Membrane Fusion with Cationic Liposomes: Effects of Target Membrane Lipid Composition†

Austin L. Bailey* and Pieter R. Cullis

Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3

Received May 16, 1996; Revised Manuscript Received October 24, 1996⊗

ABSTRACT: Determination of the mechanisms by which cationic liposomes adhere to and fuse with biological membranes is important to understanding how these lipid vesicles mediate cellular transfection. To determine what role the lipid composition of "target" membranes might have in promoting fusion with cationic liposomes, we have examined the ability of large unilamellar vesicles composed of 1,2-dioleoyl-*sn*-phosphatidylethanolamine (DOPE) and *N*,*N*-dimethyl-*N*,*N*-di-9-*cis*-octadecenylammonium chloride (DODAC) (1:1) to fuse with target liposomes of varying composition in the absence of DNA. Membrane fusion was promoted by increased negative surface charge and, for liquid crystalline lipids, by increased acyl chain unsaturation in target liposomes. However, the presence of disaturated phospholipids promoted fusion below the gel to liquid crystalline transition temperature, an effect which was eliminated by the addition of cholesterol. It was also shown that DOPE/DODAC (1:1) LUVs fused with erythrocyte ghosts and that this fusion was blocked by the presence of serum. Membrane fusion was determined by a quantitative fluorescent lipid mixing assay and qualitatively by freeze−fracture electron microscopy and fluorescence microscopy.

Cationic amphiphiles have achieved widespread use as gene transfer agents. DNA complexes with cationic lipids and complexes formed by adding DNA to liposomes prepared from cationic detergents or lipids mixed with neutral lipids have proven highly efficient for the transfection of cultured cells [for a review see Behr (1994)]. The techniques involve the formation of lipid−DNA or liposome−DNA complexes through charge interactions, followed by incubation of these complexes with the cells to be transfected. An excess of the positively charged lipid component relative to negative charge on the DNA is required, apparently to promote interactions between the complexes and the anionic residues on the cell surface. The mode by which the complexes gain entry to the cytoplasm has been the subject of extensive studies, and it is increasingly evident that this process occurs by endocytosis followed by release of the complex by destabilization of the endosomal membrane (Farhood et al., 1995; Wrobel & Collins, 1995). However, release of the DNA from the lipid complexes and its eventual delivery to the nucleus are inefficient and poorly characterized processes (Zabner et al., 1995).

A comparison of the reported cationic lipid systems is difficult. The chemical structure of the cationic lipids and the cell types that have been used to gauge transfection efficiencies are disparate. However, some systematic studies on the effects of cationic lipid structure have been made (Felgner et al., 1987; Ito et al., 1990; Leventis & Silvius, 1990; Gao & Huang, 1991). One observation is that greater levels of transfection are achieved with lipids bearing cationic charges close to the lipid−water interface. In addition, studies on transfection efficiency as a function of neutral lipid composition for DNA−cationic liposome complexes (Felgner et al., 1987; Stamatatos et al., 1988; Düzgüneş et al., 1989; Farhood et al., 1992; Zschörnig et al., 1992; Bennett et al., 1995) have demonstrated that unsaturated phophatidylethanolamines are the preferred neutral lipids for mixture with cationic lipids or detergents.

Very little is known about the influence of the composition of target cellular membranes on the extent of fusion achieved by a given cationic lipid formulation. Such information may be useful in understanding the variability in transfection among various cell lines. A first approach to understanding the possible effects of target membrane composition is to study such effects in well-defined lipid systems, liposomes, in the absence of DNA.

Fusion of liposomes has been extensively studied. Liposomal fusion can result from neutralization of charged lipid species, as induced by changes in pH or by the addition of neutralizing multivalent ions (Ticock et al., 1988; Düzgüneş et al., 1989). Fusion is also promoted by the incorporation of lipids bearing unsaturated fatty acyl chains (Tilcock & Cullis, 1982) or by lipids with small uncharged headgroups (Das & Rand, 1986). These observations led to the proposal that membrane fusion arises from the dehydration of surfaces of apposed lipid bilayers, resulting from charge neutralization, followed by the formation of nonbilayer lipid intermediates, as promoted by lipids or conditions which impart a preference for negative curvature in the contacting monolayers (Chernomordik et al., 1985; Cullis et al., 1986). These lipid properties may be important in understanding the process of lipid-mediated transfection.

† This research was supported by the Medical Research Council of Canada. A.L.B. was supported by a Graduate Research in Engineering and Technology (GREAT) Award fron the Science Council of British Columbia.

* To whom correspondence should be addressed. Current address: Laboratory of Cellular and Molecular Biophysics, NICHD, National Institutes of Health, 10 Center Drive, Room 10D-07, Bethesda, MD 20892-1855.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

S0006-2960(96)01173-7 CCC: $14.00 © 1997 American Chemical Society

To model the effects of the lipid composition of target membranes on fusion with cationic liposomes, we have studied the ability of large unilamellar vesicles (LUVs)[1] composed of 1,2-dioleoyl-*sn*-phosphatidylethanolamine (DOPE) and *N*,*N*-dimethyl-*N*,*N*-di-9-*cis*-octadecenylammonium chloride (DODAC) (1:1) to fuse with LUVs of varying composition. The effects of negative surface charge concentration, phospholipid headgroup, acyl chain saturation, and the addition of cholesterol were studied. In addition, fusion of DOPE/DODAC (1:1) with erythrocyte ghosts and the ability of serum to inhibit such fusion are demonstrated.

