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MODIFICATION OF PHOSPHOLIPID STRUCTURE RESULTS IN GREATER STABILITY OF LIPOSOMES IN SERUM *

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Prevous studies have revealed that the replacement of the C-2 ester group in phosphatidylcholine by the carbamyloxy function renders the resulting lipids, without affecting the properties of the liposomes, resistant to hydrolysis by phospholipase A_2 (Gupta, C.M. and Bali, A. (1981) Biochim. Biophys. Acta 663, 506–515). As an extension of this work, the effect of serum on the stability of liposomes, prepared from 1-palmitoyl-2-heptadec-10-*cis*-enylcarbamyloxyphosphatidylcholine (carbamylphosphatidylcholine), has been examined. The stability has been measured in terms of (a) bilayer permeability to solutes, and (b) the lipid transfer to serum proteins. Replacement of egg phosphatidylcholine in liposomes by the carbamyl analog prevented serum-induced leakage of the entrapped solutes and also inhibited the lipid (phospholipid and cholesterol) transfer. Manipulation of the cholesterol content of the liposomes had no effect on the stability. These observations indicate that the interaction of serum proteins with liposomes probably involves a highly specific binding of the proteins to the liposome surface.

Introduction

The major uptake of liposomes in vivo by liver and spleen [1] and also their enhanced rate of leakage of the entrapped solutes in plasma [2–5] restrict their successful application as drug carriers in therapy [6–8]. It has been reported [9–11] that the high-density fraction of serum lipoproteins (HDL) interacts with liposomes, resulting in transfer of phospholipid from liposomes to HDL. The latter process causes damage to the liposomal membrane, leading to an almost spontaneous leakage of the entrapped material. This enhanced leakage and the phospholipid transfer are both minimized significantly either by increasing the amount of cholesterol in liposome [12, 13] or by an appropriate choice of the phospholipid component [14–18].

Thus, it is quite evident that an appropriate tailoring of phospholipid as well as liposome structure may help to eliminate some of the major drawbacks associated with the use of liposomes as drug carriers. In this regard, it was thought appropriate to introduce minor structural changes in phosphatidylcholine molecule which would alter the binding ability of the serum constituents to the liposome surface. The preparation and properties of one of such analogs have, recently, been reported [19]. It was found that insertion of one nitrogen atom adjacent to the carbon of the C-2 ester group in phosphatidylcholines renders them, without affecting the properties of their liposomes, completely resistant to phospholipase A_2 attack [19]. The results described in the present report indicate that the replacement of egg phosphatidylcholine by carbamylphosphatidylcholine in liposomes inhibits the lipid (phospholipid and cholesterol) transfer to serum proteins and also prevents the serum-induced leakage of the entrapped solutes.

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Abbreviation: HDL, high-density lipoprotein.

Materials and Methods

All the reagents used in the study were of the highest purity. Cholesterol was obtained from Centron Research Laboratory, Bombay, India. [7(n)-^{3}H]Cholesterol (5 mCi/μmol) and [U-^{14}C]glucose (292 μCi/μmol) were purchased from the Radiochemical Centre, Amersham, U.K. and Bhabha Atomic Research Centre, Bombay, India, respectively. 6-Carboxyfluorescein was from Eastman Kodak Company. Sepharose 6B (40–120 μm beads) was bought from Sisco Research Laboratories, Bombay, India. Sephadex G-50 (20–50 μm beads) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Egg phosphatidylcholine was prepared according to the published procedure [20]. Carbamyl phosphatidylcholine, egg [*methyl*-^{14}C]phosphatidylcholine (35 μCi/μmol), and carbamyl[*methyl*-^{14}C]phosphatidylcholine (15 μCi/μmol) were synthesized as described earlier [19]. All these phospholipids exhibited single spots on silica gel G-60 TLC plates. The plates were developed in chloroform/methanol/water (65 : 25 : 4, v/v), and the spots were identified after staining the plate with iodine vapor followed by molybdenum blue spray [21]. Total phosphorus was determined by the method of Ames and Dubin [22]. The assay of radioactive isotopes was carried out in a Packard Tri-Carb 3330 liquid scintillation spectrometer with 4.0 g 2,5-diphenyloxazole, 0.2 g 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene, 60.0 g naphthalene, 20 ml ethylene glycol and 0.1 l methanol in 1,4-dioxane (total volume, 1.0 l) as the scintillator.

