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Interaction of positively-charged liposomes with blood: implications for their application in vivo

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Liposomes with positively-charged lipid components have previously demonstrated efficacy in animal models for human diseases, and are currently being evaluated in human clinical studies. Cationic lipids can improve entrapment efficiency of drugs and other substances which are negatively charged, and facilitate penetration of biological membranes *in vitro*, e.g. in transfection. However, toxic effects have also been reported for positively-charged liposomes containing stearylamine. In this report we have examined gross interactions between plasma components or erythrocytes with cholesterol-rich SUV composed of PC or DPPC and having 0–50 mol% of phospholipid replaced with positively-charged stearylamine, DOTMA, or BisHOP. Plasma interactions observed included increased turbidity of the usually clear stroma and/or formation of a clot-like mass. At plasma concentrations of 0.25 μmol/ml or more, the extent of plasma interactions depended upon the concentration of positive charge, the charge density of cationic lipid initially present in the liposomes, and to a lesser degree, the nature of the lipid providing the positive charge. At liposomal positive charge concentrations of > 0.5 μmol/ml plasma, stearylamine provoked a strong increase in plasma turbidity, whereas liposomes incorporating DOTMA or BisHOP provoked a strong clotting response. Some hemolysis of erythrocytes *in vitro* occurred on interaction with cationic liposomes where positive charge was contributed by DOTMA or stearylamine, but not BisHOP. Implications for the clinical use of liposomes containing cationic lipids, is discussed.

Introduction

Lipid which confers a net positive charge to liposomes is being used in liposome formulations intended for clinical applications [1,2], and a number of reports have described advantageous therapeutic effects promoted by liposomes which include the positively-charged lipid stearylamine (SA) [2–8]. Beneficial effects include reduction in toxicity associated with free drug [9]; this approach is currently being evaluated clinically [2] for doxorubicin in cancer chemotherapy via an intravenous infusion. The main advantage of incorporating positively-charged lipid into liposomes is to improve the proportion of negatively charged drugs including proteins, that can become associated with liposomes [10]. It is not established whether cationic lipid has intrinsic therapeutic benefit, but this could be the case where macrophages are the target for liposomal drug administration since uptake of liposomes by these cells appears to increase when positively-charged lipid is present in the liposomal bilayer [5]. On the other hand, positively-charged lipids can cause toxic effects *in vivo* and *in vitro* [11], for example, brain toxicity after direct administration to that organ [12,13], and toxicity to cells in culture [14], and to erythrocytes [15,16].

We observed that liposomes incorporating a much higher mol percent of positive charge than reported previously, i.e. liposomes composed of 50 mol% DOTMA, interacted strongly with plasma (incubated at 37°C), forming a clot-like mass on contact. The minimum amount of cationic lipid that will trigger plasma and erythrocyte interactions was therefore determined, and also the effect of the molar ratio and/or the nature of the lipid that provides the positive charge

Abbreviations: BisHOP, 2,3-dihexadecyloxyl-propyl-*N*,*N*,*N*-trimethylammonium chloride; Chol, cholesterol; DOTMA, 2,3-dioctadecenyl-oxypropyl-*N*,*N*,*N*-trimethylammonium chloride; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine (egg); SA, stearylamine.

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on these interactions. Three cationic lipids were investigated; (i) stearylamine (SA), a single-chain amine derivative of stearic acid; (ii) DOTMA, a double-chain (oleic acid-derivative) unsaturated synthetic lipid, mol. wt. 670.5; and (iii) BisHOP, a double-chain (palmitic acid-derivative) saturated synthetic lipid, mol. wt. 618.5. From the nature of the fatty acid chains, DOTMA would be expected to have a bilayer fluidity similar to PC, whereas BisHOP has a fluidity similar to DPPC. Both DOTMA and BisHOP have fatty-ether as opposed to fatty-acyl linkages. Interactions between plasma components and positively-charged liposomes containing stearylamine have been investigated previously by finger-printing adsorbed proteins isolated electrophoretically [11]. In the experiments reported here, comparison was simply made between liposomes incorporating different types and amounts of positive charge when positively-charged liposomes were incubated with either native plasma, serum or heat-treated plasma to determine whether clotting factors or intact plasma proteins are involved in gross plasma interactions visible by eye. Implications for using positively-charged liposomes *in vivo* are discussed.

