Decreased Fluidity of Red Cell Membrane Lipids in Abetalipoproteinemia

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ABSTRACT Acanthocytic red cells in patients with abetalipoproteinemia are morphologically similar to the red cells in spur cell anemia. Fluidity of membrane lipids is decreased in spur cells due to their excess cholesterol content. Acanthocyte membranes have an increased content of sphingomyelin and a decreased content of lecithin. To assess the effect of this abnormality of acanthocyte membrane lipid composition on membrane fluidity, we studied red cells from five patients with abetalipoproteinemia and four obligate heterozygote family members.

Membrane fluidity was measured in terms of microviscosity ($\bar{\eta}$) at 37°C, assessed by means of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene. It was increased from 3.2±0.1 poise in normals to 4.01–4.14 poise in acanthocytes. This was associated with an increase in the sphingomyelin/lecithin ratio from 0.84±0.08 in normals in 1.45–1.61 in acanthocytes. The $\bar{\eta}$ of acanthocyte membranes was not influenced by the degree of vitamin E deficiency. Similar changes in $\bar{\eta}$ were observed in liposomes prepared from red cell lipids. Heterozygotes had normal sphingomyelin/lecithin ratios and normal values for $\bar{\eta}$. The flow activation energy for viscosity, a measure of the degree of order in the hydrophobic portion of the membrane, was decreased from 8.3 kcal/mole in normal red cells to 7.2 kcal/mole in acanthocytes, indicating that acanthocyte membrane lipids are more ordered. Variations in the sphingomyelin/lecithin mole ratio of liposomes prepared from brain sphingomyelin and egg lecithin with equimolar cholesterol caused similar changes in both $\bar{\eta}$ and activation energy. The deformability of acanthocytes, assessed by means of filtration through 3-μm filters, was decreased.

These studies indicate that the increased sphingomyelin/lecithin ratio of acanthocytes is responsible for their decreased membrane fluidity. As in spur cells and in red cells enriched with cholesterol in vitro, this decrease in membrane fluidity occurs coincidentally with an abnormality in cell contour and an impairment in cell deformability.

INTRODUCTION

The term "acanthocyte" refers specifically to the thorny-shaped red cells found in patients with a congenital absence of low density (beta) lipoprotein, as first described by Bassen and Kornzweig (1). Cells of similar morphology, referred to as "spur cells," have been described in association with hemolysis in patients with severe liver disease (2, 3). Similarities and differences have been defined in the pathogenesis of acanthocytes and spur cells, but in both disorders abnormalities of lipid-lipid interaction within the membrane appear to be of fundamental importance. For example, the most striking membrane abnormality in spur cells is an increase in the mole ratio of cholesterol to phospholipid (C/P)[1] from normal values of 0.9–1.0 to values between 1.2 and 1.6 (4), whereas acanthocytes have normal or only slightly increased values (5, 6). On the other hand, acanthocyte membranes have an increased amount of sphingomyelin (SM) and a decreased amount of lecithin (5, 7), whereas spur cells are actually somewhat enriched with lecithin (4). Normal red cells acquire cholesterol when exposed to

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[1] *Abbreviations used in this paper*: C/P, cholesterol-to-phospholipid mole ratio; DPH, 1,6-diphenyl-1,3,5-hexatriene; P, fluorescence polarization; r, fluorescence anisotropy; SM, sphingomyelin; T, absolute temperature; ΔE, flow activation energy; $\bar{\eta}$, microviscosity; τ, lifetime of the excited state.

cholesterol-rich low density lipoprotein from patients with spur cells in vivo or in vitro (3) or to cholesterol-rich lecithin dispersions in vitro (8), and their morphology is transformed to a scalloped contour. Similarly, red cells acquire an increase SM/lecithin ratio (9) and a thorny morphology (10) during circulation in patients with abetalipoproteinemia, but these changes have not been reproduced in vitro either with abetalipoproteinemia plasma (11) or with SM-rich lipid dispersions (12).

