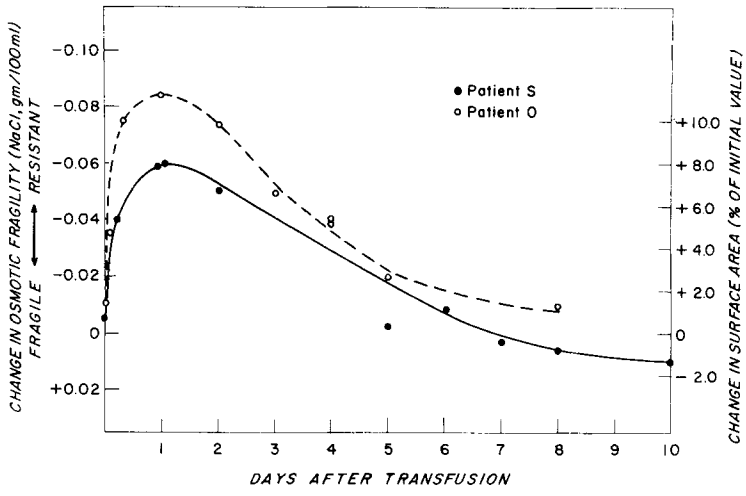

Fig 6. Changes in the surface area of normal ^{51}Cr-labeled red cells after transfusion into two patients with spur cells. Surface area was calculated from a measure of mean osmotic fragility (the concentration of hypotonic NaCl causing lysis of red cells in vitro). An initial increase in surface area resulted from the acquisition of membrane cholesterol by these normal red cells in the circulation of a patient with cholesterol-rich red cells and cholesterol-rich lipoproteins. Subsequent loss of surface area occurred as the spleen eroded the surface of these cholesterol-rich red cells (data reported, in part, in Ref 36).

CHOLESTEROL EFFECTS ON MEMBRANE FUNCTION

The enrichment of membranes with cholesterol has a number of effects on the permeability, transport, and enzymatic activities of the membrane and on the availability of membrane surface receptors. An effect of cholesterol on permeability was first demonstrated using lecithin liposomes containing various sterols, and these studies helped to establish the structural requirements for sterols in membranes [50]. Although not all studies utilizing red cells are in agreement, it appears that increases in membrane cholesterol reduce the passive permeability and facilitated diffusion of a number of electrolytes and nonelectrolytes, whereas cholesterol depletion increases permeability. In contrast, changes in membrane cholesterol do not appear to directly influence the active transport of Na or K. Thus, for example, cholesterol depletion increases the passive permeability of red cells to glycerol, acetate and Na [34, 51]. An apparent increase in active Na flux also accompanies cholesterol depletion. Although this may be explained in terms of a response to the increase in passive Na permeability rather than the primary effect on the transport mechanism [34], an effect of cholesterol on the affinity of the pump for Na has also been demonstrated [52]. In contrast, cholesterol enrichment inhibits the furosemide-sensitive diffusion of Na + K [53], as well as the diffusion of glycerol, erythritol, acetate, and proprionate [54]. Similar decreases in nonelectrolyte permeability have been observed in cholesterol-rich guinea pig red cells [55]. We have not observed any effect of cholesterol enrichment on active Na or K transport [34], although a decrease in active Na flux of very small magnitude was reported in cholesterol-rich guinea pig cells [55]. Thus, modulation of membrane cholesterol has a substantial effect on the cell's permeability to small molecules.

The human platelet has provided additional insight into the relation between membrane cholesterol content and cell function. Shattil and coworkers have shown that human platelets acquire cholesterol when they are incubated with cholesterol-rich lipid dispersions [56]. If the incubation period is short (4–5h) the change in membrane C/PL is confined to the membrane fraction. After 24 h the granule fraction is also cholesterol-enriched. Changes in membrane fluidity parallel changes in C/PL [57]. Associated with this change in membrane lipid composition, platelets become more sensitive to epinephrine and adenosine diphosphate (ADP) such that concentrations of these agents 10–50 times lower than required for inducing normal platelets to aggregate readily induce this aggregation of cholesterol-rich platelets [56] (Fig 7). It is of interest that the C/PL of the membrane (but not granule) fraction of platelets from patients with type II hyperlipoproteinemia is increased [58] and that these platelets are also more sensitive to aggregating agents [59].

