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Serum-Induced Leakage of Negatively Charged Liposomes at Nanomolar Lipid Concentrations†

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ABSTRACT: An enzyme inhibition assay was developed to determine methotrexate-γ-aspartate leakage from liposomes at lipid concentrations as low as 43 nM phospholipid. When negatively charged liposomes prepared with phosphatidylglycerol/cholesterol 67:33 or phosphatidylinositol/cholesterol 67:33 were incubated in 10% (v/v) newborn calf serum, they leaked over 90% of their contents in 2 min. In contrast, liposomes prepared from phosphatidylcholine/cholesterol 67:33 leaked 18% of their contents under the same conditions. The amount of negative charge required to induce liposome leakage was determined by preparing liposomes with varying amounts of phosphatidylglycerol and phosphatidylcholine. Extensive leakage was observed only from liposomes prepared with greater than 50 mol of phosphatidylglycerol per 100 mol of phospholipid. The effect of the phase transition temperature on leakage of negatively charged liposomes in 10% (v/v) serum was investigated by using a series of phosphatidylglycerols with varying acyl chain lengths. Liposomes prepared from distearoylphosphatidylglycerol or dipalmitoylphosphatidylglycerol leaked less than 18% of their contents in 10% serum, whereas liposomes prepared with dilauroylphosphatidylglycerol or unsaturated lipids leaked more than 70% of their contents. Lipoprotein removal from serum followed by treatment with lipid to remove residual apoproteins reduced the leakage from phosphatidylglycerol liposomes in 10% serum. Phosphatidylglycerol liposomes leaked 73% in the presence of human low-density lipoproteins, but only 29% in the presence of bovine apolipoprotein A-I, and 25% in the presence of human high-density lipoproteins. Phosphatidylglycerol/cholesterol and phosphatidylserine/cholesterol liposomes leaked 67% in 4 mg/mL bovine serum albumin purified by cold ethanol extraction. The leakage of liposomes in albumin solutions could be substantially reduced by treating the albumin with lipid. The pronounced effect of ionic strength on leakage of negatively charged liposomes in albumin solutions indicated that apolipoprotein-induced leakage of liposomes is mediated in part by electrostatic interactions.

Liposomes have been proposed as carriers for the delivery of molecules to cells (Gregoriadis & Ryman, 1972). They have proved particularly effective for promoting intracellular delivery of molecules that do not penetrate cells readily (Heath et al., 1985a). Studies of the delivery of such molecules have demonstrated that adsorptive endocytosis is the predominant mechanism of delivery, as evidenced by the inhibition of delivery by lysosomotropic agents (Heath et al., 1985b). This mechanism of delivery requires that liposomes retain their contents until endocytosis occurs. Consequently, the leakage of liposomes in serum would be expected to reduce drug delivery, and should be minimized in order to optimize liposome-mediated drug delivery.

In order to gain useful information from leakage studies, it is important to design experiments carefully so that the conditions employed for liposome leakage experiments match the conditions used during drug delivery experiments. Previously we have shown that fluoroorotic acid is a more effective inhibitor of cell division and contraction when it is encapsulated in dipalmitoylphosphatidylglycerol (DPPG)[1] liposomes than it is when encapsulated in EPG liposomes, presumably because DPPG liposomes are more stable than EPG liposomes (Heath et al., 1987). However, leakage experiments using dialysis techniques indicated that DPPG liposomes were not more stable than EPG liposomes.

The most prominent difference in the experimental conditions between liposomes leakage determinations and liposome drug delivery is the ratio of serum protein to liposomal lipids. Liposome drug delivery in vitro often uses lipid concentrations of 60–100 nM in 10% serum (Heath et al., 1985b). Lipid concentrations in leakage experiments are often micromolar to millimolar in serum or plasma (Allen & Cleland, 1980; Guo et al., 1980; Kirby et al., 1980; Heath et al., 1987; Mayhew et al., 1979; Senior et al., 1985). Therefore, to determine the effects of lipid charge and phase transition temperature on

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[1] Abbreviations: DLPG, dilauroylphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DPtPG, dipentadecanoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PG, phosphatidylglycerol; PI, soybean phosphatidylinositol; PS, bovine brain phosphatidylserine; SUV, small unilamellar vesicle(s); HDL, high-density lipoprotein(s); LDL, low-density lipoprotein(s); apo A-I, apolipoprotein A-I.

