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A STUDY OF PHOSPHOLIPID INTERACTIONS BETWEEN HIGH-DENSITY LIPOPROTEINS AND SMALL UNILAMELLAR VESICLES

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Summary

Previous observations on serum-induced leakage of liposome contents from egg phosphatidylcholine liposomes (Allen, T.M. and Cleland, L.G. (1980) Biochim. Biophys. Acta 597, 418—426) have been extended in order to examine the role of the phase transition and phospholipid backbone in leakage. The high-density lipoprotein (HDL) fraction has been purified from human serum and the rate of transfer of radioactively labelled phospholipids from sonicated liposomes to high-density lipoproteins has been examined. Results obtained from the calcein dequenching method for serum-induced leakage of liposome contents showed that as the proportion of solid phospholipid (distearoyl phosphatidylcholine, T_c = 56°C) increased, relative to the proportion of egg phosphatidylcholine, the half-time for retention of liposome contents at 37°C in the presence of serum also increased. Including increasing amounts of bovine brain sphingomyelin (T_c = 30°C) in egg phosphatidylcholine liposomes also substantially decreased leakage from liposomes in the presence of serum at 37°C. ^{14}C-labelled egg phosphatidylcholine was found to transfer readily from liposomes to purified HDL, as did ^{14}C-labelled dioleoyl phosphatidylcholine. Including cholesterol in egg phosphatidylcholine liposomes decreased the rate of transfer of phospholipid to HDL. ^{14}C-labelled distearoyl phosphatidylcholine did not exchange readily with HDL. These results are consistent with the interpretation that tightening bilayer packing prevents the apolipoprotein-mediated transfer of phospholipid to HDL and slows the leakage of liposome contents associated with this transfer. [^{14}C]Sphingomyelin also did not exchange readily with HDL. This does not appear to be a phase transition effect as the majority of sphingomyelin is above its phase transition at 37°C. The failure of sphingomyelin to exchange readily with HDL is interpreted as

Abbreviations: HDL, high-density lipoprotein(s); Tes, *N*-tris(hydroxymethyl)methylglycine

being due to intermolecular hydrogen bonding between the sphingosine backbones of the sphingomyelin molecule.

Introduction

One of the problems confronting researchers who wish to use liposome-entrapped drugs systemically in the treatment of disease is the rapid release of drug from liposomes in the presence of blood or serum. The increased permeability of liposomes in vivo over that seen in vitro in the absence of blood components is attributed to the transfer of phospholipid from liposomes to serum high-density lipoproteins (HDL). This appears to lead to loss of bilayer integrity and release of lipsomal contents [1—7]. By manipulation of liposome composition it should be possible to control the rate of leakage of liposome contents in vivo. The recent literature in this area has been reviewed by Gregoriadis [8].

Previous studies from this laboratory [7] have been extended to include a study of the effect of manipulation of phospholipid backbone and phase transition on the serum-induced leakage of liposome contents. The high-density lipoprotein fraction has been purified from human serum and the rate of transfer of several radioactively labelled phospholipids to HDL has been measured. The results appear to confirm that breakdown in liposome integrity in vivo is the result of apolipoprotein-mediated transfer of phospholipid from liposomes to HDL. Manipulations of liposomes composition which result in tightening of the bilayer reduce phospholipid transfer and increase liposome latency. Increasing the amount of sphingomyelin in liposomes has the same result, seemingly by an intermolecular hydrogen bonding effect rather than a phase transition effect.

Materials and Methods

Chromatographically pure egg phosphatidylcholine, bovine brain sphingomyelin and dioleoyl phosphatidylcholine were purchased from Avanti Biochemicals Inc. (Birmingham, AL). Cholesterol, distearoyl phosphatidylcholine and a second preparation of bovine brain sphingomyelin were purchased from Sigma Chemical Company (Saint Louis, MO). ^{14}C-labelled egg phosphatidylcholine (specific activity 60 mCi/mmol) was purchased from New England Nuclear. [^{14}C]Phosphatidylcholine (50 mCi/mmol) and ^{14}C-labelled dioleoyl phosphatidylcholine (55 mCi/mmol) were purchased from Applied Science Laboratories, Inc. Calcein was purchased from Fisher Scientific.