MATERIALS AND METHODS

Lipids and Chemicals. DODAC was provided by Inex Pharmaceuticals Corp. (Vancouver, BC). 1-Palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (POPC) was purchased from Northern lipids (Vancouver, BC). Egg phosphatidylcholine (EPC), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dioleoyl-*sn*-phosphatidylethanolamine (NBD-PE), *N*-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-*sn*-phosphatidylethanolamine (Rh-PE), and all other synthetic lipids were supplied by Avanti Polar Lipids (Alabaster, AL). Mouse serum was obtained from Caltag Laboratories (South San Francisco, CA). Cholesterol, all buffers, and miscellaneous chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Liposomes. Chloroform solutions of lipids were dried by vortex mixing under a nitrogen stream followed by the removal of residual solvent under high vacuum for 1 h. Lipids were hydrated in HEPES-buffered saline (HBS: 20 mM HEPES, 150 mM NaCl, pH 7.5) to give 20 mM multilamellar vesicle (MLV) suspensions. Five freeze−thaw cycles were used to ensure homogeneous mixture. The MLVs were extruded 10 times through two 100 nm pore-size polycarbonate filters (Costar, Cambridge, MA) in a pressure extruder (Lipex Biomembranes, Vancouver, BC) to produce large unilamellar vesicles (LUVs). All preparations were extruded at temperatures above the gel-to-liquid crystalline phase transition. The size distributions of the liposomes were determined by quasi-elastic light scattering on a Nicomp 270 Sub-micron Particle Sizer using manufacturer's software to calculate Gaussian fits to the correlation data. Lipid concentrations were determined by phosphate assay as described by Bartlett (1959). All liposome preparations were diluted with HBS to 10 mM total lipid prior to assays.

Preparation of Erythrocyte Membranes. Sealed erythrocyte ghosts were prepared by the method of Steck and Kant (1974). Briefly, 4 mL of packed cells was washed three times with HEPES-buffered saline (HBS: 5 mM HEPES, 150 mM NaCl, pH 7.5), centrifuging each time for 5 min at 750*g* with a swinging-bucket rotor. Washed cells were diluted 2-fold with HBS, lysed in 300 mL of 5 mM HEPES, and 1 mM $MgCl_2$, pH 7.5, and pelleted at 20000*g* for 20 min. Ghosts were removed from above the hard, protease-rich pellet and resuspended in 200 mL of HBS containing 1 mM $MgCl_2$. The suspension was repelleted, washed twice more, and finally resuspended in 10 mL of HBS. Phospholipid concentration was determined by phosphate assay. The absence of glyceraldehyde-3-phosphate dehydrogenase activity (Steck & Kant, 1974) was used to confirm the formation of sealed right-side-out ghosts.

Lipid Mixing Fusion Assays. The extent of membrane fusion as measured by lipid mixing was monitored by the decrease in resonance energy transfer (RET) resulting from dual fluorescent probe dilution (Struck et al., 1981). DOPE/DODAC (1:1) LUVs containing 0.5 mol % of both NBD-PE and Rh-PE were prepared in HBS, pH 7.5. Labeled vesicles were diluted to 50 μM lipid with HBS in a 3 mL quartz cuvette. Fluorescence was monitored with excitation at 465 nm, emission at 535 nm, and an emission cutoff filter at 530 nm. Temperature was maintained at 25 °C with a circulating water bath. At 30 s, target LUVs of a desired composition or erythrocyte ghosts were added in a labeled to unlabeled lipid ratio of 1:3 to give a total lipid concentration of 0.2 mM. Fluorescence was monitored over 5 min.

Each lipid mixing time course was normalized by subtracting the initial fluorescence (F_0) and dividing by the fluorescence achieved by infinite probe dilution determined by the addition of 25 μL of 100 mM Triton X-100 (F_{max}). The percent change in fluorescence was calculated as

$$\% \frac{\Delta F}{\Delta F_{max}} = 100\left(\frac{F - F_0}{F_{max} - F_0}\right)$$

These calculated values were not corrected for the effects of membrane composition or Triton X-100 on NBD-PE fluorescence.

Freeze−Fracture Electron Microscopy and Size Analysis. LUVs of four different lipid compositions were prepared in HBS, pH 7.5: DOPE/DODAC (1:1), DSPC/POPS (80:20), DOPC/POPS (80:20), and POPC (DSPC = 1,2-distearoyl-*sn*-phosphatidylcholine, POPS = 1-palmitoyl-2-oleoyl-*sn*-phosphatidylserine, DOPC = 1,2-dioleoyl-*sn*-phosphatidylcholine). Cationic liposomes were mixed with each of the three target vesicle preparations in a ratio of 1 to 3 at a total lipid concentration of 10 mM. After 5 min incubations at 25 °C, samples were mixed 1:1 with glycerol and quickly frozen. Platinum−carbon replicas were prepared as described previously (Fisher & Branton, 1974).

Fluorescence Microscopy. Lipid mixing of DOPE/DODAC (1:1) LUVs with erythrocyte ghosts was demonstrated by fluorescence microscopy using the dual fluorescent label DOPE/DODAC preparation described above for the lipid mixing assay. These LUVs contain Rh-PE at a self-quenching concentration, and the appearance of fluorescence in the ghost membranes can be used to detect membrane fusion. Labeled liposomes and ghosts were mixed in a 1:3 lipid ratio at a total lipid concentration of 1 mM in HBS, pH 7.5. After a 5 min incubation at 25 °C, 5 μL samples were mounted under large cover slips to achieve rapid immobilization, and micrographs were taken using both phase contrast and a red fluorescence filter. A sample in which the DOPE/DODAC liposomes were mixed with 1%

[1] Abbreviations: Chol, cholesterol; DODAC, *N*,*N*-dimethyl-*N*,*N*-di-9-*cis*-octadecenylammonium chloride; DOPC, 1,2-dioleoyl-*sn*-phosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-phosphatidylethanolamine; DSPC, 1,2-distearoyl-*sn*-phosphatidylcholine; EPC, egg phosphatidylcholine; ESM, egg sphingomyelin; POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylserine; HBS, HEPES-buffered saline (20 mM HEPES, 150 mM NaCl); HII, hexagonal phase; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dioleoyl-*sn*-phosphatidylethanolamine; RET, resonance energy transfer; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-*sn*-phosphatidylethanolamine; SUV, small unilamellar vesicle; % $\Delta F/\Delta F_{max}$, percent change in fluorescence.