Preparation of liposomes

Small unilamellar liposomes were prepared [19] from 40 μmol of phospholipid, 20 or 40 μmol of cholesterol and [^{14}C]glucose (62 μCi/ml) or 6-carboxyfluorescein (0.2 M) in 2.0 ml Tris-buffered saline (10 mM in 0.9% NaCl, pH 7.2) by sonication for 0.5–1.5 h followed by centrifugation at 105 000 × *g* for 30 min. In the case of carboxyfluorescein-containing liposomes, 0.6 μCi of either ^{14}C-labeled egg phosphatidylcholine or ^{14}C-labeled carbamylphosphatidylcholine and 10 μCi [^{3}H]cholesterol were also included in the lipid mixture. Separation of untrapped glucose (or carboxyfluorescein) from liposomes was carried out on a Sephadex G-50 column (20 × 1.5 cm). The elution was performed with the Tris buffer. The size of the liposomes was determined by electron microscopy [19]. The outer diameter was 25–60 nm. The leakage rate of glucose from carbamylphosphatidylcholine/cholesterol liposomes was similar to that of egg phosphatidylcholine/cholesterol liposomes [19]. Soon after the gel filtration, the liposome-enriched fractions were pooled together and used for further experiments.

Serum-induced leakage

Fresh monkey serum (1.0 ml) obtained from healthy Rhesus monkeys was incubated with 0.5 ml glucose- (or carboxyfluorescein) containing liposomes (3–4 μmol lipid phosphorus). The same amount of liposomes was also incubated with 1.0 ml buffer. Incubations were done at 37°C for 3 and 6 h. In case of glucose-entrapped liposomes, the amount of glucose released in the medium, during incubation with serum (or buffer), was separated from the liposomes by gel filtration of the incubated mixture (0.5 ml) on a Sephadex G-50 column, whereas in the case of carboxyfluorescein-containing liposomes, the gel filtration was done on a Sepharose 6B column (35 × 1.4 cm). Percent glucose release during 3 or 6 h incubation was determined by measuring the amounts of trapped and free glucose [19]. The amount of carboxyfluorescein in different fractions was measured in the presence of Triton X-100 (1% final conc.) on an Aminco SPF-500 fluorimeter using excitation and emission wavelengths of 490 and 520 nm, respectively. Percent carboxyfluorescein release was calculated from $100 \times \mathrm{Dye}_f/\mathrm{Dye}_t$, where *f* and *t* denote free and total dye, respectively. The serum-induced leakage is expressed as ratio of % release of glucose or carboxyfluorescein in the presence of serum and in buffer. Free [^{14}C]glucose and carboxyfluorescein were also separately incubated with serum and the mixtures were subjected to gel filtration on a Sephadex G-50 column. Roughly 0.5% of the total ^{14}C or 0.3% of the total fluorescence was eluted with serum proteins.

Lipid transfer to serum proteins

The egg phosphatidylcholine/cholesterol (1 : 1) and carbamylphosphatidylcholine/cholesterol (2 : 1) liposomes (doubly labeled with ^{14}C and ^{3}H) contain-

ing carboxyfluorescein were incubated with monkey serum for 0, 3 and 6 h. Subsequently, 0.5 ml of each mixture was passed through a Sepharose 6B column and 1.0 ml fractions were collected. Each fraction was analysed by measuring absorbance at 280 nm, by double label scintillation counting of ^{14}C and ^{3}H, and also by measuring fluorescence. The percentage of lipid transfer to serum proteins was estimated as the increase in radioactivity associated with the protein fractions at 3 and 6 h as compared to the sample which was chromatographed immediately (0 h). The phospholipid transfer was also measured from carbamylphosphatidylcholine (or carbamylphosphatidylcholine/egg phosphatidylcholine (1 : 1) or egg phosphatidylcholine) liposomes which were free of cholesterol and carboxyfluorescein.