Materials and Methods

Phosphatidylcholine from egg (PC, > 99% pure by thin-layer chromatography) was purchased from Avanti, Birmingham, AL. Dipalmitoyl-phosphatidylcholine (DPPC) was obtained from Nippon Fine Chemical Co Ltd., Japan. Cholesterol (Chol) was obtained from Ruger Chemical Co., and stearylamine (SA) from Sigma, St. Louis, MO. 2,3-Dioctadecenyl-oxypropyl-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), 2,3-dihexadecyloxyl-propyl-*N*,*N*,*N*-trimethylammonium chloride (BisHOP) were from Syntex Research, Palo Alto, CA. Fluorexon (calcein) was purchased from Aldrich, Milwaukee, WI. All other reagents were of analytical grade.

Preparation of liposomes

Small unilamellar liposomes (SUV) composed of PC or DPPC, and equimolar Chol, were given a net positive charge by replacing the phospholipid with DOTMA, or SA, or BisHOP, at ratios from 1 to 50 mol% of the phospholipid component. Liposomes were prepared [17,18] by removal of solvents under reduced presure to form a thin lipid film, and were subsequently hydrated with 0.2 M calcein or in 0.1 mM sodium phosphate buffer (pH 7.4) containing 0.8% NaCl and 0.02% KCl (PBS). Particle size reduction was achieved by sonication to clarity (45–60 min) using a Branson 450 sonifier (Danbury, CT, U.S.A.) fitted with a 3 inch cup-horn jacketed in cold tap water (PC preparations) or at 55–60°C (DPPC preparations). Unencapsulated calcein was separated from the liposomes by gel filtration on Sepharose 6B-CL (Pharmacia), followed by dialysis against PBS until just before use. Liposome particle size determined by laser light-scattering (Autosizer 11c, Malvern, U.K.), was independent of lipid composition. For SUV hydrated in PBS, 80–97% of particles were in the size-range 31–99 nm in diameter with a mean of 52 nm, immediately after sonication. After storage at +4°C for 44 h, the particle diameter was 34–126 nm, with a mean of 58 nm. DOTMA-containing liposomes hydrated in calcein were more variable in size (0.038–2.7 μm); with a mean diameter of 53 nm (0–30 mol%), 0.4 μm (40 mol%) and 2.2 μm (50 mol%).

Interaction with red blood cells

Packed red blood cells (RBC) were isolated from fresh, heparinised whole blood obtained from rats (Wistar, male). RBC were separated from plasma by centrifugation (1250 × *g* for 10 min) and washed three times with PBS before use. One volume of liposomes was combined with 8 volumes of packed red cells (0.4 μmol total lipid/ml) to mimic the *in vivo* ratio used for liposomes and plasma. Hemoglobin release was measured spectrophotometrically (absorbance at 540 nm) 5 min after incubation (37°C) using a modified version of the method of Reed and Yalkowsky [19]. Intact erythrocytes were recovered in the pellet after centrifugation (1250 × *g* for 10 min), washed three times in 5 ml PBS, and fully lysed in 4 ml distilled water to release all the hemoglobin. One ml of hemoglobin-rich supernatant was diluted to 5 ml with distilled water and the absorbance at 540 nm, analysed. Percent hemolysis was expressed relative to total hemoglobin released from RBC incubated in PBS, in the absence of liposomes.

Interaction with native plasma; assessment of turbidity changes and clot formation

Small unilamellar liposomes hydrated in buffer were incubated at 37 °C with rat plasma freshly obtained from heparinized blood by centrifugation at 1250 × *g* for 10 min. Samples (125 μl containing 1.5–80 μmol total lipid) of the incubation mixture, were removed one and/or thirty minute after the start of incubation and diluted into 2.5 ml PBS. Plasma turbidity was analysed spectophotometrically, at a wavelength (600 nm) where liposomes and plasma individually have minimum absorbance. In other experiments, liposomes were incubated (as above) in serum prepared from clotted whole blood, or in heat-treated plasma (56°C for 30 min). The extent of any clot formation, was estimated by weight of the dried mass. Duplicate 50 μl samples (0.04–2 μmol total lipid) of liposomes were mixed with 350 μl fresh native plasma in pre-weighed, 8 mm × 36 mm glass tubes. After 30 min incubation at 37 °C, the plasma (stroma) was removed, leaving be-

hind any clotted material. Clot weight was measured after thorough drying to a constant weight.