The effect of cholesterol on membrane enzymes has been analyzed in rats fed a corn oil diet supplemented with cholesterol [56]. Cholesterol enrichment was found to increase the sensitivity of acetylcholine esterase to fluoride inhibition, decrease the fluoride sensitivity of acetylcholine esterase to fluoride inhibition, decrease the fluoride sensitivity of the Na-K adenosine triphosphatase (ATPase), and cause no change in the fluoride sensitivity of magnesium Mg ATPase. A complex effect of cholesterol on a membrane enzyme, adenylate cyclase, was observed in human platelets [60]. The basal level of this enzyme was increased 2–3 times in cholesterol-rich platelets, and the opposite was seen in platelets with a decreased membrane cholesterol content. Moreover, the stimulation of adenylate cyclase that normally occurs with fluoride or with prostaglandin E_1 was not observed in cholesterol-rich platelets, and prostaglandin E_1 was relatively ineffective in inhibiting platelet aggregation. Effects of membrane cholesterol on membrane enzymes have also been observed in mycoplasma membranes [61, 62].

Three general concepts relate to the mechanism of these cholesterol-induced changes in membrane properties. First is the role of the influence of cholesterol on bulk membrane fluidity. Changes in fluidity may be translated across short distances and influence the range of motion or the potential volume available to a membrane enzyme and its substrate during the enzymatic process. Second is the lipid composition of the immediate environment of the enzyme. Studies with both cytochrome oxidase from mitochondria [63] and Ca-ATPase from sarcoplasmic reticulum [64] have demonstrated a specific boundary layer, or annulus, of phospholipid. Moreover, it appears that cholesterol is specifically excluded from the annulus in sarcoplasmic reticulum. Therefore, in a manner quite distinct from its bulk influence on membrane fludity, cholesterol may influence membrane enzymes by directly interacting with these boundary lipids.

A third general mechanism by which the cholesterol content of membranes may influence membrane enzyme and transport properties relates to the potential effect of membrane lipid composition on the position of proteins, such as enzymes, transport proteins, and receptors, in the plane of the membrane. Shinitzky and co-workers have recently presented evidence which suggests that enrichment of red cell membranes with cholesterol causes an increase in the exposure of membrane proteins to their aqueous environment [65, 66]. If these conclusions can be supported by other techniques, this concept provides a subtle mechanism for the modulation of many membrane events by a primary modulation of membrane lipid composition.

The effects of cholesterol on the permeability and enzymatic properties of membranes cited above emphasize the importance for normal physiology of maintaining

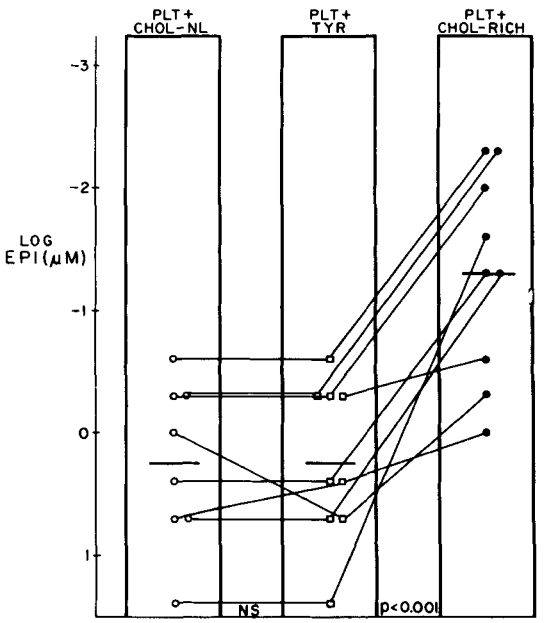

Fig 7. Epinephrine sensitivity of platelets enriched with cholesterol (chol-rich) as compared with platelets incubated with lipid dispersions which induced no change in platelet lipids (chol-nl) and control platelets incubated in tyrodes buffer (tyr). The concentration of epinephrine needed to induce platelet aggregation was decreased 10–100 times in cholesterol-enriched platelets [56].

membrane cholesterol within narrow limits. Mycoplasma membranes which have been adapted to low levels of membrane cholesterol compensate for this by increasing the amount of unsaturated fatty acids associated with their membrane phospholipids [67]. In contrast, it appears that most mammalian cell membranes adapt poorly when exposed to lipoproteins with an increased C/PL. This is evidenced by the accumulation of cholesterol in the membranes of red cells, platelets, liver cells, macrophages, and ascites tumor cells in animals fed a cholesterol-rich diet, as discussed above. These animals also accumulate cholesterol esters in their vessel walls in the form of fatty streaks and atheromatous plaques. The genesis of these lesions is undoubtedly multifactoral, and vessel injury may play an important role. Indeed, it is possible that cholesterol enrichment of endothelial cell membranes may contribute to the process of injury [68]. It is likely that a need exists within cells lining vessel walls to adapt to cholesterol-induced changes in membrane fluidity. Cholesterol esters represent an end product with no functional role. However, esterification of cholesterol does divert cholesterol from a form which is available to enter membranes to a form which is excluded from membrane structure. Cholesterol esterification may represent a form of adaptation to an excess C/PL just as cholesterol synthesis represents an adaptation to a low level of membrane C/PL [69]. In support of this hypothesis we have reported that the rate of cholesterol esterification in the mouse hepatoma cell line Fu5AH bears no relation to the cholesterol concentration of the medium; rather, it is directly related to the C/PL presented to the cells [35] (Fig 8). Thus, it appears that the elevated C/PL of lipoproteins in various animals fed cholesterol-rich atherogenic diets may call forth an adaptative process by some cells whose membranes have become less fluid due to an increase in membrane cholesterol, and that this adaptative process results in the accumulation of cholesterol esters within such cells.