Measurement of phase transitions

Phase transition temperatures of carbamyl analogs of phosphatidylcholine were determined by the use of 8-anilinonaphthalene-1-sulfonate as a fluorescent probe. The procedure used was identical to that described in the literature [23].

Results

Serum-induced leakage

As previously reported [19], the leakage rates of [^{14}C]glucose from liposomes of egg phosphatidylcholine and cholesterol were similar to that of carbamylphosphatidylcholine/cholesterol liposomes. Therefore, it was thought to be the most appropriate compound for the present study. The liposomes prepared from equimolar amounts of egg phosphatidylcholine and cholesterol, in the presence of [^{14}C]-glucose or carboxyfluorescein, were used as controls. This particular molar ratio of the two lipids was selected because of the enhanced serum-stability of the liposomes [12] as compared to liposomes containing other molar ratios [4,12,13]. In order to find out the effect, if any, of the nature of solutes on the serum-induced leakage, [^{14}C]glucose and carboxyfluorescein have been used as the neutral and charged solutes, respectively.

Leakage of the solutes from the liposomes has been measured in the presence of serum or buffer at 37°C and the serum-induced leakage is expressed as the ratio of % glucose (or carboxyfluorescein) release in the presence of serum and in buffer. The results are shown in Tables I and II.

TABLE I

SERUM-INDUCED LEAKAGE OF [^{14}C]GLUCOSE FROM LIPOSOMES

Each value is a mean of three determinations ± S.D. PC, egg phosphatidylcholine; MPC, carbamylphosphatidylcholine; Chol, cholesterol; PL, phospholipid; P_s, % glucose release in the presence of serum; P_b, % glucose release in buffer.

Liposomes	Molar ratio PL : Chol	Incubation time (h)	P_s/P_b
PC/Chol	1	3	1.90 ± 0.10
		6	1.57 ± 0.15
MPC/Chol	1	3	0.97 ± 0.05
		6	1.00 ± 0.02
MPC/Chol	2	3	1.01 ± 0.07
		6	1.00 ± 0.03

The data given in Table I indicate that leakage of glucose from carbamyl phosphatidylcholine/cholesterol liposomes was not affected by the presence of serum in the incubation mixture. On the contrary, the release of this solute from liposomes prepared from the best known combination of egg phosphatidylcholine and cholesterol [12] was nearly 2-fold enhanced in serum as compared to buffer. Moreover, reducing the cholesterol content of carbamylphosphatidylcholine liposomes to half that of the egg

TABLE II

SERUM-INDUCED LEAKAGE OF 6-CARBOXYFLUORESCEIN FROM LIPOSOMES

Each value is a mean of three determinations ± S.D. PC, egg phosphatidylcholine; MPC, carbamylphosphatidylcholine; Chol, cholesterol; PL, phospholipid; P_s, % carboxyfluorescein release in the presence of serum; P_b, % carboxyfluorescein release in buffer.

Liposomes	Molar ratio PL : Chol	Incubation time (h)	P_s/P_b
PC/Chol	1	3	3.37 ± 0.25
		6	1.98 ± 0.12
MPC/Chol	2	3	1.03 ± 0.04
		6	1.00 ± 0.03

phosphatidylcholine liposomes had no effect on the leakage rate.

The results obtained for the serum-induced leakage of carboxyfluorescein from liposomes are shown in Table II. It may be noted that the leakage of this solute from liposomes consisting of carbamylphosphatidylcholine and cholesterol (50 mol%) remained unaffected by the presence of serum, whereas in case of liposomes prepared from the equimolar mixture of egg phosphatidylcholine and cholesterol, the release of carboxyfluorescein was 2–3-fold greater in serum as compared to buffer.