Retention of entrapped fluorescent marker in plasma and PBS

Small unilamellar liposomes composed of PC or DPPC with 10–50 mol% positively-charged lipid provided by DOTMA, or SA, or BisHOP, all with equimolar cholesterol, were incubated in plasma or PBS for up to 28 h, in ratios described previously [17,18]. Marker retention, measured as latency of a self-quenching fluorescent dye [17], after dilution in PBS, was analysed using a Perkin-Elmer LS50 fluorimeter; excitation and emission wavelengths were 483 and 514 nm, respectively, with slitwidths of 2.5 nm. Structural integrity of liposomes containing 10–50 mol% cationic lipid was fully maintained in the presence of plasma or PBS at 37°C; calcein latency was > 99% of initial for up to 28 h.

Results and Discussion

The purpose of these *in vitro* experiments was to detect gross changes in plasma on contact with liposomes bearing a net positive surface charge, and to correlate interactions with type, amount, and mol percent (presumably related to charge density) of positively-charged lipid incorporated. Changes in plasma turbidity and/or formation of a 'clot' were measured in mixtures of liposomes and plasma combined in a ratio of 1:8 (vol/vol) which corresponds to a typical lipid dose of 50–400 μmol per kg body weight used in previous *in vivo* studies [20,21]. Decreasing amounts of liposomes were mixed (0.25–1.25 μmol/ml) with plasma at 37°C, to mimic the administration of progressively smaller doses *in vivo*. The interactions, which could be observed visually, were dependent on plasma concentration of liposomal positive charge.

Interaction of cationic liposomes with whole plasma

When positively-charged SUV composed of PC, equimolar cholesterol and with more than 20 mol% PC replaced by DOTMA, were mixed with plasma at 37°C in a ratio equivalent to a dose of 0.5 μmol positive charge per ml plasma, substantial turbidity occurred (Fig. 1). Plasma became turbid on contact with the liposomes (Fig. 1a), and the turbidity increased further after 30 min incubation (Fig. 1b). Subsequent measurements were therefore made after a total incubation time of 30 min. At the 30 mol% level of DOTMA incorporation corresponding to a concentration of 0.75 μmol DOTMA per ml plasma, a solid clot formed (Fig. 1b). In contrast, no clot occured when liposomes containing 30 mol% DOTMA were mixed, at the same concentration, with heated plasma or serum (Fig. 1b) suggesting that heat-sensitive proteins are involved in

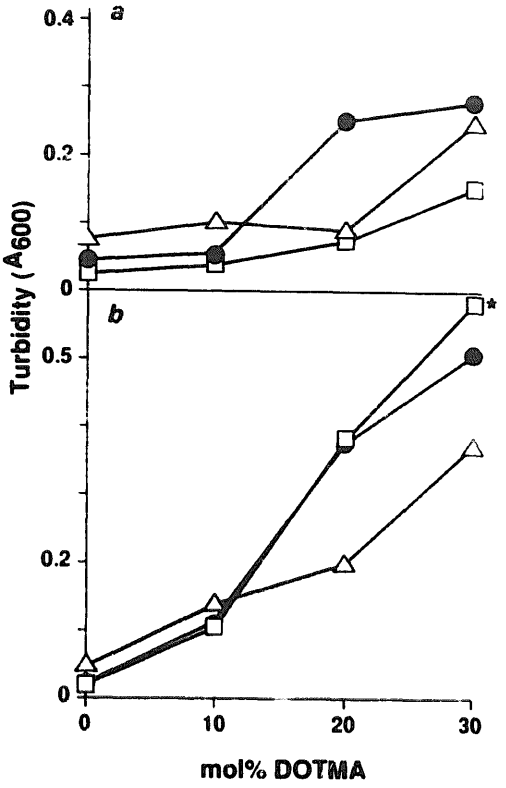

Fig. 1. Turbidity changes in plasma, heat-treated plasma and serum, combined with liposomes incorporating increasing mol% of positively-charged lipid (DOTMA). Liposomes composed of equimolar cholesterol and PC supplemented with 5–50 mol% DOTMA, were incubated at 37°C (50 μl liposomes in 350 μl plasma/serum, 0.04–2 μmol total lipid) for 1 min (a), or 30 min (b), in plasma (□); in heated plasma (●); or in serum (△). Samples (125 μl) were diluted in 2.5 ml PBS at the times indicated and A_{600} read immediately. For further detatils, see Methods. * Indicates formation of a clot.

positive charge-mediated clot formation in plasma. Quantitative assessment of plasma interactions using turbidity measurements are likely to be of limited value, however, since mechanisms leading to turbidity changes in plasma on contact with positively-charged liposomes are likely to be complex. Indeed absorbance changes probably reflect different levels of interaction between liposomes and plasma such as adsorption of negatively-charged plasma proteins, liposome-triggered plasma component aggregation and possibly further levels of agglomeration of the initially-formed aggregates, i.e. highly dynamic processes with ill-defined end-points. In contrast, formation of a distinct clot provides a readily measurable end-point.