Serum-induced leakage

Estimates for half-time of leakage of liposome contents was performed as described previously [7]. Briefly, sonicated liposomes containing entrapped 175 mM calein (pH 7.4, 280 mosM) were made from a variety of phospholipids. Liposome-entrapped calcein was separated from free calcein by chromatography on a 1 × 40 cm Sephadex G-50 column in 2 mM Tes/2 mM histidine/150 mM NaCl buffer (pH 7.4, 273 mosM). A small (10 μl) aliquot of liposomes

was pipetted into either buffer or human serum at 37°C and the fluorescence increase which accompanied calcein leakage was monitored in a Perkin-Elmer MPF-4 spectrofluorimeter at 490 nm excitation and 520 nm emission. Half-times for leakage were calculated as previously described [7] using as 100% leakage the fluorescence reading obtained following lysis of liposome samples with 1.3% deoxycholic acid. Because of the variability in half-times from preparation to preparation, the reported results are an average of at least eight independent assays.

Phospholipid exchange with HDL

Frozen pooled plasma was obtained from the Edmonton Red Cross and was fractionated by ultracentrifugation in a Beckman L2-65 ultracentrifuge at 13°C using a 60 Ti rotor at 50 000 rev./min. Very low- and low-density lipoproteins (d = 1.006—1.063 g/ml) and HDL (d = 1.063—1.21 g/ml) were isolated sequentially by centrifugation for 21 h. Solutions were adjusted to the appropriate densities with KBr in 10 mM Tris/100 mM NaCl/1 mM EDTA/0.001% sodium azide, pH 7.4. HDL was washed once at d = 1.21 g/ml and dialyzed for 48 h against several changes of the above buffer. The HDL preparation was concentrated to a final concentration of approximately 20 mg protein/ml by ultrafiltration through an Amicon PM-30 filter at 5°C and 60 lb/inch^2.

Some of the HDL was saturated with unlabelled sonicated liposomes according to the procedure of Jonas and Maine [4]. HDL was exposed to unlabelled sonicated liposomes of egg phosphatidylcholine/cholesterol (2 : 1, mol/mol), for 16 h at 37°C at a ratio of vesicle phosphatidylcholine to HDL phosphatidylcholine of 2.0. Equilibrated HDL was separated from liposomes by chromatography on a Sepharose CL-4B column (2.4 × 40 cm) and concentrated by Amicon PM-30 filtration. Non-equilibrated HDL had a final phospholipid : protein ratio (by weight) of 0.67 and equilibrated HDL had a phospholipid : protein ratio of 1.6. Protein was determined by the method of Lowry et al. [9] and phospholipid was determined according to the procedure of Dittmer and Wells [10].

Liposomes containing radioactively labelled phospholipid or cholesterol were prepared according to the following procedure. For experiments designed to study phospholipid transfer to non-equilibrated HDL the final ratio of liposome phosphatidylcholine to HDL phosphatidylcholine was 1.6. Typically, 40 mg of chromatographically pure phospholipid containing 10^6 cpm of ^{14}C-labelled lipid was taken to dryness under vacuum in a rotary evaporator. Remaining traces of organic solvent were removed by placing the dried lipid on a high vacuum pump for 1—2 h. The dried lipid was suspended in 2 ml 10 mM Tris/100 mM NaCl buffer, pH 7.4, and following mild vortexing was sonicated to clarity in a bath-type sonicator (Laboratory Supplies Co. Inc., Hicksville, NY). Equal amounts (1.5 ml) of liposome and HDL were mixed and one sample (1.0 ml) was chromatographed immediately (0 h) on a Sepharose CL-4B column (1 × 40 cm), while the other two samples of 1.0 ml each were incubated for 1 and 16 h, respectively, at 37°C, then immediately cooled to 4°C and run on Sepharose CL-4B. All fractions were analysed for protein and phospholipid as above. Radioactivity was estimated by counting 0.5 ml aliquots added to Aquasol-2 (New England Nuclear) in a Beckman LS-230

liquid scintillation counter. A procedure similar to that above was used for estimations of phospholipid exchange with pre-equilibrated HDL, except that the ratio of liposome phospholipid to HDL phospholipid was 1.0.

The percentage of phospholipid transfer to, or exchange with, HDL was estimated as the increase in radioactivity associated with the HDL peak from Sepharose CL-4B at 1 h and 16 h as compared to the sample which was chromatographed immediately (0 h). Exchange/transfer was quantitated from areas under the respective peaks.

The above procedure was repeated for a variety of phospholipid or phospholipid/cholesterol combinations for those phospholipids which we were able to obtain radioactively labelled.

Differential thermal analysis

The phase transition behavior of phosphatidylcholine/sphingomyelin combinations was examined by differential thermal analysis. Suspensions of 10 mg phospholipid, 20% by weight in water, were scanned in a Dupont 900 Thermoanalyser. Both heating and cooling scans were run at a rate of 5°C/min.