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liposome-mediated drug delivery in vitro, it is necessary to determine liposome leakage under the conditions encountered in drug delivery experiments.

In this study, we have developed an assay to determine methotrexate–γ-aspartate leakage from negatively charged liposomes diluted to as low as 43 nM phospholipid. The effects of negative charge and lipid phase transition temperature on liposome stability in the presence of serum proteins are described. Procedures to treat serum to increase the stability of dilute liposome suspensions while providing conditions suitable to study liposome-mediated drug delivery are reported.

Materials and Methods

Lipids and Other Materials. Phospholipids (Avanti, Pelham, AL) were stored in chloroform solution under argon gas in sealed ampules at −20 °C. Cholesterol (Sigma, St. Louis, MO) was recrystallized from methanol 4 times and stored under the same conditions as the phospholipids. Methotrexate–γ-aspartate was synthesized and provided by J. R. Piper, Southern Research Institite, Birmingham, AL (Piper et al., 1982). Affinity-isolated dihydrofolate reductase from bovine liver as a suspension in ammonium sulfate was obtained from Sigma (St. Louis, MO) and used without further purification. Dihydrofolic acid, reduced nicotinamide adenine dinucleotide phosphate, fraction V bovine serum albumin, salt-fractionated bovine serum albumin, human HDL, human LDL, and bovine apo A-I were supplied by Sigma (St. Lous, MO). Newborn calf serum (Gibco, Long Island, NY) was stored frozen at −20 °C until used.

Liposome Preparation. Large unilamellar liposomes were prepared under sterile conditions by reverse-phase evaporation (Szoka & Papahadjopoulos, 1978). The phospholipid:cholesterol ratio for all drug-containing liposomes was 2:1, and all preparations will be referred to subsequently by the phospholipid content alone. Methotrexate–γ-aspartate solutions were prepared for encapsulation at drug concentrations from 12.8 to 18.4 mM in 50 mM morpholinoethanesulfonic acid/50 mM morpholinopropanesulfonic acid and adjusted to pH 7.2 with sodium hydroxide. The tonicity of the drug solutions was measured with an osmometer (Wescor, Logan, UT) and adjusted to 290 mmol/kg with NaCl. Liposomes were separated from unencapsulated drug by gel chromatography on a 1 × 50 cm sterile Sephadex G-50 (Pharmacia) column. The column was eluted with sterile 50 mM morpholinoethanesulfonic acid/50 mM morpholinopropanesulfonic acid/1 mM EDTA/NaCl, pH 7.3, at the same tonicity as the encapsulated drug solution. Drug concentrations in the liposomes were determined by solubilizing a portion of the liposome suspension in 1:3:1 chloroform/methanol/water and measuring the absorbance at 370 nm using a molar extinction coefficient of 7943. Lipid content was measured by phosphorus analysis (Bartlett, 1959). Sonicated liposomes for serum or albumin treatment were prepared without cholesterol under sterile conditions by drying lipid onto a 13-mm test tube under argon, followed by adding 10 mM phosphate-buffered saline, pH 7.4, 290 mmol/kg, and the mixture was sonicated for 1 h at 23 °C in a bath-type sonicator (Laboratory Supply Co., Hicksville, NY).

Lipoprotein Removal from Serum. Solid NaBr was added to serum containing 0.04% EDTA, 0.01% Thimerosal, and 0.05% NaN_3 to give a solution density of 1.25 g/mL at 23 °C. The serum solutions were centrifuged in 5-mL centrifuge tubes at 53000 rpm in Beckman SW 55 Ti rotor for 24 h at 26 °C. The top 1.5 mL of serum from each centrifuge tupe was aspirated and discarded. The bottom 3.5 mL of serum from each centrifuge tube was collected with a pasteur pipe and pooled.