In recent years a number of physical tools have been used to assess lipid-lipid interactions in natural and artificial membranes. These include differential scanning calorimetry (13), X-ray diffraction (14), and probes which are capable of assessing molecular motion in the hydrophobic core of the membrane (15, 16). We have demonstrated a decreased mobility of two fluorescent probes in cholesterol-rich human red cell membranes (12, 17), reflecting an increase in membrane microviscosity ($\bar{\eta}$). Similar results have been obtained in cholesterol-rich guinea pig red cells with an electron paramagnetic resonance probe (18). We have proposed that this change in membrane fluidity is responsible for the abnormal morphology and survival of spur cells (17). Shinitzky and Barenholz have reported that the $\bar{\eta}$ of pure SM vesicles is markedly greater than that observed in lecithin vesicles (19). We, therefore, questioned whether the enrichment of red cell membranes with SM would cause them to have an abnormal fluidity. The present study examines the effect of the increased SM/lecithin ratio of acanthocytes on the $\bar{\eta}$ of acanthocyte membrane.

METHODS

Lipid analyses. For measurement of membrane lipids, red cells were thrice washed with 0.155 M NaCl, and 0.2 ml of packed red cells were extracted with 80 vol of isopropanol: chloroform (5:3) (20). Cholesterol (21) and lipid phosphorous (22) were determined colorimetrically. Phospholipid weight was taken to equal lipid phosphorous × 25. After the addition of the antioxidant, 2,3-di-tert-butyl-4-methyl phenol, lipid extracts were taken to dryness with N_2, and phospholipids were separated on silica gel H (23). Plasma vitamin E levels were measured by Dr. Herbert Kayden (24).

Lipid dispersions. Brain sphingomyelin and egg lecithin were obtained from Lipid Products, Ltd., South Nutfield, England, and each was shown to be greater than 99% pure by thin-layer chromatography. After being taken to dryness in vacuo, 40 mg of phospholipid was sonified alone or with equimolar cholesterol (Sigma Chemical Co., St. Louis, Mo.) in 10 ml of 0.155 M NaCl for 30 min in a fluted metal cup maintained under N_2 in a constant temperature bath at 45°C at a power setting of 85 W with a Branson sonifer (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) equipped with a standard tip, as described previously (8, 12). No degradation of phospholipids was observed when these sonicated mixtures were examined by thin-layer chromatography. To disperse lipids extracted from red cells, the lipids from 0.2 ml of packed cells were taken to dryness and sonified as above. Dispersions were centrifuged at 21,800 *g* for 30 min to sediment titanium released from the tip and trace amounts of undispersed lipid.

Analysis of the fluidity of red cell membranes and lipid dispersions. The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich Chemical Co., Inc., Milwaukee, Wis.), was used to label red cell ghosts and lipid dispersions (free of serum). Measurements of fluorescence polarization and fluorescence intensity were performed with an Elscint MV-1 Microviscosimeter (Elscint Inc., Hackensack, N. J.) as described by Shinitsky and co-workers (15, 19, 25, 26). This instrument is equipped with a 200 W mercury arc which generates a 366 nm band, an emission cut-off filter for wavelengths below 390 nm (aqueous 2M $NaNO_2$), Glan Thompson polarizer, dual photomultipliers to simultaneously record emission intensities parallel and perpendicular to the plan of excitation ($I_\parallel$ and $I_\perp$), and a thermo-regulated sampler chamber. Temperature was measured with an electronic thermistor (Cole-Parmer Instrument Co., Chicago, Ill.).

DPH was kept as a stock solution in tetrahydrofuran at a concentration of 2 mM. Immediately before use, it was diluted 1:2,000 in 0.155 M NaCl with vigorous mixing. 1 vol of the dilute DPH dispersion was added to 1 vol of red cell ghosts suspended in 0.155 M NaCl at a ghost concentration of 1×10^8 per ml or to 1 vol of a 1:40 dilution of lipid dispersion in 0.155 M NaCl, and the mixture was incubated at 37°C for 30 min. The amount of DPH dissolved in red cell membranes was equivalent to molecule per 100 molecules of membrane lipid (12).