Results

Serum-induced leakage

The half-times for leakage of 175 mM calcein from osmotically stable liposomes in the presence of buffer or serum at 37°C was examined and the results are presented in Table I. As previously reported [7], when liposomes composed of pure egg phosphatidylcholine were incubated in an excess of serum at 37°C, greatly increased leakage of contents from liposomes was noted relative to buffer incubation. Inclusion of cholesterol in the liposomes at a molar ratio of egg phosphatidylcholine : cholesterol of 2 : 1 decreased leakage by 2—3-fold in serum, and approximately 1.3-fold in buffer. The effect of incorporating increasing amounts of distearoyl phosphatidylcholine in the liposomes

TABLE I

HALF-TIME FOR LEAKAGE AT 37°C FOR SONICATED EGG PHOSPHATIDYLCHOLINE/DISTEAROYL PHOSPHATIDYLCHOLINE LIPOSOMES

The figures in parentheses represent the number of assays for each sample. Half-time (h ± S.D.) for leakage was estimated from the increase in fluorescence accompanying release of quenched calcein from liposomes according to the technique of Allen and Cleland [7]. PC, egg phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; CHOL, cholesterol.

	Buffer	Serum
PC only	23.7 ± 7.2 (8)	1.0 ± 0.5 (8)
PC/CHOL (2 : 1)	32.0 ± 8.6 (8)	2.6 ± 0.8 (8)
PC/DSPC (4 : 1)	26.5 ± 8.9 (8)	1.7 ± 0.6 (8)
PC/DSPC/CHOL (8 : 2 : 5)	38.6 ± 17.7 (8)	5.4 ± 2.3 (8)
PC/DSPC (1 : 1)	21.4 ± 7.0 (8)	2.3 ± 0.3 (8)
PC/DSPC/CHOL (1 : 1 : 1)	25.8 ± 5.9 (8)	5.5 ± 2.4 (8)
PC/DSPC (1 : 4)	7.9 ± 3.3 (8)	3.4 ± 0.2 (8)
PC/DSPC/CHOL (2 : 8 : 5)	30.2 ± 4.1 (8)	4.8 ± 2.0 (9)
DSPC only	8.8 ± 3.1 (8)	15.0 ± 5.1 (8)

was also examined; this lipid (T_c = 56°C), when pure, is in the gel phase at 37°C. Incorporation of up to equimolar amounts of distearoyl phosphatidylcholine in egg phosphatidylcholine liposomes did not substantially change their leakage behavior in buffer at 37°C. The presence of cholesterol at a phosphatidylcholine : cholesterol ratio of 2 : 1 in egg phosphatidylcholine/distearoyl phosphatidylcholine (4 : 1) and (1 : 1) liposomes resulted in slightly longer half-times for leakage in buffer. At a molar ratio of 1 : 4, liposomes showed a higher leakage rate, similar to that seen for distearoyl phosphatidylcholine alone. The presence of cholesterol in egg phosphatidylcholine/distearoyl phosphatidylcholine (1 : 4) liposomes markedly reduced leakage in buffer and resulted in a $t_{1/2}$ for leakage similar to that seen for higher ratios.

Incorporation of increasing amounts of distearoyl phosphatidylcholine in liposomes resulted in a slightly increased $t_{1/2}$ in the presence of serum at 37°C as compared to egg phosphatidylcholine alone (Table I). Inclusion of cholesterol in these liposomes conferred a marked to slight protective effect against serum-induced leakage, the magnitude of the effect decreasing with increasing distearoyl phosphatidylcholine content. A large increase in $t_{1/2}$ for leakage in the presence of serum was observed for liposomes composed of distearoyl phosphatidylcholine alone. This result was similar to the leakage of calcein from distearoyl phosphatidylcholine liposomes in buffer.

The effect of incorporating increasing mole fractions of sphingomyelin in egg phosphatidylcholine liposomes was also examined, and the results are presented in Table II. There was a tendency of $t_{1/2}$ for leakage in the presence of buffer at 37°C to increase with increasing sphingomyelin content of the liposomes up to a phosphatidylcholine : sphingomyelin molar ratio of 1 : 1. The incorporation of 35 mol% sphingomyelin has been reported by Finkelstein and Weissmann [11] as increasing the stability of liposomes in the presence of serum. Incorporation of cholesterol into the liposomes at a phospholipid : cholesterol molar ratio of 2 : 1 resulted in some interesting observations. Cholesterol had a protective effect against leakage in buffer for pure

TABLE II

HALF-TIME FOR LEAKAGE AT 37°C FOR SONICATED EGG PHOSPHATIDYLCHOLINE/SPHINGOMYELIN LIPOSOMES

The figures in parentheses represent the number of assays for each sample. Half-time (h ± S.D.) for leakage was estimated from the increase in fluorescence accompanying the release of quenched calcein from liposomes according to the technique of Allen and Cleland [7], PC, egg phosphatidylcholine; SM, sphingomyelin; CHOL, cholesterol.