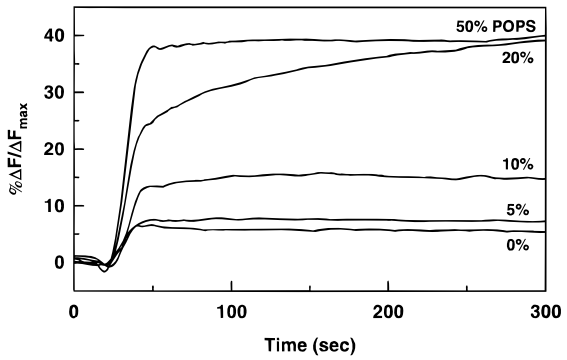

FIGURE 1: Effect of negative charge concentration in DOPC LUVs on lipid mixing with DOPE/DODAC (1:1). At 30 s, DOPC/POPS liposomes prepared with POPS concentrations ranging from 0 to 50 mol % were added to DOPE/DODAC liposomes labeled with 0.5 mol % each of NBD-PE and Rh-PE. The ratio of anionic to cationic liposomes was 3:1, and the total lipid concentration was 0.2 mM. NBD-PE fluorescence was normalized to the value obtained by the addition of Triton X-100 detergent as described in Materials and Methods.

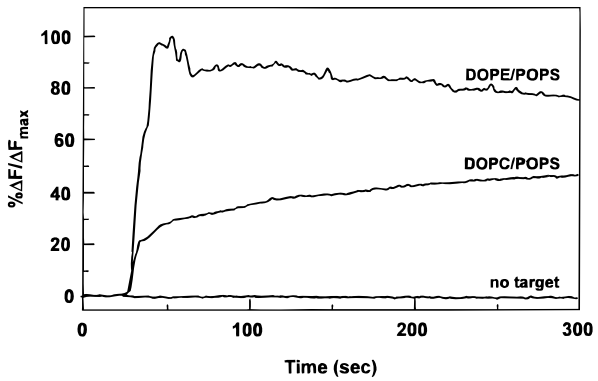

FIGURE 2: Comparison of lipid mixing for DOPE/POPS (80:20) and DOPC/POPS (80:20) with DOPE/DODAC (1:1). The control fluorescence time course was recorded without adding target liposomes.

mouse serum prior to incubation with erythrocyte ghosts was also used.

RESULTS

DOPE/DODAC LUVs. LUVs composed of DOPE/DODAC (1:1) were used throughout these studies. Pure DOPE does not form bilayers when dispersed in aqueous buffer but rather adopts a highly hydrophobic hexagonal (H_{II}) phase. Addition of as little as 15 mol % DODAC to DOPE can induce the formation of bilayer phase in water. However, membranes composed of DOPE and cationic lipids can be destabilized in the presence physiological levels of salt (Stamatatos et al., 1988). In our system, at least 30 mol % DODAC is required to form liposomes with DOPE in HBS, and even at this level rapid increase in vesicle size is observed (data not shown). With 1:1 DOPE/DODAC, the liposomes are more stable and undergo limited size increases over a period of several hours (see light scattering data and freeze−fracture below). In all experiments described here, the DOPE/DODAC (1:1) vesicles were prepared on the day the experiment was performed.

Effect of Negative Charge on Lipid Mixing. To determine the effect of negative charge concentration in target liposomes on the extent of membrane fusion, we examined lipid mixing between DOPE/DODAC (1:1) LUVs and DOPC LUVs containing POPS in the range of 0−50 mol % (Figure 1). A small increase in fluorescence was observed with DOPC vesicles in the absence of negative charge ($\Delta F/\Delta F_{max} \sim 6\%$). This increase was approximately doubled by the addition of 10 mol % POPS to the target vesicle population. At 20 mol % POPS extensive lipid mixing occurred and continued to occur for the duration of the assay, reaching a value of $\Delta F/\Delta F_{max} \sim 40\%$ at 5 min. This result was used as a benchmark in subsequent experiments in which the lipid compositions of the target vesicles were varied while maintaining POPS content at 20 mol %.

When vesicles composed of equimolar DOPC and POPS were added to DOPE/DODAC (1:1), the increase in fluorescence was very rapid over the first few seconds and then arrested at an apparent maximum of $\Delta F/\Delta F_{max} \sim 40\%$. This value probably corresponds to the maximum possible extent of lipid mixing between these vesicle populations. We were unable to extrude a lipid preparation composed of DOPC/POPS/DOPE/DODAC (60:15:12.5:12.5) to verify this. The attenuation of NBD-PE fluorescence by high concentrations of negatively charged POPS can account for the relatively low level of fluorescence observed.