The microviscosimeter simultaneously analyzes $I_\parallel$ and $I_\perp$, and calculates and displays the degree of fluorescence polarization (P) according to the formula (27):

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} = \frac{I_\parallel/I_\perp - 1}{I_\parallel/I_\perp + 1}. \quad (1)$$

Fluidity is expressed in terms of microviscosity, calculated according to the Perrin equation (27):

$$\frac{r_0}{r} = 1 + C(r)\frac{T \cdot \tau}{\bar{\eta}}, \quad (2)$$

where r is the fluorescence anisotropy which is obtained from P by the relationship (19):

$$r = \frac{I_\parallel - I_\perp}{I_\parallel - 2I_\perp} = \frac{I_\parallel/I_\perp - 1}{I_\parallel/I_\perp + 2} = \frac{2P}{3 - P}. \quad (3)$$

τ is the lifetime of the excited state, T is the absolute temperature, and $\bar{\eta}$ is the microviscosity. r_0 is the upper limit of r. For DPH $r_0 = 0.362$ (19). C(r) is a structural parameter of the probe, which varies slightly with r and was calibrated for DPH with paraffin oil as a reference solvent (25). The microviscosity so obtained represents the harmonic mean of the effective viscosity opposing the rotational diffusion of the probe in all possible directions, other than that around an axis equal to the dipole, and it is expressed in macroscopic units (poise). The flow (or fusion) activation energy for microviscosity (ΔE) was calculated from the slope of the plot, log $\bar{\eta}$ vs. 1/T, fit by the method of least squares (28, 29).

Excited state lifetimes (τ) were estimated from the relative fluorescence intensities at each temperature ($I_\parallel + 2I_\perp$) and from the upper limit value of r_0 of 11.4 ns (19). These corresponded to independent lifetime measurements made at 24–25°C with an Ortec photon-counting fluorescent lifetime instrument (Ortec Inc., Oak Ridge, Tenn.) equipped with an air spark-gap type flash lamp and appropriate filters. The decay of fluorescence was defined by 265 data points which were anlyzed by computer (17). The time frame of

TABLE I
Red Cells in Abetalipoproteinemia

Patients	Abetalipoproteinemia R. I.	A. M. V.	M. S.	R. B.	G. B.	Heterozygotes (4)*	Normals (6)*
Hematocrit	40	38	43	41	43	ND	40–44
Reticulocytes	1.8	1.0	3.0	0.8	1.0	ND	0.6–1.2
Red cell lipids							
Cholesterol, *mg/10⁸ cells*	15.1	16.5	17.4	15.8	17.2	14.2±0.7	14.6±0.7
Phospholipid, *mg/10⁸ cells*	30.3	32.3	33.6	27.8	32.0	32.6±0.8	31.2±1.6
Cholesterol/phospholipid, *mol/mol*	1.02	1.02	1.04	1.08	1.14	0.87±0.06	0.95±0.06
Sphingomyelin/lecithin, *mol/mol*	1.52	1.45	1.61	1.60	1.61	0.92±0.08	0.84±0.08

* Numbers in parentheses refer to numbers of subjects studied.

this fluorescence lifetime is sufficiently short so as not to be influenced by Brownian motion of cells.

Filtration studies. For measurement of their filterability, red cells were washed three times and resuspended at a hematocrit of 2% in Ringer's solution containing 0.25 g/100 ml of human serum albumin and 12 mM Tris-hydrochloride, pH 7.4. Filterability was taken to be the time for 2 ml of this cell suspension to pass through membranes of 3 μm pore size (Nuclepore Corp., Pleasanton, Calif.) under negative pressure equal to 10 cm of water (30).

Subjects. Five patients with abetalipoproteinemia were studied. Each of these patients has been the subject of previous reports: R.I. (31), A.M.B. (7), M.S. (11), G. B. (32), and R.B. (32). The father, mother, brother, and sister of R.I., all of whom are obligate heterozygotes, were also studied.

RESULTS

Hematocrit levels were within the normal range in all patients (Table I). Reticulocytes were elevated slightly in two patients but normal in the other three. The morphology of red cells was abnormal in all five patients, with over 50% of acanthocytes in each case. Red cells were of normal morphology in the four obligate heterozygotes studied.