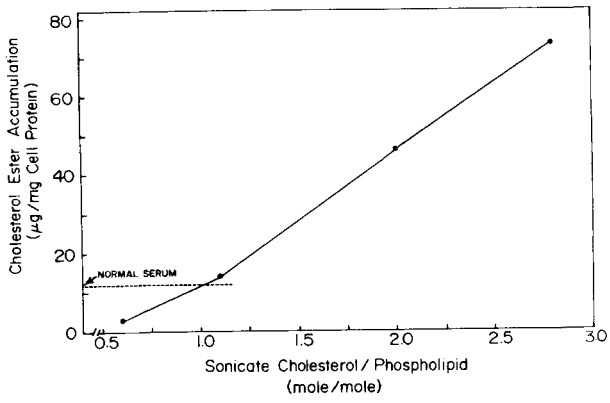

Fig 8. Effect of the cholesterol/phospholipid mole ratio of the medium on cholesterol esterification accumulation in FuSAH hepatoma cell cultures. The degree of cholesterol ester accumulation correlated directly with the C/PL of lipids in the growth medium. Sonicated lipids with a C/PL of 1.0 caused a degree of cholesterol ester accumulation equivalent to that observed with normal serum [35].

TRANSFER OF CHOLESTEROL PLUS LECITHIN TO RED CELLS

In most patients with liver disease, cholesterol-rich spur cells are not observed. Rather, red cells appear as target cells due to the acquisition of an excess of both cholesterol and phospholipid in proportional amounts [33–70] (Fig 9). Although this has been observed in various forms of liver disease, including hepatitis, cirrhosis, and obstructive jaundice, the changes are most striking in patients with obstructive jaundice or with other forms of liver disease with an obstructive component. The cholesterol increase may be as great as 75% but more commonly ranges between 25 and 50% above normal. Although there is variability from patient to patient, the percentage increase in phospholipid is approximately 60% of the percentage increase in cholesterol, resulting in an increase in the C/PL of approximately 15%. The phospholipid increase is not distributed among the various phospholipids, but rather is confined to lecithin. Thus, cholesterol and lecithin are not only the most exchangeable of the major red cell lipids, but their membrane compartments also undergo the greatest quantitative change in liver disease. In several patients with target cells studied by us thus far, membrane fluidity is normal or actually increased slightly as a result of the acquisition of both cholesterol, which tends to decrease fluidity, and lecithin, which is a very fluid lipid.

It is of interest that a similar abnormality in red cells has been observed in patients with a congenital absence of the serum enzyme lecithin-cholesterol acyltransferase (LCAT) [71]. LCAT deficiency of a variable degree is also quite common in patients with liver disease. However, we have not been able to find any correlation between the serum LCAT activity of patients with liver disease and the abnormalities of lipids within their red cells [33].

Two processes appear to participate in determining the amounts of cholesterol and lecithin in target cells. First is a process analogous to, if not identical with, the isolated transfer of cholesterol from lipoproteins to red cells as dictated by the C/PL of the lipo-

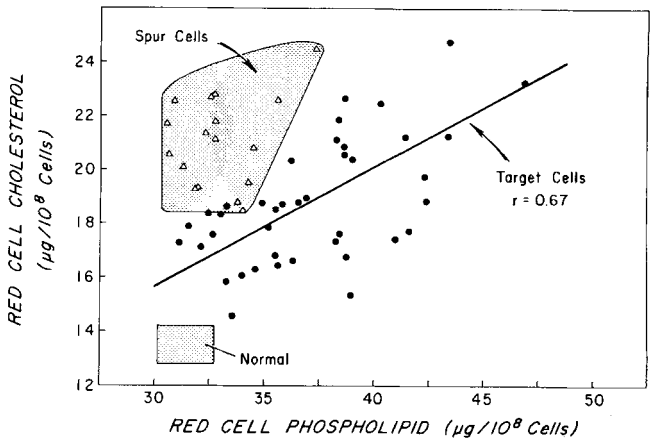

Fig 9. Cholesterol and phospholipid content of red cells from patients with liver disease. Spur cells have a selective increase in cholesterol content, whereas target cells are enriched in both cholesterol and phospholipid [33].

protein. Since the C/PL of low density lipoproteins (LDL) in most patients with liver disease is increased only mildly [33], this accounts for only a small amount of the additional membrane cholesterol. However, this process is readily demonstrable in vitro [33], and it accounts for the increased C/PL of target cells in liver disease [72].