The fluorescence assay used here has been previously shown to be insensitive to vesicle aggregation (Düzgüneş et al., 1987) and specifically measures dilution of fluorescent lipid probes. However, the assay does not distinguish between fusion and hemifusion (lipid mixing of only the outer monolayers of apposed vesicles), and it does not provide any information on the fate of the contents of the two vesicle populations, i.e., whether the contents mixing occurs or membrane destabilization gives leakage of the vesicle contents. Previous attempts to use contents mixing assays with cationic liposome systems have failed (Düzgüneş et al., 1989). We had similar difficulties with the DOPE/DODAC (1:1) system. The available contents mixing probes (calcein, aminonaphthalenetrisulfonic acid, and dipicolinic acid) are all multivalent anions that destabilize the cationic liposomes at millimolar concentrations in HBS. Without being able to encapsulate these probes, we have relied on lipid mixing, particle size analysis, and electron microscopy to determine the extent of membrane fusion.

Effects of the Phospholipid Headgroup. Substitution of DOPE for DOPC in negatively charged target vesicles (20 mol % POPS) resulted in much more rapid lipid mixing with DOPE/DODAC vesicles (Figure 2). The lipid mixing was followed by the formation of large aggregates that gave rise to increased noise and a slow decrease in the fluorescence as the aggregates precipitated. The increased lipid mixing is attributed to the mutual membrane destabilization caused by charge neutralization between DOPE/DODAC and DOPE/POPS vesicles compared to destabilization of only the DOPE/DODAC membranes when incubated with a DOPC/POPS target. DOPE/POPS liposomes containing 10−50 mol % POPS all gave similar lipid mixing behavior (only 20 mol % shown), although higher levels of aggregation were observed at the lower levels of POPS. Replacing DOPC with DOPA or DOPG also gave very rapid lipid mixing with DOPE/DODAC (not shown). Since both DOPA and DOPG are negatively charged phospholipids, the increase in mixing probably results more from increased neutralization of the DODAC charge than from any effect of phospholipid headgroup geometry.

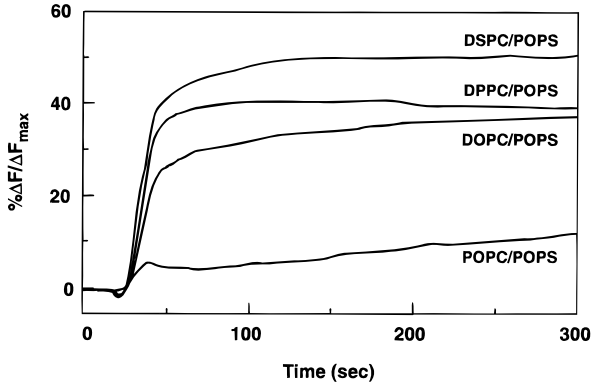

FIGURE 3: Effect of target vesicle acyl chain saturation on lipid mixing with DOPE/DODAC. DOPC, POPC, DPPC, and DSPC vesicles were all prepared with 20 mol % POPS and added to DOPE/DODAC (1:1) liposomes at 30 s.

Effects of Acyl Chain Saturation. To determine the effects of fatty acyl chain saturation in the target membranes on lipid mixing with DOPE/DODAC (1:1), we prepared target vesicles composed of 20 mol % POPS in various synthetic phosphatidylcholines. It was expected that increased chain saturation would give reduced lipid mixing since increasing chain order should stabilize the target bilayers. This was the case for POPC/POPS (80:20), which gave a much lower rate and extent of lipid mixing compared to DOPC/POPS (Figure 3). However, the disaturated phosphatidylcholines, DPPC and DSPC, gave higher levels of lipid mixing than DOPC. These unexpected results can be attributed to the phase behavior of the disaturated lipids. DPPC and DSPC both exist in the gel state at 25 °C while DOPC and POPC are liquid crystalline at this temperature. Membranes composed of gel state lipids can contain defects between planar lipid domains, and the presence of hydrophobic regions at these defects may be responsible for increased membrane destabilization and lipid mixing with DOPE/DODAC LUVs. Higher levels of lipid mixing with these disaturated systems relative to DOPC occur even in the absence of negatively charged POPS (data not shown).

The mixtures of DOPE/DODAC and target vesicles described above were subjected to size analysis by quasi-elastic light scattering (Figure 4). The results indicate very different interactions for DOPE/DODAC (1:1) with DSPC/POPS (80:20) compared to those observed with DOPC/POPS (80:20). Addition of negatively charged LUVs containing the disaturated lipid DSPC to DOPE/DODAC gave particles larger than could be measured by light scattering. In contrast the bis-monounsaturated lipid DOPC gave a smaller, measurable size increase of about 75% with an accompanying increase in size distribution. POPC vesicles without any negatively charged lipid gave no significant size increase when mixed with DOPE/DODAC. The size of the DOPE/DODAC (1:1) liposomes prior to addition of target vesicles is also given. Note that while the cationic liposomes are larger than expected for the given extrusion conditions due to their instability in HBS, they were combined with target vesicles in a ratio of 1:3 as for the lipid mixing assays. Therefore, the size increase observed for the mixture with DOPC/POPS is not simply a result of mixing vesicle populations of two different sizes. This is demonstrated by the POPC control, where no such increase is observed.