	Buffer	Serum
PC only	23.7 ± 7.2 (8)	1.0 ± 0.5 (8)
PC/CHOL (2 : 1)	32.0 ± 8.6 (8)	2.6 ± 0.8 (8)
PC/SM (4 : 1)	32.7 ± 11.6 (8)	1.5 ± 0.2 (8)
PC/SM:CHOL (8 : 2 : 5)	53.8 ± 12.6 (8)	3.9 ± 2.3 (8)
PC/SM (1 : 1)	59.1 ± 15.3 (8)	5.4 ± 0.7 (8)
PC/SM:CHOL (1 : 1 : 1)	54.9 ± 13.1 (8)	8.7 ± 3.0 (8)
PC/SM (1 : 4)	43.8 ± 27.4 (9)	34.7 ± 7.9 (8)
PC/SM/CHOL (2 : 8 : 5)	33.1 ± 14.5 (9)	20.5 ± 8.1 (8)
SM only	9.9 ± 3.9 (8)	6.7 ± 1.1 (16)

egg phosphatidylcholine and egg phosphatidylcholine/sphingomyelin (4 : 1), but did not appear to have any effect on leakage at egg phosphatidylcholine/sphingomyelin (1 : 1), and had a tendency to increase leakage at egg phosphatidylcholine/sphingomyelin (1 : 4). Liposomes composed of sphingomyelin alone had a significantly higher rate of leakage in buffer than egg phosphatidylcholine/sphingomyelin combinations. The leakage rate for sphingomyelin alone was comparable to that seen for distearoyl phosphatidylcholine alone.

Half-times for leakage of calcein from egg phosphatidylcholine/sphingomyelin liposomes in the presence of serum increased with increasing sphingomyelin content. The $t_{1/2}$ for leakage from egg phosphatidylcholine/sphingomyelin (1 : 4) was significantly higher than that seen for higher egg phosphatidylcholine : sphingomyelin ratios. The leakage at this liposome composition was not significantly different from that seen in buffer. Cholesterol conferred a protective effect against serum-induced leakage up to a egg phosphatidylcholine : sphingomyelin ratio of 1 : 1, but as also seen in the presence of buffer, the inclusion of cholesterol in egg phosphatidylcholine/sphingomyelin (1 : 4) liposomes caused increased leakage. Leakage from pure sphingomyelin liposomes in the presence of serum was similar to that seen in the presence of buffer.

Because we were unsure of the phase transition behavior of our particular sphingomyelin preparations, differential thermal analysis of egg phosphatidylcholine/sphingomyelin combinations was performed. Pure sphingomyelin obtained from both Avanti and Sigma had similar scans. Pure sphingomyelin showed a major peak near 31°C, with a shoulder in the vicinity of 37°C where our leakage assays were performed. Pure sphingomyelin is thus predominately in the liquid-crystalline state at 37°C. Egg phosphatidylcholine/sphingomyelin (1 : 4) had a single broad peak at 31°C, and incorporating increasing amounts of phosphatidylcholine into the preparations at the same total lipid concentration resulted in a gradual decrease of the enthalpy of transition (ΔH) and a shift of the transition peak to lower temperatures. Egg phosphatidylcholine/sphingomyelin (4 : 1) liposomes exhibited a second, broad exotherm centered near −10°C, indicating that a phase separation of egg phosphatidylcholine and egg phosphatidylcholine/sphingomyelin phases is occurring at high phosphatidylcholine : sphingomyelin ratios at low temperatures. Those results are similar to those reported previously by Untracht and Shipley [19].

Phospholipid exchange with HDL

The results of a typical pre-equilibration experiment with HDL are shown in Fig. 1. Sonicated phosphatidylcholine/cholesterol (2 : 1) liposomes were incubated with HDL, at a 2 : 1 molar ratio of liposome to HDL phospholipid, for 16 h at 37°C. A sample of liposomes which were used for the HDL saturation was run over the same Sepharose CL-4B column, prior to running the liposome/HDL sample. Liposomes alone (open circles, Fig. 1) showed a large peak of small, sonicated liposomes, and a smaller peak of multilamellar liposomes at the void volume. After incubation, the phospholipid associated with the sonicated liposome peak decreased (solid circles, Fig. 1) while the phospholipid associated with HDL protein (solid squares, Fig. 1) increased.