Red cell cholesterol and total phospholipid were in the normal range in all patients, although the values for cholesterol were in the high normal range and values for phospholipid were among the low normal values (Table I). Therefore, the C/P was slightly higher in patients with abetalipoproteinemia than in normal controls. The SM/lecithin ratio was sharply elevated in all five patients. Obligate heterozygotes had normal values for total cholesterol and phospholipid and for both the C/P and SM/lecithin ratios.

Red cell membrane $\bar{\eta}$ at 37°C, measured by the fluorescence polarization of DPH in ghost membranes, was higher in patients with abetalipoproteinemia than in normals, and this increase was out of proportion to their level of C/P (Fig. 1). Shown for comparison is the curve reported by us describing the relationship between C/P and $\bar{\eta}$ (12). Obligate heterozygotes were in the normal range. The mean value for membrane $\bar{\eta}$ at 37°C in abetalipoproteinemia was 4.06 poise, as compared with a mean value of 3.20 in normals and 3.15 poise in the four heterozygotes studied. The fluidity of liposomes prepared from lipids extracted from normal red cells and acanthocytes was assessed in terms of the r of DPH at 28°C. With the red cell lipids from three patients, r was 0.254, 0.262, and 0.263, respectively, as compared with the normal value of 0.245. Thus, the abnormal membrane fluidity of acanthocytes appeared to be associated with an abnormality in the lipid portion of the membrane.

Microviscosity is a measure of molecular motion, or flow, in the membrane. For flow to occur, a hole of a certain size must be available into which the given unit of flow can move. The energy of formation of a hole the size of a molecule is represented by ΔE (28, 29). It correlates with the degree of order within

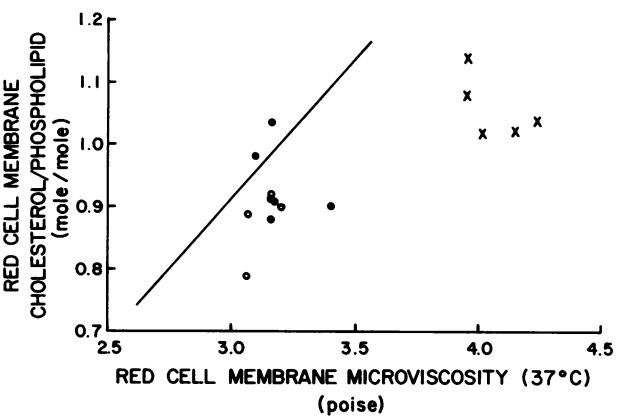

FIGURE 1 Cholesterol/phospholipid (C/P) mole ratio and microviscosity of red cell membranes. ● = normal; X = abetalipoproteinemia; ○ = family members heterozygous for abetalipoproteinemia. The curve represents the relationship between red cell membrane C/P and microviscosity obtained after modifying the C/P of normal red cells in vitro (12). Microviscosity is increased in abetalipoproteinemia beyond that which would result from the small increase in membrane C/P.

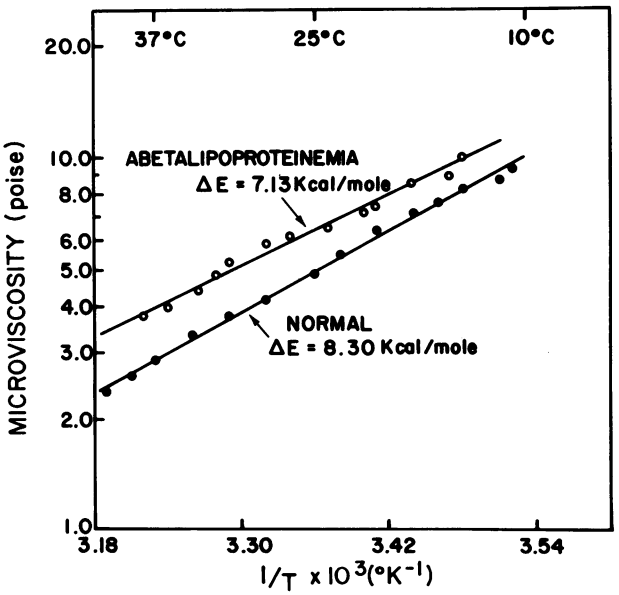

FIGURE 2 Temperature dependence of microviscosity in normal and abetalipoproteinemia red cell membranes. ΔE, the flow activation energy for viscosity, was calculated from the slope of this relationship. Microviscosity was increased and ΔE was decreased in abetalipoproteinemia.

the hydrophobic core of the membrane (26). ΔE is calculated from the slope of the curve describing the relationship between the log of the microviscosity and the reciprocal of the absolute temperature (Fig. 2). Acanthocyte membranes had a lower value for ΔE than normal red cell membranes, indicating that their lipid bilayer was more ordered than that of normal red cells.