Lipid Treatment of Serum or Albumin. Newborn calf serum, from which lipoprotein had been eliminated by centrifugation, was incubated with sonicated EPC liposomes (153 g of protein/g of lipid) for 21 h at 37 °C in order to remove residual apolipoproteins. Alternatively, lipoprotein-depleted newborn calf serum was incubated with sonicated DMPC liposomes (4.75 g of protein/g of lipid) for 21 h at 23 °C. To remove the liposomes from the serum, the density of the serum mixtures was adjusted to 1.25 g/mL with solid NaBr, and the samples were centrifuged at 53000 rpm for 12 at 26 °C. The bottom 3.5 mL was recovered from the centrifuge tubes and dialyzed against Dulbecco's phosphate-buffered saline containing 0.1 mM EDTA, 0.01% Thimerosal, and 0.05% NaN_3. The protein content of the serum samples was determined by the method of Lowry using bovine serum albumin as standard. Serum samples were filter-sterilized (Nalge, Rochester, NY) and refrigerated until use.

Cold ethanol-extracted bovine serum albumin was incubated with large unilamellar EPC liposomes, without cholesterol (50 g of protein/g of lipid), or with sonicated EPC liposomes (80 g of protein/g of lipid) for 21 h at 37 °C. To remove the liposomes, the density of the albumin samples was adjusted to 1.25 mg/mL with solid NaBr, centrifuged at 53000 rpm for 12 h at 26 °C, and dialyzed against phosphate-buffered saline as above.

Enzyme Inhibition Assay for Methotrexate–γ-Aspartate. Methotrexate–γ-aspartate is a potent competitive inhibitor of dihydrofolate reductase (Piper et al., 1982) and was measured by its ability to inhibit the dihydrofolate-dependent oxidation of NADPH catalyzed by dihydrofolate reductase. Oxidation of NADPH was monitored through the reduction of absorbance at 340 nm. Methotrexate–γ-aspartate that is encapsulated in liposomes is unable to bind to dihydrofolate reductase. Therefore, this assay determines the amount of drug that has leaked from liposomes without separating free drug from liposome-encapsulated drug. All reagents were dissolved in 10 mM phosphate-buffered saline, pH 7.4, 290 mmol/kg, which contained 10% v/v serum or 4 mg/mL albumin, as appropriate. In all cases, samples were determined at the same time as a series of standards containing between 1 and 6 pmol of drug. Effective drug concentration was measured from the plot of absorbance at 340 nm vs concentration of methotrexate–γ-aspartate, which was linear up to 6 nM drug (not shown). Incubation times were controlled by adding reagents to successive tubes in 15-s intervals. Ten microliters of liposome suspension or drug standard containing 1–6 pmol of drug was diluted with 790 μL of buffer at 22 °C and vortexed. One hundred microliters of buffer containing 2 milliunits of dihydrofolate reductase and 150 nmol of NADPH was added to the liposome suspension and vortexed. The mixture was allowed to incubate for 2 min in order to allow the drug, which is a slow, tight-binding inhibitor, to bind to the enzyme. One hundred microliters of buffer containing 150 nmol of dihydrofolic acid was added to the liposome suspension, and the mixture was vortexed and placed in a 37 °C water bath for 8 min. The reaction was stopped by adding 100 μL of 0.1 mM methotrexate (this reagent did not contain proteins). The absorbance of the samples was measured at 340 nm in a Beckman DU-64 spectrophotometer. Validation studies, in which the change of absorbance with time was followed continuously, established that the rate of oxidation of NADPH was constant (zero order) for the entire 8 min of the assay.

Results

Liposome Leakage in 10% Serum. Table I shows the effect of 10% newborn calf serum, the concentration of serum nor-

Table I: Leakage of Methotrexate–γ-Aspartate from Liposomes in the Presence of Serum Proteins

liposome[a]	% leakage mean	s^b	n^c	[lipid] (nM)
(1) 10% Newborn Calf Serum				
EPG	94	13	29	53
DOPG	70	2	4	nd[d]
DLPG	84	4	3	230
DMPG	53	3	4	163
DPtPG	35	1	4	198
DPPG	17	2	7	234
DSPG	17	2	7	234
PI	90	11	17	71
PS	78	9	24	43
EPC	18	4	17	85
(2) 4 mg/mL Bovine Serum Albumin				
EPG	67	5	16	53
PI	47	6	3	71
PS	57	3	4	43
DPPG	3	0	4	234

[a] Liposomes were prepared from phospholipid/cholesterol 67:33. Liposomes were diluted in 10 mM phosphate-buffered saline, pH 7.2, containing 10% (v/v) serum or 4 mg/mL albumin for 2 min prior to leakage measurements. [b] Standard deviation of leakage measurements. [c] Number of determinations. [d] Not determined.