Lipid transfer to serum proteins

Carboxyfluorescein-containing liposomes, prepared in the presence of trace amounts of ^{14}C-labeled phospholipid and [^{3}H]cholesterol, were incubated with serum at 37°C for 3 and 6 h. After each incubation, a portion of the mixture was chromatographed over a Sepharose 6B column and the fractions were analysed for protein, lipids and carboxyfluorescein as described in Materials and Methods. The percentage of lipid transfer to serum proteins was estimated as the increase in radioactivity associated with protein peaks from the column at 3 and 6 h as compared to the sample which was chromatographed immediately (0 h). The lipid transfer was calculated from areas under the respective peaks. The areas under the peaks were expressed as % of total eluted counts (or protein). A typical elution pattern of the 0, 3 and 6 h incubation mixtures for carbamylphosphatidylcholine/cholesterol (2 : 1) liposomes (broken line) and egg phosphatidylcholine/cholesterol (1 : 1) liposomes (solid line) is shown in Fig. 1 (A–C). The distribution of ^{3}H is not shown in the figure. When liposomes were incubated in buffer (in the absence of serum) and a portion of the mixture was then passed through the column, all the ^{14}C and ^{3}H were recovered in fractions 16–20. Free carboxyfluorescein was recovered in fractions 37–48. In one experiment, serum alone was chromatographed as above and the fractions were analysed for proteins (Fig. 1D). Recoveries of lipids and protein from the column were at least 90%. A summary of the data for transfer of lipids to serum proteins is given in Table III. The values shown are derived from three independent experiments.

Fig. 1 (A–C, solid line) shows that the amount of

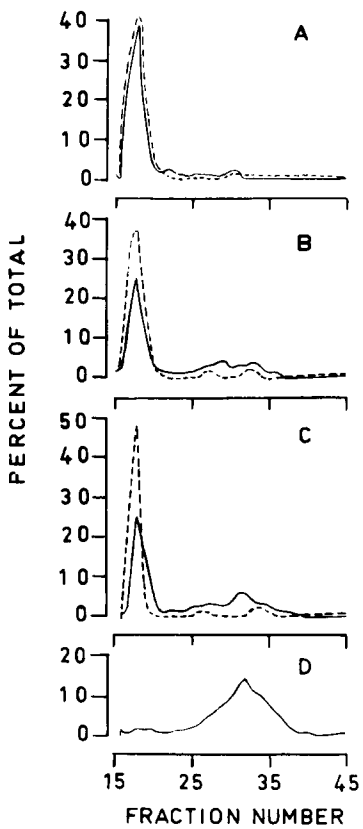

Fig. 1. The egg phosphatidylcholine/cholesterol (1 : 1) and carbamylphosphatidylcholine/cholesterol (2 : 1) liposomes (doubly labeled with ^{14}C and ^{3}H, see text) containing carboxyfluorescein were incubated at 37°C with monkey serum for 0 (A), 3 (B) and 6 h (C). Subsequently, a portion of each mixture was passed through a Sepharose 6B column (35 × 1.4 cm) and fractions were collected. Each fraction was analysed for lipids, proteins and carboxyfluorescein. In one experiment, serum alone was chromatographed as above and fractions were analysed for protein (D). Solid line, egg [^{14}C]-phosphatidylcholine; broken line, carbamyl[^{14}C]phosphatidylcholine.

^{14}C eluting in the protein-enriched fractions increased consistently with an increase in the time of incubation of egg phosphatidylcholine/cholesterol (1 : 1) liposomes with serum. The amount of the phospholipid transfer from these liposomes to serum proteins was 32–38% in 6 h. On the other hand, no such increase with an increase in the incubation time was observed in the case of carbamylphosphatidylcholine/cholesterol (2 : 1) liposomes (Fig. 1, A–C, broken line). Transfer of the phospholipid to protein in this case was 3–5% in 6 h. As may be seen in Table III,

TABLE III

PERCENT TRANSFER OF LIPIDS FROM LIPOSOMES TO SERUM PROTEINS IN 6 h AT 37°C

The percentage was estimated from the amount of radioactivity transferred from liposomes to serum proteins following incubation at 37°C for 6 h. The values of lipid transfer shown here are obtained after subtracting the amounts of the radioactivity associated with protein fractions at 0 h and are expressed as a range from three experiments. PC, egg phosphatidylcholine; MPC, carbamylphosphatidylcholine; Chol, cholesterol; PL, phospholipid.