Vitamin E levels were measured in three patients with abetalipoproteinemia. Patient R.I. was known to have ceased vitamin E supplements more than 1 yr before study, and his plasma level was 0.03 μg/ml. Patient M.S. took vitamin E sporadically and had a plasma level of 0.11 μg/ml. Patient A.M.V. regularly took vitamin E, and her plasma level was 0.77 μg/ml. Thus, plasma levels of vitamin E correlated well with the history of vitamin E ingestion. Despite this range of vitamin E levels, all patients had similar values for membrane $\bar{\eta}$. Moreover, when patient R.I. was restudied 6 mo after the institution of vitamin E supplements, his membrane $\bar{\eta}$ at 37°C was the same as at the time of vitamin E deficiency. Thus, a sensitivity to lipid peroxidation due to vitamin E deficiency did not appear to underlie the abnormality of membrane fluidity.

The deformability of acanthocytes was assessed in terms of the ease with which they traversed 3-μm Nucleopore filters (Table II). The ratio of red cells to filter pores was approximately 100:1. Filtration time was prolonged in acanthocytes as compared with normal control red cells; however, there was a substantial variation in the degree of prolongation among the five patients studied. In three of the patients all of the red cells traversed the filter during the observation period, whereas, in two, only 75% were filtered by 200 s.

To further assess the role of SM and lecithin in the abnormal fluidity of acanthocytes, liposomes were prepared containing either brain SM or egg lecithin together with equimolar cholesterol. The $\bar{\eta}$ of SM-cholesterol was greater than that of lecithin-cholesterol throughout the temperature range of 10–40°C (Fig. 3). Although pure SM exhibits a phase transition at approximately 34°C (19, 33), equimolar SM-cholesterol does not undergo a phase transition. SM-cholesterol was more ordered than lecithin-cholesterol, as evidenced by a ΔE value of 3.83 kcal/mole for SM-cholesterol as compared with 7.51 kcal/mole for lecithin-cholesterol. In mixed SM-lecithin liposomes a direct relationship was observed between the SM/lecithin ratio and the $\bar{\eta}$ of liposomes at 37°C (Fig. 4). This was also true when liposomes containing SM and lecithin were prepared with equimolar cholesterol. The ΔE of SM:lecithin:cholesterol (1:1:2 mole ratio) was 6.35 kcal/mole.

DISCUSSION

These studies demonstrate that acanthocyte membrane lipids have an increased microviscosity and an increased degree of order. Moreover, these physical characteristics appear to be imparted to the membrane by the presence of excess SM relative to lecithin. Similar physical properties are imparted to spur red cells by the presence of excess cholesterol (10, 16, 17). The similarity in morphology of these two abnormal red cells, each of which has acquired an increase in $\bar{\eta}$ from a different abnormality of membrane lipid composition, suggests that the change in cell shape may relate to either localized or generalized changes in the dynamics of lipids within the membrane bilayer. It should be noted that cholesterol-rich red cells with an increased membrane microviscosity and with a morphology similar to acanthocytes and spur cells are also observed in animals such as guinea pigs (34), rabbits

TABLE II
Red Cell Filtration

Patient	Filtration time (SM)	Percentage of cells filtered
	s	%
R. I.	17	100
A. M. V.	40	100
M. S.	91	100
G. B.	200	75
M. B.	200	75
Normals	14±5	100%