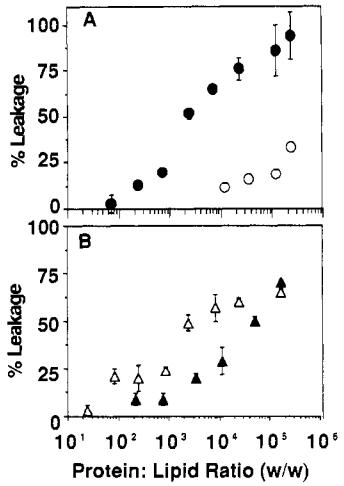

FIGURE 1: Effect of the protein to lipid ratio on liposome leakage. EPG/cholesterol (2:1) liposomes containing methotrexate–γ-aspartate were diluted to 53 nM lipid in 10 mM phosphate-buffered saline, pH 7.2, containing serum proteins. (Panel A) The protein to lipid ratio was achieved by varying the concentration of serum; (solid circles) leakage from EPG liposomes in buffer containing untreated serum; (open circles) EPG leakage in buffer containing serum which was delipidated and treated with DMPC SUV (4.8 g of protein/g of lipid). (Panel B) Buffer contained albumin; (open triangles) EPG leakage in buffer with 4 mg/mL albumin and varying amounts of empty EPG/cholesterol (2:1) liposomes; (solid triangles) EPG leakage in buffer with varying concentrations of albumin.

mally used for cell culture, on liposome leakage. Several types of negatively charged liposomes diluted to about 60 nM in 10% serum leak their contents rapidly and extensively. Leakage is most extensive from EPG liposomes (94%) and from PI liposomes (90%). In contrast, neutral liposomes prepared from EPC leak only 18% of their contents under similar conditions. The inclusion of 2 mM EDTA in 10% serum did not affect the level of leakage observed from EPG liposomes. This demonstrates that serum-mediated leakage is not caused by calcium nor mediated by phospholipases.

The effect of fatty acyl chain composition or leakage from EPG liposomes is shown in Table I. Of the two liposome types that contain unsaturated fatty acyl chains, the greatest leakage was observed from the EPG liposomes. DOPG liposomes

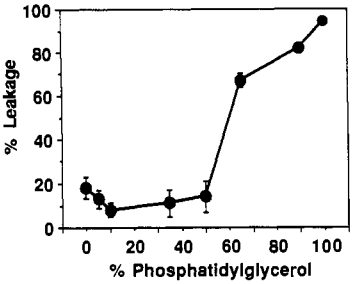

FIGURE 2: Effect of negative charge on liposome leakage in 10% serum. Liposomes containing methotrexate–γ-aspartate were prepared with varying amounts of EPG and EPC (33 mol of cholesterol per 100 mol of phospholipid). Liposomes were diluted in 10 mM phosphate-buffered saline containing 10% (v/v) serum. Lipid concentrations ranged from 53 to 62 nM.

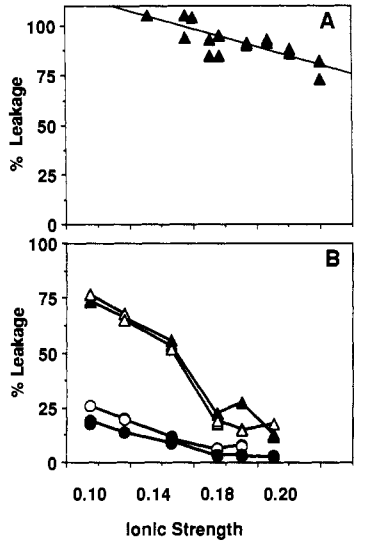

FIGURE 3: Effect of ionic strength on liposome leakage. Phospholipid/cholesterol (67:33) liposomes containing methotrexate–γ-aspartate were diluted in 10 mM phosphate-buffered saline, pH 7.2. The ionic strength was adjusted with NaCl. (Panel A) Buffer contained 10% serum: (solid triangles) leakage from EPG liposomes. (Panel B) Buffer contained 4 mg/mL albumin: (solid triangles) leakage from EPG liposomes; (open triangles) leakage from PS liposomes; (open circles) leakage from EPC liposomes; (solid circles) leakage from DPPG liposomes. Lipid concentrations were the same as in Table II.

leaked less than EPG liposomes despite having a similar lipid transition temperature, and a higher level of unsaturation. Leakage from saturated PG liposomes is proportional to chain length. DLPG liposomes leak almost as much as EPG liposomes. DSPG liposomes are very stable, and leak less than neutral (EPC) liposomes.