The effect of lipid saturation in target vesicles on fusion with DODAC/DOPE LUVs was also determined by freeze−

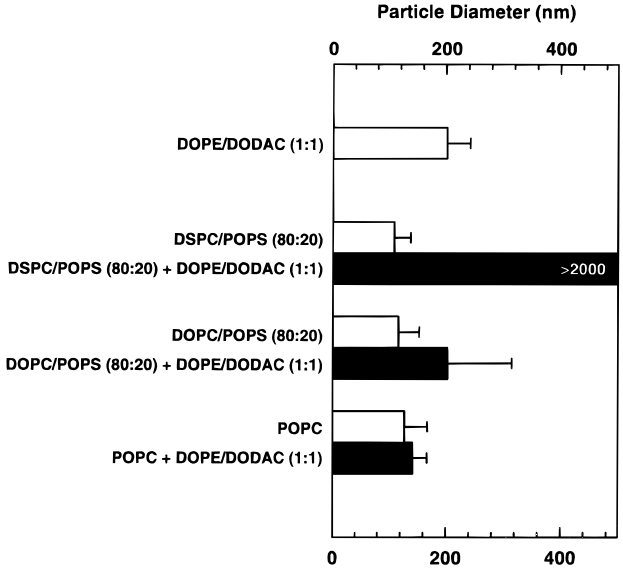

FIGURE 4: Size distributions of DOPE/DODAC (1:1) liposomes and 1:3 mixtures of DOPE/DODAC (1:1) with DSPC/POPS (80:20), DOPC/POPS (80:20), and POPC determined by quasi-elastic light scattering. Data accumulation was begun 5 min after addition of target vesicles and continued for 5 min with a photopulse rate of 350-400 kHz.

fracture electron microscopy (Figure 5). Addition of DSPC/POPS (80:20) vesicles to DOPE/DODAC resulted in very large aggregated structures with complex networks of continuous membrane. This correlates well with the high degree of membrane fusion indicated by the lipid mixing assay and the formation of very large particles as determined by light scattering. Addition of DOPC/POPS (80:20) target vesicles to DOPE/DODAC (1:1) gave numerous, distinct, large lipid vesicles (approaching 1 μm), indicating extensive fusion of liposomes without the formation of large aggregates, again in agreement with lipid mixing and light scattering results. The surfaces of these large vesicles are mostly smooth with some small indentations. These indentations, called "lipidic particles", have previously been observed in fusing liposomal systems and have been attributed to the existence of nonbilayer fusion intermediates (Verkleij et al., 1980). The freeze−fracture micrograph of the control mixture of POPC liposomes with DOPE/DODAC (1:1) indicated no membrane fusion with mostly 100 nm LUVs and some larger vesicles (∼200 nm) which can be attributed to DOPE/DODAC vesicles (see Size Analysis).

Effect of Cholesterol on Disaturated Lipid Vesicles. The presence of cholesterol in DSPC or DPPC membranes results in the disappearance of the gel state and the formation of an ordered liquid crystalline phase (Bloom et al., 1991). At 25 °C, this ordered lamellar phase can be detected with the addition of 3−4 mol % cholesterol, and at concentrations greater than 25 mol % no gel state domains remain. To demonstrate that the existence of the gel phase lipid was responsible for the extensive lipid mixing observed for the disaturated phospholipid target vesicles, we studied the effect of adding increasing levels of cholesterol to DSPC vesicles while maintaining the level of negatively charged POPS at 20 mol % (Figure 6). Addition of 5−10 mol % cholesterol gave small increases in lipid mixing, but at 20 mol % cholesterol, the level of fluorescence attained was diminished and at 45 mol % cholesterol it was reduced to less than half. Interestingly, the decreases in lipid mixing observed at the

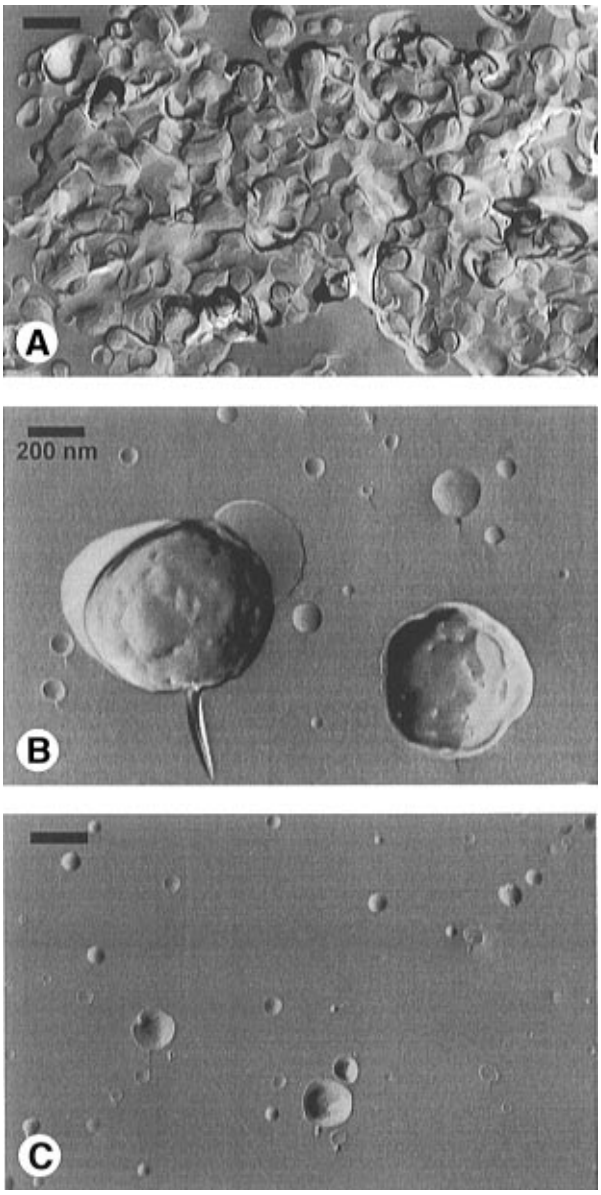

FIGURE 5: Freeze−fracture electron micrographs of DOPE/DODAC (1:1) liposomes mixed in a 1:3 lipid ratio with liposomes composed of (A) DSPC/POPS (80:20), (B) DOPC/POPS (80:20), and (C) POPC. The total lipid concentration was 10 mM. Samples were incubated for 5 min at 25 °C and mixed 1:1 with glycerol prior to preparation of platinum−carbon replicas. Original magnification was 20000×, and bars represent 200 nm.

higher levels of cholesterol appeared to be accompanied by an increase in vesicle aggregation as evidenced by the decrease in the fluorescence over time and by the turbid appearance of the sample.