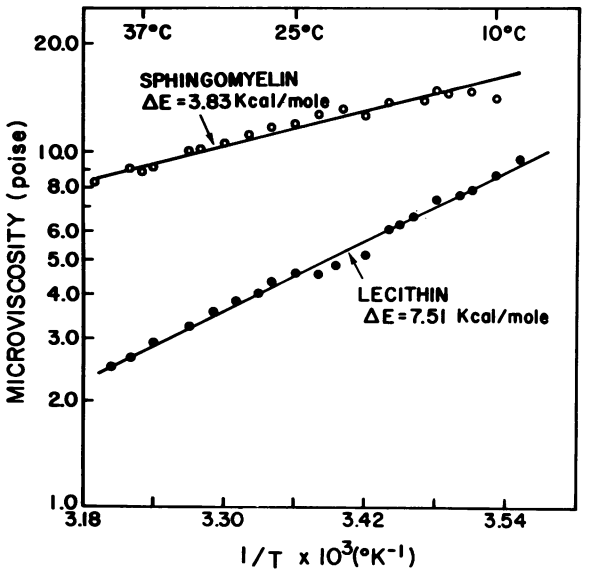

FIGURE 3 Temperature dependence of microviscosity and ΔE in equimolar cholesterol-egg lecithin and cholesterol-brain sphingomyelin liposomes. Cholesterol-sphingomyelin liposomes were more ordered (lower value for ΔE) and less fluid (increased microviscosity).

(35), and dogs (36) fed diets supplemented with cholesterol.

Red cells are unable to synthesize lipids de novo, although they can lengthen fatty acid chains and acylate lysophospholipids (37, 38). Lecithin and SM, which are the only major phospholipid classes in the outer leaflet of the membrane bilayer (39, 40), exchange slowly with their counterparts in plasma lipoproteins. Reed has estimated that the turnover of SM is 8% per day (41). High density lipoproteins are the only plasma lipoproteins present in abetalipoproteinemia. They are normal in terms of concentration and in terms of both their C/P and their ability to accept additional cholesterol (11). However, they are abnormal in terms of their SM/lecithin ratio (5, 7), and this appears to underly the abnormality in red cells. The fact that SM exchanges slowly probably accounts for the inability to reproduce the observation during the incubation of normal red cells in abetalipoproteinemia serum for periods of 24–48 h.

Ways and Dong have demonstrated that the SM/lecithin ratio of older acanthocytes is greater than that of younger cells (9). Therefore, the microviscosity of older cells is probably greater, as well. It is likely that premature destruction of these older cells causes the somewhat shortened red cell survival observed in abetalipoproteinemia (5, 42). Nonetheless, red cell survival in abetalipoproteinemia is not shortened as profoundly as in spur cell anemia (2, 3). We have previously shown that the spleen conditions spur cells, transforming them from a scalloped contour to a thorny shape and then destroying them (30). It is likely that the same process affects acanthocytes. However, patients with spur cells have the congested, hyperplastic spleens of portal hypertension. In contrast, neither splenomegaly nor portal hypertension are features of abetalipoproteinemia probably explaining the longer survival of these physically similar red cells.

It should be noted that membrane fluidity, as reported herein, and membrane viscoelasticity, as measured with micropipettes, are different physical properties (43, 44). Fluidity reflects the rotational diffusion of molecules within the membrane bilayer. It is a three-dimensional process (27). Viscoelasticity applies to plastic flow in the plane of the membrane. It is a two-dimensional process, influenced primarily by membrane proteins (44). It is difficult to compare these two properties directly. Both appear to be important characteristics of membranes.

A poor filterability of acanthocytes has been reported in one previous study (6), but in another filterability was normal (32). However, in this latter study the pore-to-cell ratio was 1.0, and only the ability of cells to pass through the filter without regard to filtration time was measured. In contrast to specific physical measurements such as fluidity and viscoelasticity, filterability simply measures the deformability of whole cells as they flow through small orifices which are meant to mimic the spleen filter. Filterability is known to be influenced by cell surface viscosity as well as by hemoglobin viscosity, and it is sensitive to changes in the