The effect of serum concentration on leakage from 53 nM EPG liposomes is seen in Figure 1A. Under these conditions, liposomes are highly sensitive to serum-induced leakage. As little as 2.5×10^2 g of protein/g of lipid will induce significant leakage. This corresponds to a serum concentration of only 0.01%. At 2.5×10^3 g of protein/g of lipid (0.1% serum), 50% leakage of liposome contents occurs. This demonstrates that extensive leakage occurs at all serum concentrations useful for cell culture.

Figure 2 shows the effect of surface charge on serum-induced leakage of liposome contents. EPG has no effect of serum-induced leakage when 50 mol/100 mol of phospholipid or less is present in the liposome bilayer. When 65 mol of EPG/mol of phospholipid or more is present, leakage is extensive (67%) and increases to a maximal value as the mole fraction of EPG is increased to 1. These effects must be caused

Table II: Effect of Treating Serum Proteins with Lipid on Liposome Leakage

treatment	% leakage from EPG[a] mean	s^b	n^c
(1) 10% Newborn Calf Serum			
none	94	13	29
delipidated[d]	71	3	3
delipidated + EPC[e]	44	3	4
delipidated + DMPC[f]	33	1	4
(2) 4 mg/mL Bovine Serum Albumin			
none	67	5	16
EPC[g]	23	9	5
EPC[h]	7	2	4
salt-fractionated[i]	14	1	4

[a] Methotrexate–γ-aspartate encapsulated in EPG/cholesterol (2:1) liposomes was diluted to 53 nM lipid in 10 mM phosphate-buffered saline, pH 7.2, containing serum proteins for 2 min before leakage measurements. [b] Standard deviation. [c] Number of samples. [d] Serum was delipidated by increasing the serum density to 1.25 g/mL with solid NaBr and centrifugation. [e] Delipidated serum was treated with SUV without cholesterol (150 g of protein/g of EPC) for 21 h at 37 °C. [f] Delipidated serum was treated with SUV, without cholesterol (4.8 g of protein/g of DMPC), at 23 °C for 21 h. [g] Large unilamellar vesicles (without cholesterol) were incubated with albumin (50 g of protein/g of EPC) for 21 h at 37 °C. [h] SUV were incubated with albumin (80 g of protein/g of EPC) for 21 h at 37 °C. Lipid used to treat delipidated serum or albumin was removed by flotation from a solution density of 1.25 g/mL created with NaBr before leakage determinations. [i] 4 mg/mL bovine serum albumin initially purified by salt-fractionation.

Table III: Leakage of Methotrexate–γ-Aspartate from Liposomes in the Presence of Lipoproteins and Apolipoprotein A-I

liposome[a]	% leakage HDL[b] mean	s^c	LDL[d] mean	s	apo A-I[e] mean	s
EPG	29	2	73	3	25	8
EPC	13	0	20	1	19	5
DPPG	10	2	16	2		
DOPG			57	1		

[a] Liposomes were prepared from phospholipid/cholesterol 67:33 and were diluted in 10 mM phosphate-buffered saline, pH 7.2, containing serum proteins for 2 min at 23 °C prior to leakage measurements. Lipid concentrations were the same as in Table I. [b] 0.3 mg of HDL protein/mL. [c] Standard deviation. [d] 0.1 mg of LDL protein/mL. [e] 0.05 mg of apo A-I/mL.

by the negative charge of the EPG, because EPG and EPC have identical fatty acyl compositions. Figure 3A shows the effects of ionic strength on serum-mediated leakage from EPG liposomes. Increasing ionic strength decreases the extent of leakage to about 75% at an ionic strength of 0.22. This observation is compatible with the concept that leakage is induced by an electrostatic interaction between serum proteins and negatively charged liposomes.