Within a range of liposomal positive charge to plasma of 0–1.25 μmol/ml plasma, clot weight depended upon the type of molecule contributing the positive charge, and the mol% of cationic lipid incorporated (Fig. 2). The weight of clots formed was measured after plasma was incubated at 37°C for 30 min, with liposomes incorporating positive charge provided by different types of molecules, the 30 min incubation time allowing time for any clot to develop and gave an

end-point from which comparisons could be made. Liposomes with 20–50 mol% DOTMA or BisHOP present in the bilayer induced definite clot formation, whereas SA-containing liposomes, only stimulated greater turbidity. This latter observation may indicate that stearylamine molecules can leave the liposomal bilayer and interact directly with negatively-charged plasma proteins. However, for each source of positively-charged lipid incorporated into the liposomal bilayer, a total positive charge concentration of greater than 0.25 μmol positive charge/ml plasma (Fig. 2) appears to be required before macroscopic intereactions are observed.

Interestingly, this threshold of cationic lipid concentration also appears to hold when liposomes with 1 : 1 molar ratio of cationic lipid to neutral phospholipid, which caused the maximum clot formation, were mixed with plasma. When SUV having 50 mol% of phospholipid replaced by cationic lipid, were added to plasma in a volume which gave a total plasma concentration of 0.25 μmol cationic lipid/ml (Fig. 3), the liposomes interacted with plasma similarly to neutral liposomes at the same total lipid concentration. Thus liposomes administered intravenously at a dose of total positive charge below the threshold concentration, would be expected to be cleared from plasma at a similar rate to neutral liposomes at the same total lipid concentration, if no other factors were acting. However, it has been shown previously that positively-charged liposomes can have a shorter plasma half-life than neutral liposomes

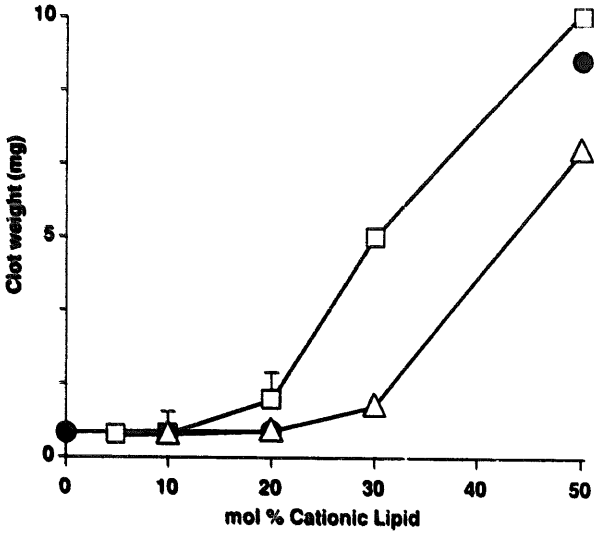

Fig. 2. Effect of mol% of positively-charged lipid incorporated into liposomes: clot formation in plasma. Liposomes (SUV) composed of equimolar cholesterol and PC or DPPC with 5–50 mol% of the phospholipid replaced by DOTMA (□); or SA (△); or BisHOP (●); were mixed with rat plasma (50 μl liposomes and 350 μl plasma, 0.125–1.25 μmol cationic lipid per ml plasma) and incubated (37 °C for 30 min). Uncoagulated plasma was completely removed, and any remaining clot dried and weighed (see Methods). Background clot weight of plasma in the absence of liposomes was 0.25–0.33 mg, and in the presence of neutral liposomes was 0.31 ± 0.05 mg (S.D.), $n = 6$.