The larger amount of cholesterol and lecithin which is acquired by red cells may involve an independent transfer of lecithin followed by a process of equilibrium during which the C/PL of the red cell (now transiently decreased because of this added lecithin) comes into equilibrium with the increased C/PL of LDL by means of a transfer of cholesterol from LDL to cells. In this regard, it is of interest that the normal phospholipid/protein weight ratio of LDL is approximately 1.0, but it is increased 25% in patients with liver disease [33]. Although an in vitro system has not yet been established to test the importance of phospholipid/protein interactions on the transfer of phospholipid from LDL to cell membranes, it appears likely that the elevated phospholipid/protein of LDL in liver disease underlies the increased lecithin content of target cells. The LDL in patients with spur cells also has a high phospholipid/protein ratio and lecithin also accumulates in spur cells. The fact that the total phospholipid content is not increased in spur cells in vivo appears to result from a loss of membrane phospholipid together with other membrane components as these red cells are conditioned during circulation in vivo [36, 48]. Only the relative increase in lecithin compared with other phospholipids remains in spur cells [33]. Thus, the total content of cholesterol in red cell membranes appears to be a function of 1) the membrane phospholipid content (possibly as influenced by the phospholipid/protein of LDL); 2) the fraction of phospholipid available within each membrane for solubilizing cholesterol (as determined by the amount of membrane phospholipid which is in the form of boundary lipids and other structures strongly related to proteins within the membrane but excluding cholesterol); and 3) by the C/PL of LDL which is in equilibrium with these membranes.

An alternative mechanism for cholesterol and lecithin accumulation in target cells has been suggested by DeGier and his co-workers [70]. This mechanism takes cognizance of the fact that many patients with liver disease, particularly of the obstructive variety,

have a discrete lipoprotein (LP-X) which is discoidal in appearance and which is composed primarily of cholesterol and lecithin in equimolar amounts [73, 74]. Similar structures have been observed in LCAT deficiency [75]. DeGier and associates have proposed that these lipoprotein vesicles fuse with the red cell membrane and in this way transfer equivalent quantities of cholesterol and lecithin to the red cell membrane. Moreover, these workers have presented electron micrographs which are interpreted as showing fusion occurring within 2 h in vitro. Although fusion of artificial vesicles with red cells has also been reported, it appears that the presence in the vesicle of a negatively charged phsopholipid, such as phosphatidylserine, is an essential requirement for fusion to occur [76]. Lecithin-cholesterol vesicles in liver disease and in LCAT deficiency are neutral. Furthermore, the time course of lipid acquisition by target cells in vivo is slow, with a $T_{1/2}$ of 24 h [33], whereas fusion leading to a 15% increase in surface area was observed with LP-X in vitro in only 2 h [70]. Finally, the process of target cell formation is totally reversible in vivo [33], whereas a fusion process would be irreversible. Thus, it appears unlikely that vesicle fusion with red cells represents a major mechanism whereby a large excess of lecithin is acquired by target cells, and direct transfer of lecithin, followed by equilibration with cholesterol, appears more likely. Further work is needed to resolve this problem.

CONCLUSIONS

The organization of lipids in cell membranes requires specific molecular interactions. Many cell processes occur within the fluid environment created by these membrane lipids. As evolution has proceeded from prokaryotes to eukaryotes and from poikilotherms to homeotherms, cells have developed a decreased tolerance of fluctuations in membrane fluidity. Adaptative mechanisms have developed in order to maintain a normal cell membrane fluidity under conditions which might cause it to change, such as variations in the lipid composition or temperature of the cell's environment. Disease states result when human cells fail to adapt. Red cells are particularly vulnerable to abnormalities in membrane fluidity since they lack specific adaptative mechanisms of their own and must depend for adaptation on the lipid composition of plasma lipoproteins. Exposure of red cells to a lipid environment rich in cholesterol relative to phospholipid leads to an enrichment of red cell membrane cholesterol. This is associated with derangements in membrane fluidity and function and with abnormalities of cell contour and cell survival in vivo.

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