Table II shows the effects of serum treatments known to remove lipoproteins on serum-mediated liposome leakage. Removal of lipoproteins by flotation from serum, elevated to a density of 1.25 g/mL by NaBr addition, reduces serum-mediated leakage from EPG liposomes to 71%. Delipidated serum may contain as much as 10% of the total apo A-I (Patsch & Gotto, 1987). In order to eliminate apolipoproteins, we incubated the delipidated serum with small unilamellar vesicles prepared from EPC or DMPC and centrifuged the mixture to eliminate liposomes and lipid-bound proteins by flotation. This treatment further reduced leakage to as little as 33%. These results demonstrate that lipoproteins are a major cause of the serum-mediated leakage of negatively charged liposomes. Figure 1A shows the leakage that is induced by lipid-treated serum as a function of the protein:lipid ratio. Comparison of the curve with that obtained for untreated serum suggests that greater than 99% of serum components that induce leakage have been eliminated by this treatment.

Liposome Leakage in the Presence of Lipoproteins. Table III shows the leakage of liposomes induced by purified HDL, LDL, and apoprotein A-I at concentrations comparable to those found in 10% newborn calf serum. EPG liposomes leaked 29% of encapsulated drug in the presence of human HDL, 73% in the presence of human LDL, and 25% in the presence of apo A-I. Therefore, LDL appears to be the largest identified cause of serum-induced EPG liposome leakage. The effects of LDL on leakage from liposomes of different phospholipid composition are similar to the effects of serum on the leakage of such liposomes. Leakage is much lower from neutral liposomes, and from DPPG liposomes. DOPG liposomes show extenisve leakage that is less than is seen from EPG liposomes. Both HDL and apo A-I induce a much lower level of leakage from negatively charged liposomes than LDL. Moreover, the difference between neutral and negatively charged liposomes leakage is much less in the presence of apo A-I compared to LDL.

Liposome Leakage in Albumin Solutions. Recently, a number of investigators have developed chemically defined, serum-free culture media prepared with albumin, transferrin, insulin, linoleic acid, and other factors required for cell growth (Barnes, 1987). Therefore, we wished to investigate whether liposomes are stable in the presence of albumin, so that we might create a defined growth medium that does not induce liposome leakage. Table I shows that the leakage observed with ethanol-extracted albumin is 67% for EPG liposomes, and much lower for neutral, DPPG, and DSPG liposomes. These observations closely parallel those obtained with serum, suggesting that albumin is contaminated with serum proteins that cause leakage.

Albumin-mediated leakage from EPG liposomes is strongly dependent on ionic strength. This is in marked contrast to the effects of ionic strength on serum-mediated leakage from EPG liposomes, wherein only a partial inhibition of leakage occurred when the ionic strength was increased to 0.22. This may indicate that not all of the serum components that cause leakage from negatively charged liposomes are present in albumin. Albumin-mediated leakage from EPC and DPPG liposomes is also dependent on the ionic strength, although the overall amount of leakage is much lower than for EPG liposomes. Figure 1B shows the effect of the albumin:lipid ratio on leakage from EPG liposomes. With constant lipid concentration, EPG liposomes leak 20% of their contents at a protein:lipid ratio of 3.5×10^3 (w/w). This corresponds to an albumin concentration of 0.04 mg/mL, which is 100 times lower than the albumin concentration found in 10% serum. With a constant albumin concentration, 20% leakage from EPG liposomes occurs at a protein:lipid ration of about 210 (w/w), which corresponds to a lipid concentration of 24 μM. Our previous study of fluoroorotic acid leakage from EPG liposomes used a lipid concentration of 1 mM (Heath et al., 1987). Therefore, our current observations demonstrate that the lipid concentration used in earlier work was too high to observe serum-mediated EPG liposome leakage.

Table II shows the effects of lipid treatment of albumin on albumin-induced leakage from EPG liposomes. Treatment of albumin with EPC SUV followed by their removal with centrifugation reduces leakage to only 7%. This is comparable to the amount of leakage that we observed when no proteins were present (results not shown). Endotoxin-free albumin that

is initially purified by salt fractionation has recently become commercially available. EPG liposomes leak only 14% in this albumin, suggesting that this material is only slightly contaminated with the lytic agents found in serum.