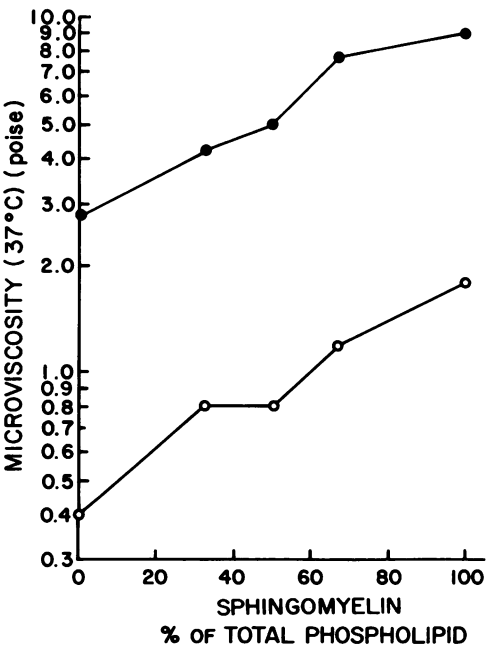

FIGURE 4 Microviscosity at 37°C in liposomes containing varying quantities of egg lecithin and brain sphingomyelin, either alone (○) or with equimolar cholesterol (●). Sphingomyelin caused an increase in the microviscosity of these mixed lipid liposomes.

surface area-to-volume ratio of cells (43). Since cell surface viscosity is primarily a function of membrane proteins and is influenced only minimally by membrane lipid fluidity, it seems unlikely that a decreased deformability of acanthocytes results from a change in the fluidity of lipids, per se. Nor is there reason to believe that the hemoglobin of acanthocytes has abnormal physical properties. Finally, when evaluated in terms of osmotic fragility, the surface area-to-volume ratio of acanthocytes is normal. It is of interest that filtration time is also prolonged within both spur cells (3) and with cells enriched with cholesterol in vitro (8), and membrane fluidity is decreased in both of these red cell abnormalities (12, 17). In the case of cholesterol-enriched red cells, the surface area-to-volume ratio is actually increased, a factor which theoretically should aid rather than impair deformability. It is possible that the poor filterability of acanthocytes, spur cells and cholesterol-enriched red cells is related to the distorted membrane contour which is present under each condition. However, the mechanism by which filterability is decreased in red cells in which membrane fluidity is also decreased remains to be defined.

Acanthocytes have small but abnormal amounts of long chain polyunsaturated fatty acids (45) which would tend to decrease membrane microviscosity. That the decrease in fluidity observed does not relate to peroxidation of these fatty acids is suggested by the similar results obtained in patients protected by vitamin E and deficient in vitamin E and the lack of improvement in microviscosity after many months of vitamin E supplementation. The fatty acid composition of bovine brain SM, studied here, is similar to that of human red cell SM (33, 45, 46), which has a predominance of palmitic (16:0) and nervonic (24:1) acids. Sphingosine provides a second saturated hydrocarbon chain. In terms of its transition temperature, pure SM behaves like a saturated lecithin, the upper limit of transition being similar to that of dipalmitoyl lecithin. Similarly, changes in surface area per lipid molecule upon hydration are similar for SM and dipalmitoyl lecithin (32). In terms of microvisocity, SM also resembles dipalmitoyl lecithin, the $\bar{\eta}$ at 37°C of SM-cholesterol being 9.0 poise as compared with 8.8 poise for dipalmitoyl lecithin-cholesterol (12). Thus, the substitution of SM for lecithin effectively increases the amount of viscous, ordered lipid within the membrane.

The control of membrane fluidity appears to be an important property of many, if not all, biological systems. For example, fluidity is modified in several ways under conditions of changing temperature. In poikilothermic animals, such as fish (47, 48), frogs (49), and crustacean plankton (50), adaptation to lower temperatures is associated with an increase in membrane unsaturated fatty acids and a decrease in saturated fatty acids. Ground squirrels increase the lysophosphatide content of mitochondrial membranes in order to maintain fluidity during hibernation (51); whereas hamsters accomplish the same goal in brain membranes by decreasing the C/P (52). Since mammalian red cells lack the ability to synthesize lipids de novo (39), the state of their fluidity depends to a large extent on the control of lipoprotein lipid composition. While this serves adaptive and homeostatic roles under normal conditions, abnormalities in lipoproteins, such as in the patients studied here, lead to abnormalities in the lipid composition and thereby in the fluidity and function of red cell membranes.

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