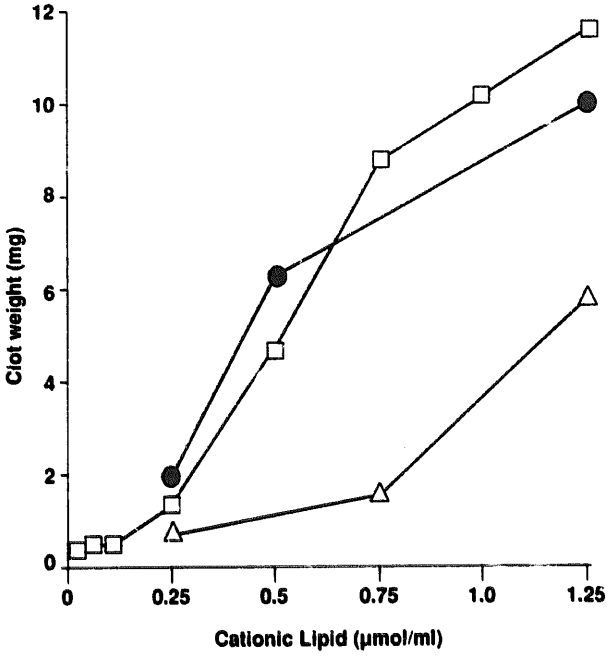

Fig. 3. Clot formation in plasma incubated with liposomes containing positively-charged lipid incorporated at 50 mol%. Liposomes composed of equimolar cholesterol and PC or DPPC with 50 mol% of phospholipid component replaced by DOTMA (□); or SA (△); or BisHOP (●); were mixed with rat plasma at 37°C (0.04–2 μmol total lipid per 350 μl plasma), incubated at the same temperature for 30 min, and assayed as described in the legend to Fig. 2. Clot weights were obtained by mixing 1–50 μl SUV with plasma (cationic-lipid concentrations were 0.025–1.25 μmol/ml).

[21] suggesting that other factors act *in vivo* to determine plasma clearance rates of positively-charged liposomes: These factors could include; micro-interactions (i.e. adsorption of plasma factors which do not result in visible changes in plasma, i.e. turbidity or clotting); positive charge-sensitive interactions with cells *in vivo*; or a combination of these.

While the extent of interaction between positively-charged liposomes and plasma components appears to be dependent upon the concentration of liposomal positive charge in the plasma, we wished to determine whether mol percent of positive charge present in the bilayer, presumably determining the surface charge-density, can also influence the magnitude of turbidity and clot formation. Positive charge was provided by vesicles containing 10–50 mol% positively-charged lipid, and specific concentrations of positive charge (i.e. 0.25, 0.5, or 0.75 μmol/ml plasma) was achieved by adding an appropriate volume of liposomes (Fig. 4). The extent of clotting after incubation with plasma for 30 min at 37°C increased as surface charge-density increased (Fig. 4). Effects were most pronounced for DOTMA and BisHOP and least for SA (Fig. 4), supporting the assertion that fewer positively-charged groups are exposed on the liposome surface when positive charge is provided by SA than by DOTMA in equivalent liposomes (PC/Chol) and conditions. The