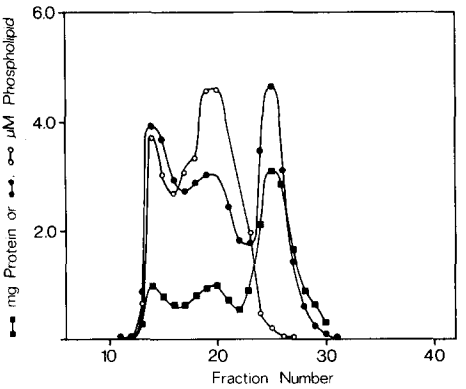

Fig. 1. Pre-equilibration of high density lipoprotein from human plasma with sonicated egg phosphatidylcholine liposomes containing 33 mol% cholesterol. The ratio of liposome phosphatidylcholine to HDL phosphatidylcholine was 2.0. Samples were chromatographed over a 2.4 X 40 cm Sepharose CL-4B column. ○———○, liposome phosphate before incubation with HDL. ●———●, liposome and HDL phosphate after 16 h incubation at 37°C, ■———■, HDL protein after 16 h incubation at 37°C.

Little or no decrease was observed in the amount of phospholipid associated with the multilamellar peak. Some HDL protein was observed to be associated with both the multilamellar and the small unilamellar liposome peaks (solid squares, Fig. 1). Pre-equilibration of HDL resulted in an increase of phospholipid : protein in HDL of from 0.67 in non-equilibrated HDL to 1.6.

The results of a typical experiment for radioactively labelled phospholipid exchange with HDL are shown in Fig. 2. Fig. 2 shows the results for incubation

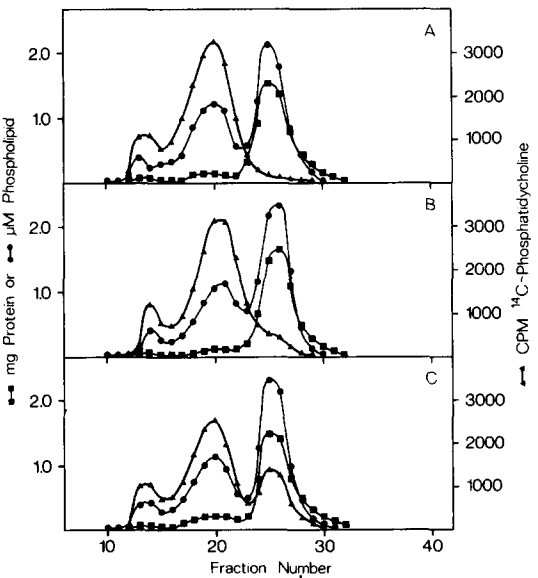

Fig. 2. Incubation of sonicated phosphatidylcholine : cholesterol, 2 : 1 molar ratio with pre-equilibrated HDL at a ratio of liposome phosphatidylcholine to HDL phosphatidylcholine of 1.0. A, mixed and immediately chromatographed over a 1 X 40 cm Sepharose CL-4B column; B, incubated for 1 h at 37°C prior to chromatography; C, incubated for 16 h at 37°C prior to chromatography. ●———●, liposome and HDL phosphate; ▲———▲, [^{14}C]phosphatidylcholine; ■———■, HDL protein.

TABLE III

PERCENT EXCHANGE/TRANSFER OF PHOSPHOLIPIDS WITH HDL AT 37°C

The percentage was estimated from the amount of radioactivity transferred from small unilamellar liposomes to HDL following incubation at 37°C for the times indicated. PC, egg phosphatidylcholine; SM, sphingomyelin; CHOL, cholesterol; DSPC, distearoyl phosphatidylcholine; DOPC, dioleoyl phosphatidylcholine.

	Time (h)	Non-equilibrated HDL	Equilibrated HDL
PC	1	13.0	5.6
PC	16	57.0	28.0
PC/CHOL (2 : 1) (^{14}C-PC)	1	4.2	3.4
PC/CHOL (2 : 1) (^{14}C-PC)	16	23.8	19.0
PC/SM (1 : 4) (^{14}C-SM)	1	1.8	0
PC/SM (1 : 4) (^{14}C-SM)	16	12.7	1.9
PC/SM (1 : 4) (^{14}C-PC)	1	3.3	N.D.
PC/SM/CHOL (2 : 8 : 5) (^{14}C-SM)	1	6.2	
PC/SM/CHOL (2 : 8 : 5) (^{14}C-SM)	16	28.9	
SM	1	3.2	
SM	16	15.0	
PC/DSPC (1 : 4) (^{14}C-DSPC)	1	3.8	
PC/DSPC (1 : 4) (^{14}C-DSPC	16	12.5	
DSPC	1	0	
DSPC	16	8.1	
PC/DOPC (1 : 4) (^{14}C-DOPC)	1	14.9	
PC/DOPC (1 : 4) (^{14}C-DOPC	16	39.6	
DOPC	1	17.1	
DOPC	16	39.7	