Liposomes	Molar ratio PL : Chol	Lipid transfer (%)	
		PL	Chol
PC/Chol	1	32–38	55–63
MPC/chol	2	3– 5	12–15
MPC	–	4– 8	–
PC/MPC (1 : 1)	–	72–76	–
PC	–	77–81	–

the phospholipid transfer from liposomes prepared from carbamylphosphatidylcholine alone (free of cholesterol) is not significantly different (4–8%) from that of carbamylphosphatidylcholine/cholesterol (2 : 1) liposomes.

A consistent increase, with an increase in the incubation time, in the amount of cholesterol transferred from both types of liposome to serum proteins was observed. In 0 h, 5–8% of the total cholesterol was found to be associated with protein-enriched fractions. The transfer of this lipid from egg phosphatidylcholine/cholesterol (1 : 1) liposomes was 55–63% in 6 h (Table III), whereas this amount was only 12–15% in the case of carbamylphosphatidylcholine/cholesterol (2 : 1) liposomes.

To ascertain whether the phospholipids transferred from liposomes to proteins become associated mainly with lipoproteins or with albumin (or with some other serum proteins) enriched fractions, pure HDL and albumin from monkey serum were separately chromatographed on the column. The HDL was recovered in fractions 28–34, whereas albumin was eluted in fractions 30–38. As may be seen in Fig. 1C), the maximum amount of protein-associated phospholipids appeared to elute in fractions which match the elution profile of HDL. This is quite in agreement with the earlier findings [9–13].

Discussion

A number of reports describe that the transfer of lipids from liposomes to HDL is a major factor responsible for the disruptive effect of serum on the integrity of liposome bilayer [9–13,18]. The process of this lipid transfer seems to be mediated by apolipoproteins [11,24–26].

The ability of cholesterol to tighten the packing of phospholipids in the bilayer [27] leads to the greater stability of liposomes in blood or serum [3,4,12,13, 25]. It has been speculated that the increased phospholipid packing resists the insertion of apolipoprotein in the bilayer matrix and, therefore, reduces the lytic effect of serum on liposomes [4,28]. The reported poor transfer of saturated phosphatidylcholines, having thermal melting transition temperature (T_m) greater than 37°C, from the liposomes to HDL [14–18] provides confirmatory evidence to the above postulate. However, the poor transfer observed in the present study is not due to the tighter phospholipid packing in liposomes because carbamyl phosphatidylcholine undergoes phase transition at a temperature (under 0°C) which is much below 37°C.

The leakage of entrapped solutes from sphingomyelin/cholesterol liposomes remains unaffected by the presence of blood or serum [15–18]. These observations are in accordance with the recent finding that the phospholipid transfer from these liposomes to HDL is almost negligible [18]. The failure of sphingomyelin transfer to HDL is attributed to intermolecular hydrogen bonding between the sphingosine backbones of the sphingomyelin molecule [18].

Although a possibility of intermolecular hydrogen bonding between NH and C = 0 of the C-1 ester exists in carbamylphosphatidylcholine bilayers, this was considered as a least likely reason for the lack of lipid transfer. If such bonding exists in these bilayers, this should result in tighter phospholipid packing and, therefore, in reduced leakage as compared to egg phosphatidylcholine bilayers. Since the permeability behavior of the two is similar [19], the possibility of the intermolecular hydrogen bonding is precluded.

From the above discussion, it becomes obvious that the situation in carbamylphosphatidylcholine seems different from that of phosphatidylcholines and sphingomyelins. As the basic structural difference between phosphatidylcholines and the carbamyl

analog lies in the C-2 ester region, it is not unreasonable to conclude that this part of the phospholipid molecule probably plays some important role in the process of binding of lipoproteins to liposome. This region in phosphatidylcholines is known to form a part of the liposome surface [29–31] and thus it is likely that lipoproteins first interact with the surface of liposome prior to their insertion in the hydrophobic domain of the bilayer. This means that even a partial modification of the phospholipid structure exposed on the interface of the bilayer would prevent the binding of protein to liposome surface and, therefore, the lipid transfer.