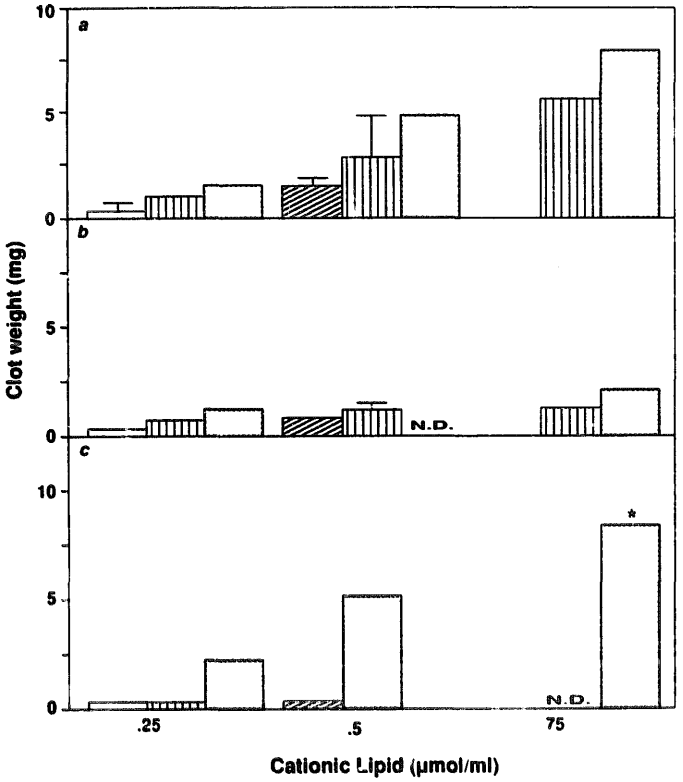

Fig. 4. Effect of charge density of positively-charged liposomes: clot formation in plasma incubated with SUV containing increasing mol% of cationic lipid. Liposomes composed of equimolar cholesterol and PC or DPPC with 10–50 mol% of phospholipid replaced by DOTMA (a); or SA (b); or BisHOP (c); were incubated (37 °C, 30 min) in the ratio of 10–50 μl liposomes to 350 μl plasma, to give a final concentration of 0.25, or 0.50, or 0.75 μmol cationic lipid/ml plasma. The amount of positively-charged lipid incorporated into liposomes (10–50 mol%) was; 10%, (open bars); 20%, (cross hatched); 30%, (vertical lines); 50%, (dots). S.D are means of 4–7 replicate experiments, other values are means of duplicate experiments.

strong plasma interactions, especially at 50 mol% suggest that both DOTMA and BisHOP were efficiently incorporated into and retained in the bilayer, and that BisHOP was incorporated into a more rigid bilayer structure (DPPC/Chol, 1 : 1). Positive charge may be differently disposed on the surface of SA-containing liposomes, or the single-chain molecule SA may be less readily retained in liposomes in the presence of plasma. The extent to which the SA molecules were retained is undetermined at this stage. The higher mol percent/plasma concentration of positively-charged lipid may trigger a more intense plasma interaction by binding negatively-charged plasma proteins more avidly, or simply in greater amounts, than the lipsomes with less positive charge on their surface.

Interaction of cationic liposomes with RBC

Positively-charged SUV also interacted with RBC in accordance with the source of lipid positive charge. DOTMA and SA-containing SUV caused some hemolysis in a standard red-cell toxicity assay [20], whereas BisHOP-containing liposomes did not (Fig. 5). Cationic liposomes and red blood cells (Fig. 5) interacted when liposomal positive charge exceeded 0.25 μmol DOTMA, or 0.5 μmol SA, and was not detected up to 1.25 μmol BisHOP/ml packed red cells. Up to 8% of total hemoglobin was released from RBC, when positively charged lipid was incorporated at 10 and 20 mol% for DOTMA and SA, respectively. Liposomes were less interactive with the RBC when positive charge was present in a relatively rigid bilayer structure ie BisHOP in DPPC/Chol liposomes (Fig. 5), compared with DOTMA or SA in a less rigid bilayer. Hemolysis is a complex process, involving a variety of factors such as pH, lipid solubility, charge, and molecular dimensions of the solute molecule [22]. Isolated RBC were sensitive to the concentration of SA in liposomes, possibly caused by fusion of liposomes on contact with the RBC [16], which may also occur with DOTMA-containing liposomes. However, *in vivo*, liposomes and erythrocytes interact in a milieu of plasma components. It is therefore likely that liposomes, which acquire a coating plasma components on contact with blood [11], do not interact as readily with RBC *in vivo* as *in vitro*.