of egg phosphatidylcholine/cholesterol (2 : 1) with pre-equilibrated HDL. It can be seen that increasing amounts of ^{14}C-labelled egg phosphatidylcholine (solid triangles) become associated with the HDL protein peak (solid squares) after 1 h incubation (Fig. 2, B) and 16 h incubation (Fig. 2, C), as compared with zero time (Fig. 2, A). A small amount of HDL protein (solid squares) is associated at all incubation times with the liposome peaks, but this does not seem to increase substantially at the longer incubation time. Also, unlike the case where liposomes are incubated with non-equilibrated HDL (e.g., Fig. 1). very little net transfer of phospholipid is seen from the sonicated liposome peak (solid circles) to the HDL protein peak.

It is assumed, therefore that the results seen with the pre-equilibrated HDL represent a phospholipid exchange process [3], and the results obtained with non-equilibrated HDL represent a combination of phospholipid transfer to, and exchange with, HDL.

A summary of the data for exchange or exchange plus transfer of phospholipid with HDL is given in Table III for a variety of phospholipids. The results for percent exchange/transfer of phospholipids with HDL parallel the results seen with serum-induced leakage, i.e., the samples with high leakage rates correspond to those with high rates of phospholipid exchange, and vice versa. Exchange/transfer of phospholipids to non-equilibrated HDL was highest for the liquid-crystalline phosphatidylcholines, i.e., egg phosphatidylcholine, dioleoyl phosphatidylcholine and egg phosphatidylcholine/dioleoyl phosphatidylcholine (1 : 4). Inclusion of 33 mol% cholesterol in the egg phosphatidylcholine liposomes reduced the level of exchange/transfer of ^{14}C-labelled egg

phosphatidylcholine approximately 3-fold, a reduction similar to that seen for half-time of serum-induced leakage of egg phosphatidylcholine liposomes in the presence of cholesterol (Table I). Exchange of egg phosphatidylcholine or egg phosphatidylcholine/cholesterol (2 : 1) with equilibrated HDL was lower than that seen with non-equilibrated HDL, as would be expected if the transfer or saturation component of the exchange was reduced by the pre-equilibration procedure.

Phosphatidylcholines below the gel to liquid-crystalline phase transition temperature showed greatly reduced exchange/transfer as compared to those above. Distearoyl phosphatidylcholine liposomes showed no exchange of ^{14}C-labelled distearoyl phosphatidylcholine with non-equilibrated HDL after 1 h incubation at 37°C. Exchange after 16 h was also considerably reduced. Inclusion of 20 mol% egg phosphatidylcholine in distearoylphosphatidylcholine liposomes increased the level of exchange/transfer of ^{14}C-labelled distearoyl phosphatidylcholine, but it still remained considerably below that seen for egg phosphatidylcholine or dioleoyl phosphatidylcholine. These results again parallel the observations for serum-induced leakage from distearoyl phosphatidylcholine-containing liposomes.

Another parallel between the results for HDL exchange and serum-induced leakage was noticed for sphingomyelin-containing liposomes. The liposomes with the longest $t_{1/2}$ for leakage, egg phosphatidylcholine/sphingomyelin (1 : 4), also had the lowest exchange/transfer to non-equilibrated HDL for [^{14}C]-sphingomyelin. Incubation of these liposomes with equilibrated HDL reduced the exchange rate even further. However, the exchange rate for ^{14}C-labelled egg phosphatidylcholine from these liposomes was high (3.3% in 1 h), particularly if one considers that egg phosphatidylcholine was only 20% of the total phospholipid. If this result was extrapolated to 100% egg phosphatidylcholine the exchange rate would be 16.5% in 1 h (3.3% × 5), which is similar to the exchange rate seen for egg phosphatidylcholine alone (13% in 1 h) or dioleoyl phosphatidylcholine alone (17% in 1 h). Liposomes containing sphingomyelin alone had higher exchange/transfer rates than egg phosphatidylcholine/sphingomyelin (1 : 4) liposomes, and decreased half-times for serum-induced leakage. Similarly, inclusion of 33 mol% cholesterol in egg phosphatidylcholine/sphingomyelin (1 : 4) liposomes increased the rate of exchange/transfer of [^{14}C]-sphingomyelin as well as increasing the leakage rate of calcein from these liposomes (i.e. decreasing $t_{1/2}$ for leakage).