To examine the effect of carbamylphosphatidylcholine on the egg phosphatidylcholine transfer from liposomes, carbamylphosphatidylcholine/egg phosphatidylcholine (1 : 1) liposomes were incubated with serum and the transfer of egg phosphatidylcholine to serum proteins was measured. The results given in Table III indicate that the presence of carbamylphosphatidylcholine in liposomes had no significant effect on the egg phosphatidylcholine transfer. This observation is explained by considering the possibility of transfer of one phospholipid molecule in each collision of lipoprotein and liposome. This would mean that the surface-exposed structure of the phospholipid molecule will first undergo specific binding with the pre-existing complementary sites on the lipoprotein followed by penetration of the hydrophobic tail of the protein into bilayer, leading to sequestration of phospholipid molecule from liposome. This mechanism may readily explain the failure of carbamylphosphatidylcholine to inhibit the transfer of egg phosphatidylcholine from liposomes. Since most of the structure of cholesterol is buried within the bilayer [27], the surface structure of carbamylphosphatidylcholine/cholesterol liposomes should be similar to that of carbamylphosphatidylcholine liposomes. This should explain the reduced amount of cholesterol transfer from carbamylphosphatidylcholine/cholesterol liposomes.

Scherphof et al. [25] have explored the involvement of the lipid phase transition in serum-induced dissolution of multilamellar liposomes. The maximum dissolution was observed at or close to the transition temperature. The values obtained for the phase transition temperatures of 1-palmitoyl-2-pentadecanylcarbamyloxyphosphatidylcholine and 1-palmitoyl-2-heptadecanylcarbamyloxyphosphatidylcholine are approximately 40 and 52°C, respectively. These values are quite close to the transition temperatures of dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine [32], indicating that insertion of one nitrogen atom adjacent to the carbon of the C-2 ester group in the latter phospholipids does not perturb the phospholipid packing in the bilayer. Although we could not accurately determine the transition temperature for carbamylphosphatidylcholine, this should be closer to the value obtained for 1-palmitoyl-2-oleoylphosphatidylcholine [33]. Thus, the value, under 0°C, determined for carbamylphosphatidylcholine is compatible with the expected value (−5°C). As there is only a small difference in the melting transition temperatures of 1-palmitoyl-2-oleoylphosphatidylcholine and egg phosphatidylcholine [32], it is expected that carbamylphosphatidylcholine and egg phosphatidylcholine may not undergo phase separation in their mixed bilayers. The possibility of selective removal of egg phosphatidylcholine from egg phosphatidylcholine/carbamylphosphatidylcholine bilayers due to phase separation is, therefore, excluded [25].

Finally, it may be argued that the reduced amount of transfer of cholesterol from carbamylphosphatidylcholine/cholesterol liposomes could be due to the stronger interactions between this phospholipid and cholesterol. The stronger interactions between phospholipids and cholesterol are known to result in the tighter packing and, therefore, the reduced permeability of bilayer to solutes [27]. Since the effect of cholesterol on the leakage of glucose from the carbamylphosphatidylcholine liposomes is comparable to that from egg phosphatidylcholine liposomes [19], the possibility of stronger interactions between cholesterol and carbamylphosphatidylcholine is thus precluded.

In order to investigate further the specificity in the interaction of liposomes with serum proteins, similar studies with phosphatidylcholine analog in which the phosphate and the quarternary ammonium groups are separated by three methylene groups are in progress in this laboratory.

Conclusion

A complete replacement of egg phosphatidylcholine in liposomes by carbamylphosphatidylcholine

prevents the serum-induced leakage of the entrapped solutes and also inhibits phospholipid as well as cholesterol transfer to serum proteins. This finding has been explained in terms of lack of interaction of serum lipoproteins with the liposome surface. On the basis of this study, it is proposed that only one phospholipid molecule is transferred in each collision of lipoprotein and liposome. Moreover, it is also speculated that the interacting lipoprotein may have pre-existing complementary binding sites for the surface-exposed portion of the phospholipid to be transferred.

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