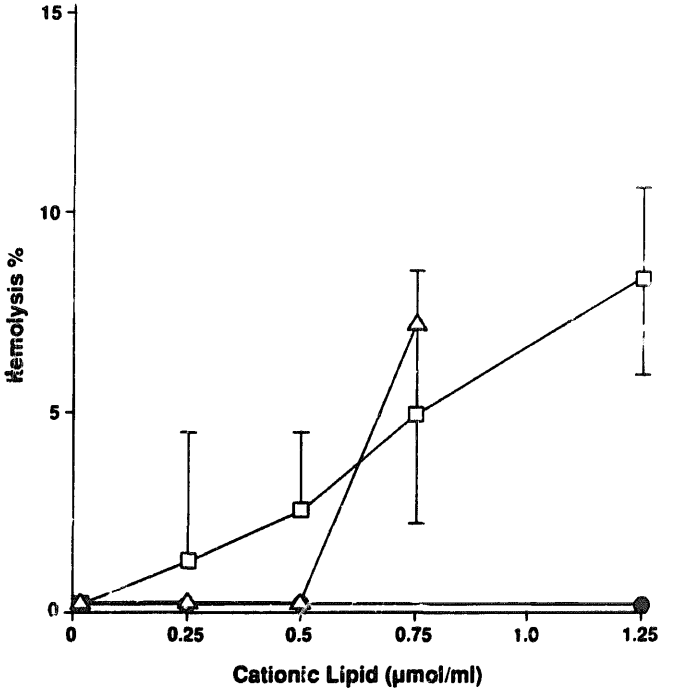

Fig. 5. Extent of hemolysis of erythrocytes exposed to liposomes containing positively-charged lipid. Liposomes composed of equimolar cholesterol and PC or DPPC (40 μmol total lipid/ml) with 0–50 mol% of phospholipid replaced by DOTMA (□); or SA (△), or, BisHOP (●); were mixed (see Methods) with washed, packed, red blood cells (rat) as described in Materials and Methods. After 10 min incubation with liposomes or in physiological buffer, intact red blood cells were recovered by centrifugation. Total hemoglobin in each batch of recovered cells was released by lysis in pure water, and absorbance compared for buffer-incubated and liposome-incubated cells. Red cell lysis in the presence of liposomes was expressed as percent absorbance obtained for buffer-incubated cells.

Implications for use of positively-charged liposomes in therapy

The studies described here for positively-charged liposomes hydrated in buffer at pH 7.4 containing no drug, show that such liposomes display substantial charge- and concentration-dependent interactions with plasma and isolated erythrocytes. A threshold concentration of 0.25 μmol positively-charged lipid per ml of rodent plasma was required before interactions were detected macroscopically. The significance of these findings for *in vivo* applications of liposomes bearing positive charge are hard to predict, but cationic liposomes administered at a concentration above the positive charge threshold are likely to undergo rapid clearance due to aggregation phenomena. Previous studies [21] showed that liposome clearance was accelerated when liposomes were administered at a concentration of positive charge at or below a level where (based on our *in vitro* studies) little or no 'macroscopic' plasma interaction would be expected. Indeed, interactions with plasma components take place in addition to those detected in this study [11,23,24]. For example, liposomes of various compositions and net charge acquire a coating of negatively-charged proteins on exposure to plasma, especially those with a net positive charge [11,23]. Accelerated clearance of positively-charged liposomes in vivo may therefore be (a) due to increased net size of the liposomes mediated by negatively-charged plasma factors, (b) effected by charge-sensitive cell-mediated uptake responsive to liposomal positive charge or more likely sensitive to the coating of negatively-charged proteins on the liposome surface, or (c) a combination of these. The effect of positive surface charge on *in vivo* interactions has been examined in a separate study (Senior, J.H., Trimble, K. and Maskiewicz, R., submitted).

In conclusion, limits of gross plasma interactions have been defined for positively-charged liposomes hydrated in buffer; intact (dye-retaining) liposomes were formed in the presence of calcein, a well-characterized system for establishing liposome formation and stability. Differences in plasma interactions occurred when positive charge was provided by either SA, DOTMA or BisHOP, and these differences correspond to whether positive charge was provided by a single-chain or double-chain molecule in the bilayer. The extent of plasma interactions was also dependent on charge density and bilayer fluidity. Blood components involved in the interactions were active in whole plasma but were inactive or incompletely active in serum and/or heated plasma, suggesting that heat-sensitive factors present in plasma but not serum (such as negatively-charged proteins of in the clotting cascade) may be involved. However, interactions between liposomes bearing a net positive charge and blood components need not necessarily solely involve aggregate formation via a specific mechanism such as the clotting cascade. Formation of molecular scale aggregates eventually leading to macroscopic agglomerates or 'clots' could be due at least in part, to electrostatic interactions between oppositely-charged polyelectrolytes, the liposomal particle being analogous to cationic polymer molecules [25]. Since a great many plasma components have a net negative charge, more experimental work would be required before speculating further on the identity of protein(s) involved. Substantial interactions detected *in vitro* between liposomes bearing a net surface positive charge and blood components, would probably lead to rapid clearance of liposomes from circulating blood if they occur *in vivo*, and could lead to trapping of liposomes in lung capillaries. Since positive charge-mediated effects were not observed below 0.25 μmol charge/ml plasma regardless of source of charge, doses of positively-charged liposomes administered to humans well-below the saturating doses given to mice, are unlikely to reach the threshold for macroscopic liposome-plasma aggregation where adverse reactions such as embolism, could occur.

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