No attempt was made to remove the traces of multilamellar liposomes present in our sonicated presentations prior to exchange/transfer experiments. Phospholipid associated with multilamellar liposomes was in no case greater than 10% of the total phospholipid and in most cases less than 5%, as ascertained from phospholipid analysis of the void volume peak from Sepharose CL-4B. It was not felt that this small contamination would substantially effect the results for exchange/transfer with HDL.

Discussion

A good correlation exists between the ability of serum to perturb the integrity of liposome bilayers and the extent to which phospholipid is capable of

exchanging with purified serum high density lipoprotein. These observations appear to confirm the theory that apolipoprotein-mediated transfer of liposomal phospholipid to high density lipoprotein is the major factor leading to loss of liposomal integrity and thus loss of their ability to retain entrapped substances [1–7].

The ability of cholesterol to reduce the leakage of liposome contents induced by the presence of serum or blood has been described previously [5,7,12]. This has been attributed to a tightening of the phospholipid bilayer by cholesterol, which in turn inhibits the association of apolipoproteins with the bilayer by reducing the number of defects, phase boundaries or insertion sites for apolipoprotein in the lipid matrix [7,13]. The observation (reported in this communication) of reduced ability of fluid phosphatidylcholine bilayers containing 33 mol% cholesterol to exchange with HDL provides confirmatory evidence for this theory.

The involvement of lipid phase transition in the plasma-induced dissolution of liposomes has been explored by Sherphof et al. [5] for multilamellar phosphatidylcholine liposomes. They report maximum dissolution of multilamellar vesicles at or close to the phase-transition temperature with no disintegration above or below that temperature. The rate of dissolution of liposomes at the phase transition appeared to decrease with increasing fatty acid chain length. In the present study no dissolution of egg phosphatidylcholine multilamellar liposomes was seen at 37°C (Fig. 1, void volume peak) but substantial phospholipid transfer/exchange was seen for sonicated egg phosphatidylcholine liposomes and for dioleoyl phosphatidylcholine liposomes at 37°C. The explanation probably lies in the amount of surface area available for insertion of apolipoproteins. For multilamellar vesicles the surface area presented to the lipoprotein is small as compared to the total amount of phospholipid present in the liposomes. The surface area of the outer monolayer of a multilamellar liposome has been estimated to be only 5–8% of that of a large unilamellar liposome containing the same total amount of lipid [14]. If, as postulated by Jonas and Maine [4], only the outer monolayer phospholipids are available for exchange, then relatively little exchange would be seen for multilamellar vesicles as compared to sonicated liposomes. Substantial transfer/exchange of phosphatidylcholine from sonicated liposomes above their phase transition (egg phosphatidylcholine, dioleoyl phosphatidylcholine) has been observed in this report. Sonicated distearoyl phosphatidylcholine (C18 : 0) liposomes, which have the same fatty acid chain length as dioleoyl phosphatidylcholine (C18 : 1) liposomes but which are below their phase transition at 37°C, show much diminished exchange rates, as well as increased retention of contents in the presence of serum. It would seem that the tighter packed, gel phase lipid is even more resistant to insertion of apolipoproteins than is liquid-crystalline phospholipid with 33 mol% cholesterol. In the presence of buffer, however, distearoyl phosphatidylcholine liposomes at 37°C are more leaky than liquid-crystalline liposomes such as sonicated egg phosphatidylcholine liposomes. This could be attributed to the leakage of calcein through grain boundaries in the tightly packed, gel phase liquid [15] but apparently these grain boundaries do not permit insertion of apolipoprotein to any extent, as the leakage of calcein from distearoyl phosphatidylcholine liposomes at

37°C in serum is not significantly different from the leakage in buffer. These liposomes were made at 60°C, above their phase transition, and, following sonication, were brought to room temperature and allowed to sit overnight (in order to anneal the liposomes) prior to separation of free calcein [16].