Discussion

This study demonstrates that negatively charged liposomes prepared from low phase transition temperature lipids leak their contents extensively at nanomolar lipid concentrations when in the presence of serum or some of its components. This is in marked contrast to previous studies of leakage from similar liposomes at millimolar lipid concentrations, from which leakage was much lower, and not appreciably different from the leakage of liposomes prepared from negatively charged lipids with a high phase transition temperature (Heath et al., 1987).

Leakage from EPG liposomes in 10% serum appears to be mediated in part by lipoproteins. Purified human LDL induced the greatest leakage from EPG liposomes. Treatment of serum to remove lipoproteins and apolipoproteins reduced leakage from EPG liposomes. Similarly, when albumin was treated with empty liposomes in a manner which has been shown to remove contaminating apolipoproteins, the leakage from EPG liposomes was minimal. The rapid leakage of methotrexate–γ-aspartate from EPG liposomes also indicates that the leakage is mediated by lipoproteins. Leakage of carboxyfluorescein from liposomes in the presence of serum was observed to occur within seconds (Guo et al., 1980; Scherphof & Morselt, 1984). With our assay, 94% of encapsulated methotrexate–γ-aspartate leaked from EPG liposomes after 2-min incubation in 10% serum. Using a two-compartment growth inhibition assay, Ng and Heath (1989) recently demonstrated that 95% leakage of methotrexate–γ-aspartate from EPG liposomes occurs in medium containing 10% fetal calf serum during the early phase (0–5 h) of a 72-h growth inhibition assay. Therefore, the serum-mediated leakage of EPG liposomes is a transient event that is observed at high protein:lipid ratios, and continued incubation of the liposomes with serum proteins only slightly increases the leakage of methotrexate–γ-aspartate.

Leakage from phosphatidylcholine liposomes and lipid transfer to lipoproteins have been shown to depend on the plasma to liposome ratio (Scherpof & Morselt, 1984). As phosphatidylcholine is transferred to the HDL, the particles increase in size proportional to the protein to lipid ratio (Tall & Green, 1981). Leakage from EPG liposomes may result from lipid transfer to serum lipoproteins. The nonlinear relationship between EPG leakage and serum concentration (Figure 2) may result from a relative increase in lipid transfer to lipoproteins as the lipid to protein ratio increased. This may explain why a 100-fold decrease in serum concentration resulted in only a slight decrease in leakage from EPG liposomes. Alternatively, extensive leakage from EPG liposomes may occur without the concomitant transfer of lipid to HDL, which has been observed with large unilamellar EPC liposomes (Scherphof, et al., 1984).

The extensive leakage of EPG liposomes appears not to be due to apolipoproteins associated with HDL, since this lipoprotein fraction induced only 29% leakage. Purified bovine apo A-I, which is the major protein of HDL, induced similar leakage from EPG and EPC liposomes, indicating that this protein is not responsible for the great difference in the stability between EPC and EPG liposomes in calf serum. Apolipoproteins E and B which are associated with LDL may be disruptive to EPG liposomes. When bound to lipids, both apolipoproteins E and B display regions rich in basic amino acids (Innerarity et al., 1983; Knott et al., 1985). These positively charged regions of the apolipoproteins may disrupt negatively charged liposomes with low phase transition temperatures. Although apolipoprotein E is also associated with HDL which were not as disruptive to EPG liposomes as the LDL fraction, the binding of HDL to negatively charged liposomes may be impaired by the C apolipoproteins. C apolipoproteins bound to lipoproteins have been shown to oppose the uptake of the particles by the perfused rat liver without the displacement of apolipoprotein E (Windler & Havel, 1985). Therefore, if the C apolipoproteins interfere with the electrostatic attraction between the positively charged region of apolipoprotein E and a region of the LDL receptor known to be negatively charged (Yamamoto et al., 1984), the presence of C apolipoproteins on HDL may also oppose the association of apolipoprotein E with negatively charged liposomes. Serum proteins other than lipoproteins may also contribute to the instability of EPG liposomes. Cholesterol ester transfer protein binds to phosphatidylcholine liposomes only after the inclusion of 30% negatively charged PI (Pattnaik & Zilversmit, 1979). Bovine liver phospholipid transfer protein has a greater affinity for PS liposomes than neutral vesicles (Machida & Ohnishi, 1980; Wirtz et al., 1976). Human plasma transfer protein has been shown to transfer pyrenyl-PG and PS between model lipoproteins (Massey et al., 1985).