Lipid Mixing with Erythrocyte Ghosts. The destabilization of DOPE/DODAC vesicles by the addition of liposomes containing negatively charged phospholipids is a crude model to study the interaction of cationic liposomes with biological membranes. The outer leaflets of cell membranes normally have very low levels of negatively charged phospholipids (Houslay & Stanley, 1982). The negative surface charge of cells arises primarily from the presence of acidic residues on membrane proteins and glycolipids. It is assumed that the observed interactions of cationic liposomes with cells involve charge attraction to anionic residues near the cell surface.

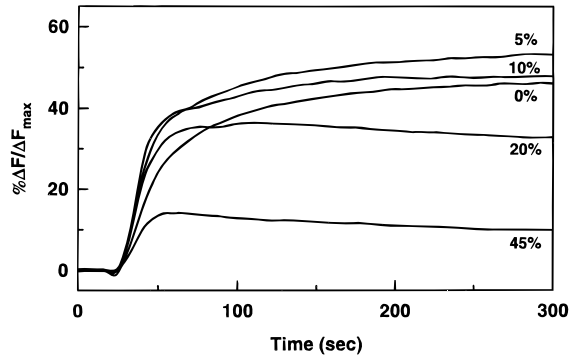

FIGURE 6: Effect of cholesterol concentration on lipid mixing of DOPE/DODAC (1:1) and DSPC/cholesterol. At 30 s, DSPC/Chol liposomes prepared with Chol concentrations ranging from 0 to 45 mol % were added to fluorescently labeled DOPE/DODAC liposomes.

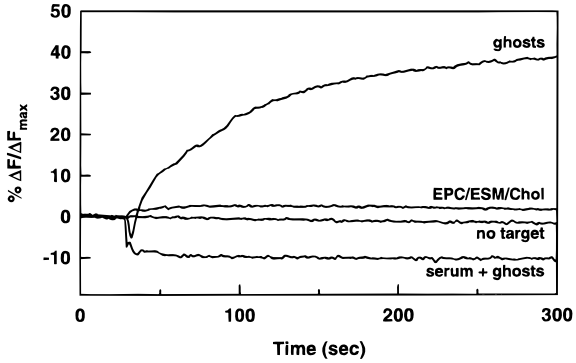

FIGURE 7: Fusion of DOPE/DODAC (1:1) liposomes with erythrocyte ghosts. Fluorescently labeled liposomes and ghosts were mixed in a 1:3 lipid ratio (0.2 mM total lipid) at 30 s in either the absence or presence of 1% (v/v) mouse serum. For comparison, EPC/ESM/Chol (1:1:2) was assayed as a model of the lipid composition of the outer leaflet of the erythrocyte membrane. Also shown is a control without the addition of ghosts or target vesicles.

Erythrocyte ghosts were added to DOPE/DODAC (1:1) liposomes in HBS to demonstrate lipid mixing of this cationic lipid system with a biological membrane (Figure 7). An initial drop in NBD-PE fluorescence occurred due to light scattering of the relatively large ghosts. Fluorescence then increased over the duration of the assay, reaching a value of $\Delta F/\Delta F_{max} \sim 40\%$ at 5 min. The high rate of fusion observed is presumably due to anionic residues present on the exterior of the erythrocyte membrane. While it has been shown previously that glycolipids can reduce fusion between liposomes (Hoekstra & Düzgüneş, 1986; Maggio & Yu, 1992) presumably through steric effects, ionic interactions between cationic liposomes and the anionic surface residues appear to negate any steric effect. The importance of the negatively charged residues was demonstrated by mixing DOPE/DODAC liposomes with vesicles composed of EPC/ESM/cholesterol (1:1:2; EPC = egg phophatidylcholine, ESM = egg sphingomyelin) which approximates the lipid composition of the outer leaflet of the erythrocyte membrane (Houslay & Stanley, 1982). Very little lipid mixing was observed.

The high level of lipid mixing between DOPE/DODAC (1:1) and erythrocyte ghosts is in contrast to previous reports that cationic liposomes do not fuse with the plasma membranes of cultured cells *in vitro* and that endocytosis of lipid−DNA complexes is necessary for transfection (Wrobel & Collins, 1995). However, the presence of as little as 1%