The results seen with preparations containing sphingomyelin are more difficult to interpret due to the complex phase transition behavior of this phospholipid. Bovine brain sphingomyelin preparations had heat capacity maxima at 30 and 37°C for sphingomyelin from Avanti and 32 and 36°C for sphingomyelin from Sigma in our hands (not shown). These values are similar to those reported by others for bovine brain sphingomyelin [17,18]. The effect of increasing the mole fraction of egg phosphatidylcholine is initially to eliminate the high temperature peak and then to shift the resulting single endotherm to lower temperatures. This behavior is similar to that described by Untracht and Shipley [19]. These authors report that at 37°C in excess water sphingomyelin may be incorporated into the egg phosphatidylcholine bilayer up to a maximum of 90 mol%, after which lipid phase separation of gel phase sphingomyelin occurs. At an egg phosphatidylcholine : sphingomyelin ratio of 1 : 4 the two phospholipids are totally miscible and in the liquid-crystalline phase [19]. This is the composition where we see maximum stability of liposomes to the action of serum, and a low rate of exchange of sphingomyelin with HDL. This is a different situation than seen for the phosphatidylcholines, which showed high serum-induced leakage and rapid phospholipid exchange/transfer when in the liquid-crystalline state. Obviously some other explanation than phase-transition behavior is needed, although this could account for the behavior of pure sphingomyelin (see below). The increased stability of phosphatidylcholine/sphingomyelin (1 : 4) could lie in the presence of inter- and intramolecular hydrogen bonds formed between sphingomyelin molecules. Considerable evidence for the formation of these bonds has been advanced by Schmidt et al. [20], who note that hydrogen bonding (and other effects) tend(s) to make sphingomyelin-containing bilayers more rigid. The effect of inter- and intramolecular hydrogen bond formation in bilayers containing a high mol% of sphingomyelin seems to be to prevent insertion of apolipoproteins, leading to the increased stability of these liposomes. Small amounts of phosphatidylcholine (i.e. 20 mol%), the glycerol backbone of which does not allow it to participate in hydrogen bonding to the same extent as sphingomyelin, is relatively easily exchange (Table III), as would be expected on the basis of this hypothesis. Increasing amounts of egg phosphatidylcholine in the liposomes decreases the extent to which sphingomyelin can participate in intermolecular bonding with a resulting increase in membrane fluidity and increased destabilization of the membrane in the presence of serum (Table II).

In the case of pure sphingomyelin, the situation is complicated by the presence of an order-disorder transition at 37°C [18,21]. Pure sphingomyelin is more sensitive to the action of serum HDL than sphingomyelin with 20 mol% egg phosphatidylcholine. A likely explanation for these observations, therefore, is the greater ease of insertion of apolipoprotein into the disordered phase of pure sphingomyelin at 37°C. The ability of pure sphingomyelin to participate in intermolecular hydrogen bonding at its phase transition

has not been explored, but presumably pure sphingomyelin would be very resistant to the action of serum proteins at temperatures above or below its phase transition. Increased phospholipid exchange with HDL was seen for pure sphingomyelin at 37°C, as was increased leakage in the presence of serum.

The behavior of egg phosphatidylcholine/sphingomyelin (1 : 4) in the presence of 33 mol% cholesterol is puzzling. Below this ratio cholesterol shows a protective effect in the presence of serum or buffer, as would be expected from its ability to tighten bilayers above the phase transition. However, for liposomes at egg phosphatidylcholine : sphingomyelin molar ratios of 1 : 4, chlesterol has a disordering effect and increases leakage in the presence of both buffer and serum, as well as increasing the rate of phospholipid exchange with HDL. Perhaps the explanation lies in the ability of cholesterol to interfere with intermolecular hydrogen bonding at high sphingomyelin molar ratios, leading to a fluidization of the membrane. At high egg phosphatidylcholine : sphingomyelin molar ratios, where phosphatidylcholine may already be interfering with the ability of sphingomyelin to hydrogen-bond, then cholesterol seems to have a bilayer tightening effect.

In summary, it is possible, by manipulating phospholipid fatty acid side chain, head group or cholesterol content, to confer increased stability on liposomes in the presence of blood, serum, or high density lipoproteins, and increase considerably their ability to retain solutes in vivo. The mechanism of serum-induced dissolution of liposomes appears to be related to the ability of apolipoproteins from high density lipoproteins to associate with lipid bilayers and transfer phospholipid to, or exchange phospholipid with, HDL. The insertion of apolipoproteins into bilayers is more easily accomplished at lipid phase transitions, at phase boundaries and in liquid-crystalline lipid. Those factors which tighten the bilayer, such as increasing cholesterol content (except in the case of sphingomyelin — see above), using gel phase phospholipid or using phospholipid which can participate in inter- and intramolecular hydrogen bonding (such as sphingosine-containing phospholipids), increase the stability of liposomes in the presence of serum and will, therefore, improve our ability to control leakage rates for optimal therapeutic use of liposome-entrapped drugs.

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