Ethanol-extracted albumin preparations have been shown to contain apo A-I and traces of C apolipoproteins (Guo et al., 1980; Fainaru & Deckelbaum, 1979). As discussed above, neither of these HDL components seems a likely cause of the leakage that we have observed. Other apolipoproteins and other leakage-inducing proteins may be present at concentrations undetected by these methods. The leakage of EPG in albumin depended on how the protein:lipid ratio was achieved. Generally, EPG leakage was lower when the protein to lipid ratio was obtained by diluting the albumin and may have resulted from apolipoprotein binding to the glass surface. The effects of the protein to lipid ratios on albumin-mediated leakage of EPG were similar to the results obtained for leakage in the presence of serum. An increase in the protein:lipid ratio might increase the size of PG–apolipoprotein complexes, in a manner similar to what has been observed for apo A-I and phosphatidylcholines (Jonas et al., 1980). This may produce the nonlinear relationship between EPG leakage and albumin concentration.

The effect of ionic strength on EPG leakage in albumin solutions indicates that apolipoprotein interactions with PG liposomes are mediated in part by electrostatic interactions. The effect of ionic strength on EPG leakage was much more pronounced in albumin solutions compared to the effect in 10% newborn calf serum. This suggests that some of the leakage inducers in serum may not be present in the albumin.

The effect of surface charge density on serum-mediated leakage of liposomes becomes apparent when greater than 50% of the phospholipid is negatively charged. Other studies have also reported that 10% or 20% negatively charged lipid had little effect on liposome leakage in the presence of serum proteins (Allen & Cleland, 1980; Mayhew et al., 1979; Senior et al., 1985).

The extensive leakage of methotrexate–γ-aspartate from 53 nM EPG liposomes in 10% serum has important implications for liposome-mediated drug delivery. Most notably, the potency of drugs encapsulated in negatively charged liposomes in 10% serum may be much less than it would be if no leakage occurred. Further, the extensive leakage of liposomes in 10% serum suggests that in vitro drug delivery experiments may

be better studied in a chemically defined media which does not contain serum proteins. We hope to investigate this in future studies.

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pH-Dependent Interaction of Amphiphilic Polypeptide Poly(Lys-Aib-Leu-Aib) with Lipid Bilayer Membrane

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ABSTRACT: A sequential polypeptide, poly(Lys-Aib-Leu-Aib) (Aib represents 2-aminoisobutyric acid), was synthesized, and the interaction with lipid membrane was studied. Poly(Lys-Aib-Leu-Aib) was designed to take an amphiphilic structure upon the formation of α-helix. Circular dichroism of poly(Lys-Aib-Leu-Aib) in an aqueous solution showed a negative Cotton effect due to α-helix. The content of α-helix increased when the pH was raised above 7.5 or in the presence of small unilamellar vesicles composed of egg yolk lecithin. On the other hand, α-helical conformation was broken by increasing the ionic strength of solution. Carboxyfluorescein leakage from dipalmitoylphosphatidylcholine (DPPC) vesicles induced by binding of poly(Lys-Aib-Leu-Aib) to the lipid membrane was facilitated in an alkaline solution and/or in a solution of low ionic strength. These phenomena can be related to the α-helix content of the polypeptide. It was shown that poly(Lys-Aib-Leu-Aib) induced fusion of DPPC vesicles in an alkaline solution below the phase-transition temperature of the membrane. It was further shown that the aggregation and fusion of the neutral lipid membrane was regulated by changing the pH of solution.

An amphiphilic structure found in polypeptide molecules is considered to be one of the most important structural units for biological activities (Massey et al., 1981). Kaiser and Kezdy (1984) have pointed out that apolipoproteins and peptide toxins potentially form an amphiphilic α-helix, which is supposed to be favorable for the interaction with phospholipid vesicles and other amphiphilic surfaces (Segrest et al., 1973; Mao et al., 1981). It has been shown that many peptide hormones likewise take amphiphilic secondary structure, which is essential in interactions with receptor. Schwyzer (1986) has

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