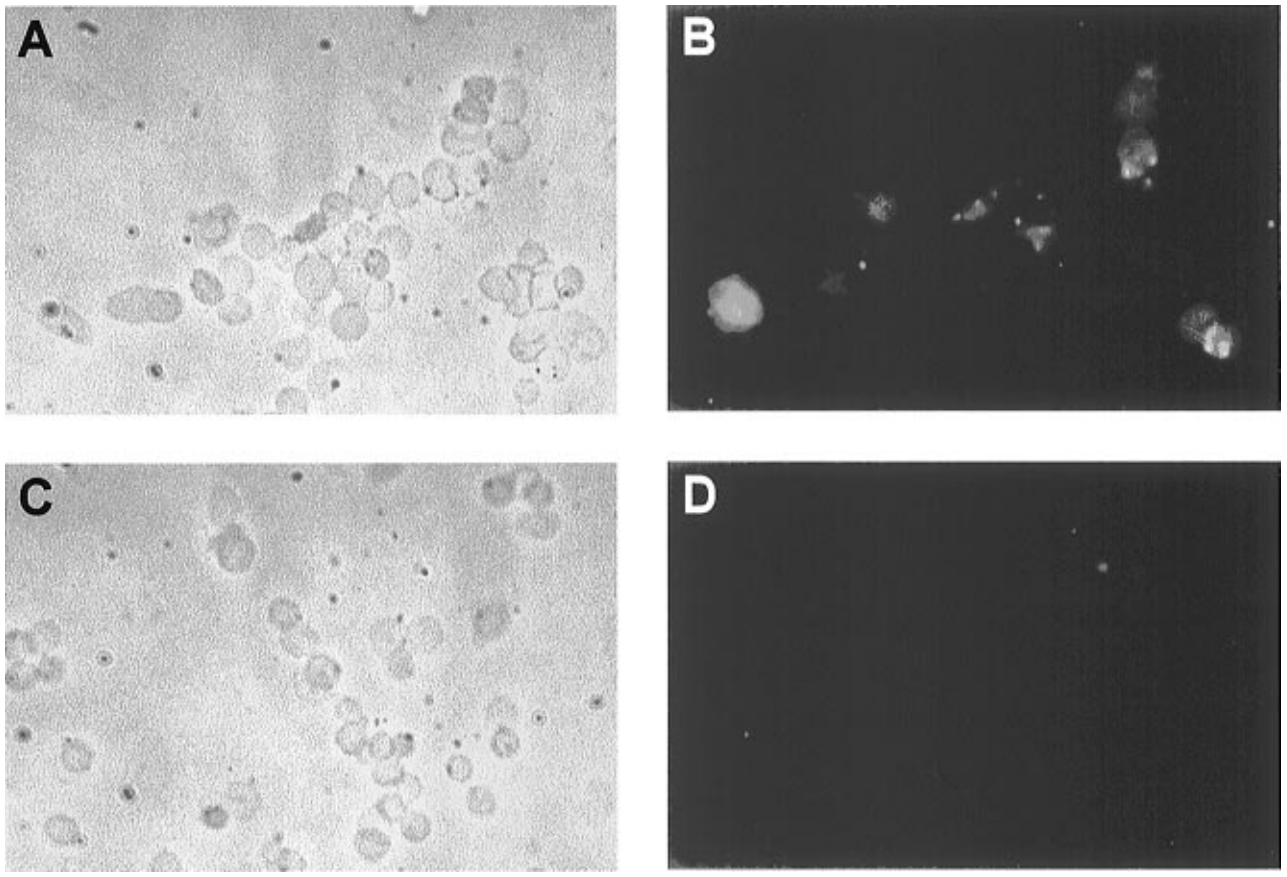

FIGURE 8: Fluorescence micrographs showing the appearance of Rh-PE in erythrocyte ghosts upon lipid mixing with labeled DOPE/DODAC (1:1). Liposome and ghosts were mixed in a 1:3 lipid ratio (1 mM total lipid): (A) phase contrast and (B) Rh-PE fluorescence in HBS; (C) phase contrast and (D) Rh-PE fluorescence when liposomes were preincubated with 1% (v/v) mouse serum prior to addition of ghosts. Original magnification 200× (reproduced at 70% of original size).

serum eliminates lipid mixing between DOPE/DODAC and erythrocyte ghosts (Figure 7) due to interactions of serum proteins with the cationic liposomes, if not also with the ghost membranes. Serum, which is normally included in the medium of cultured cells, would therefore be expected to inhibit fusion of cationic liposomes with cell membranes. In a similar experiment, adding 1% serum to a mixture of DOPE/DODAC (1:1) and DOPC/POPS (80:20) also prevented lipid mixing (data not shown). Furthermore, serum prevents self-fusion of DOPE/DODAC (85:15) vesicles in low salt buffers.

Lipid mixing of fluorescently labeled DOPE/DODAC (1:1) liposomes and erythrocyte ghosts as well as the effects of serum was also observed by fluorescence microscopy (Figure 8). Prior to lipid mixing, the Rh-PE is present in the DOPE/DODAC at self-quenching concentrations (Arbuzova et al., 1993). Five minutes after the addition of erythrocyte ghosts, many of the ghosts exhibit Rh-PE fluorescence. In the presence of 1% serum, however, no labeling of ghosts occurs.

DISCUSSION

Fusion of cationic liposomes composed of DOPE/DODAC (1:1) with a population of target membranes is dependent on the concentration of negative charge in the target. The negative charge has several probable roles. First, it promotes close contact between the two membrane surfaces by charge attraction. This results in a mutual surface-charge neutralization which reduces the hydrophilic nature of the apposed membranes and promotes intimate contact by the loss of water previously bound through hydrogen bonding. Finally, the neutralization of the DODAC promotes membrane destabilization by virtue of the preference of DOPE to adopt nonbilayer structures, in the same way that addition of multivalent anions or high salt concentrations destabilize these systems (Düzgüneş et al., 1989).

While in our model systems the negative charge is provided by an anionic phospholipid, it appears that the negative charges associated with cell membranes such as acidic residues on glycolipids or proteins can also fulfill this role. Erythrocyte membranes do not have negatively charged phospholipids exposed on the outer leaflet, yet they readily fuse with DOPE/DODAC liposomes in the absence of serum.

The complete inhibition of fusion in the presence of as little as 1 mol % serum is an important observation. It is consistent with reports that DNA transfection with cationic liposomes occurs only after endocytosis in *in vitro* cell culture where serum is normally included at higher concentrations. That serum components interact with both negatively and positively charged liposomal surfaces *in vitro* and *in vivo* has been previously documented (Chonn et al., 1992; Senior et al., 1991). How the stabilizing effects of the serum components might be negated within the endosome to allow fusion of cationic liposome complexes with the endosomal membrane remains unexplored.

Destabilization of DOPE/DODAC liposomes was promoted by the presence of high concentrations of DOPE in the target membranes, which is again attributed to the

preference of this lipid to adopt a hexagonal phase. Fusion was also promoted by the disaturated lipids, DPPC and DSPC, which do not form hexagonal structures. This unexpected effect is attributed to the presence of defects in LUVs composed of saturated phosphatidylcholines at temperatures below the gel to liquid crystalline transition temperature. As shown by Nayar et al. (1989), these defects can be visualized by freeze−fracture electron microscopy as interfaces between planar regions on the LUV surface. The ability of cholesterol to inhibit gel phase formation and the corresponding loss of fusion with DODAC/DOPE LUVs suggest a role for the hydrophobic defects present in gel state LUVs in the fusion events. In similar work, fusion of liposomes promoted by the formation of defects upon freezing was demonstrated by Hui et al. (1981).

A complete understanding of the processes by which DNA complexes with cationic liposomes achieve cellular transfection must include an understanding of the role of the cellular membranes in these processes and how their interactions with cationic liposomes are influenced by membrane composition. It is clear from this work that destabilization by and fusion with cationic liposomes depend not only on charge attraction leading to contact with the target membrane but also on the composition of the target membranes which determines the propensity of the target membrane to undergo fusion.

ACKNOWLEDGMENT

We are grateful to Dr. Myrna Monck for providing additional data on the effect of serum in preventing fusion in cationic lipid systems.

REFERENCES

Arbuzova, A., Korte, T., Müller, P., & Herrmann, A. (1993) *Biochim. Biophys. Acta 1190*, 360−366.
Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.
Behr, J.-P. (1994) *Bioconjugate Chem. 5*, 382−389.
Bennett, M. J., Nantz, M. H., Balasubramaniam, R. P., Gruenert, D. C., & Malone, R. W. (1995) *Biosci. Rep. 15*, 47−53.
Bloom, M., Evans, E., & Mouritsen, O. G. (1991) *Q. Rev. Biophys. 24*, 293−397.
Chemomordik, L. V., Kozlov, M. M., Milikyan, G. B., Abidor, I. G., Markin, V. S., & Chizmadzhev, Y. A. (1985) *Biochim. Biophys. Acta 812*, 643−655.
Chonn, A., Semple S. C., & Cullis, P. R., (1992) *J. Biol. Chem. 267*, 18759−18765.
Cullis, P. R., Tilcock, C. P. S., & Hope, M. J. (1986b) Lipid Polymorphism, in *Membrane Fusion* (Wilschut, J., & Hoekstra, D., Eds.) pp 35−64, Marcel Dekker, New York.
Das, S., & Rand, R. P. (1986) *Biochemistry 25*, 2882−2889.
Düzgüneş, N., Allen, T. M., Fedor, J., & Papahadjopoulos, D. (1987) *Biochemistry 26*, 8435−8442.
Düzgüneş, N., Goldstein, J. A., Friend, D. S., & Felgner, P. L. (1989) *Biochemistry 28*, 9179−9184.
Farhood, H., Bottega, R., Epand, R. M., & Huang, L. (1992) *Biochim. Biophys. Acta 1111*, 239−246.
Farhood, H., Serbina, N., & Huang, L. (1995) *Biochim. Biophys. Acta 1235*, 289−295.
Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., & Danielsen, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7413−7417.
Fisher, K., & Branton, D. (1974) *Methods Enzymol. 32*, 35.
Gao, X., & Huang, L. (1991) *Biochem. Biophys. Res. Commun. 179*, 280−285.
Hoekstra, D., & Düzgüneş, N. (1986) *Biochemistry 25*, 1321−1330.
Houslay M. D., & Stanley, K. K. (1982*) Dynamics of biological membranes: influence on synthesis, structure, and function*, Wiley, New York.
Hui, S. W., Stewart, T. P., Boni, L. T., & Yeagle, P. L. (1981) *Science 212*, 921−923.
Ito, A., Miyazoe, R., Mitoma, J., Akao, T., Osaki, T., & Kunitake, T. (1990) *Biochem. Int. 22*, 235−241.
Leventis, R., & Silvius, R. J. (1990) *Biochim. Biophys. Acta 1023*, 124−132.
Maggio, B., & Yu, R. K. (1992) *Biochim. Biophys. Acta 1112*, 105−114.
Nayar, R., Hope, M. J., & Cullis, P. R. (1989) *Biochim. Biophys. Acta 986*, 200−206.
Senior, J. H., Trimble, K. R., & Maskiewicz, R. (1991) *Biochim. Biophys. Acta 1070*, 173−179.
Stamatatos, L., Leventis, R., Zuckermann, M. J., & Silvius, J. R. (1988) *Biochemistry 27*, 3917−3925.
Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 31*, 172−180.
Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093−4099.
Tilcock, C. P. S., & Cullis, P. R. (1982) *Biochim. Biophys. Acta 684*, 212−218.
Tilcock, C. P. S., Cullis, P. R., & Gruner, S. M. (1988) *Biochemistry 27*, 1415−1420.
Verkleij, A. J., van Echteld, C. J. A., Gerritsen, W. J., Cullis, P. R., & de Kruiff, B. (1980) *Biochim. Biophys. Acta 600*, 620−624.
Wrobel, I., & Collins, D. (1995) *Biochim. Biophys. Acta 1235*, 296−304.
Zabner, J., Fasbender, A. J., Moninger, T., Poellinger, K. A., & Welsh, M. J. (1995) *J. Biol. Chem. 270*, 18997−19007.
Zschörnig, O., Arnold, K., Richter, W., & Ohki, S. (1992) *Chem. Phys. Lipids 63*, 15−22.

BI961173X