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Fusion of cationic liposomes with mammalian cells occurs after endocytosis

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Abstract

The interaction of cationic liposomes prepared using either dioleoyltrimethylammonium propane (DOTAP) or 3β-(*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl)cholesterol (DC-CHOL) with model membranes and with cultured mammalian cells was examined using an assay developed for monitoring virus-cell fusion (Stegmann et al. (1993) Biochemistry 32, 11330–11337). Lipid mixing between cationic liposomes and liposomes composed of DOPE/dioleoylphosphatidylglycerol (DOPG) or dioleoylphosphatidylcholine (DOPC)/DOPG was insensitive to pH in the range of pH 4.5–7.0 and was not affected by sodium chloride concentration in the range of 0–150 mM. Lipid mixing was dependent on dioleoylphosphatidylethanolamine (DOPE), since cationic liposomes prepared using dioleoylphosphatidylcholine (DOPC) were incapable of lipid mixing with DOPC/DOPG liposomes. The interaction of cationic liposomes with Hep G-2 and CHO D^- cells was also studied. For both cell types, liposome-cell lipid mixing was rapid at 37° C, beginning within minutes and continuing for up to 1 hour after uptake. The extent of lipid mixing was decreased at 15° C, especially at later (≥ 20 min) time points. This suggests that at least part of the observed lipid mixing occurred after reaching cellular lysosomes. No lipid mixing was seen at 4° C. Monensin inhibited lipid mixing between cationic liposomes and the cells, despite having no effect on liposome uptake. Inhibition of endocytic uptake of liposomes, either by incubation in hypertonic media or by depletion of cellular ATP with sodium azide and 2-deoxyglucose abolished liposome-cell fusion in both cell types. These data demonstrate that binding to the cell surface is insufficient for cationic liposome-cell fusion and that uptake into the endocytic pathway is required for fusion to occur.

Keywords: Membrane fusion; Endocytosis; Cationic liposome; Liposome

1. Introduction

Cationic liposomes have proven to be useful tools for delivery of plasmid DNA and RNA into cells [1–7]. Improved delivery of antisense oligonucleotides using cationic liposomes has also been demonstrated [8]. Recently, cationic liposomes have been shown to mediate delivery and expression of plasmid DNA after i.v. injection in the mouse model [9]. Despite these repeated successes, very little is understood about the events which take place when cationic liposomes interact with mammalian cells or the processes which result in the delivery of nucleic acids. There are three current models for the interaction: (A) liposome-cell fusion within or destabilization of the endosome [2,4,6], (B) direct fusion with the plasma membrane [1], and (C) transfer of the lipid-DNA complex across cellular membranes into the cytosol, with migration of the complex into the nucleus [10]. However, no definitive evidence in support of any particular model has been presented.

The present work is a first step in attempting to understand the interaction of cationic liposomes with mammalian cells and the implications of the interaction for oligonucleotide and plasmid DNA delivery. Since cationic liposomes have been shown to fuse with model anionic membranes [4,11,12] and with erythrocyte ghosts [11], and since fusion is one of the important putative mechanisms of the liposome–cell interaction, we have examined the fusion of cationic liposomes with two types of mammalian

Abbreviations: DOTAP, dioleoyltrimethylammonium propane; DC-CHOL, 3β-(*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl)cholesterol; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; Pyr-PC, 3-palmitoyl-2-(1-pyrenedecanoyl)phosphatidylcholine; Pyr-PE, *N*-pyrenyl egg phosphatidylethanolamine; F-PE, *N*-(5-fluoresceinthiocarbamoyl)dipalmitoylphosphatidylethanolamine; [^{3}H]CE, palmityl[1,2-^{3}H(N)]cholesteryl ether.

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cultured cells. Our findings demonstrate that cationic liposomes fuse with mammalian cells after endocytosis.

2. Materials and methods

2.1. Materials

All lipids except DC-CHOL and Pyr-PC were purchased from Avanti Polar Lipids (Alabaster, Al) and were evaluated periodically by thin-layer chromatography. DC-CHOL was synthesized as described [7]. Pyr-PC was purchased from Molecular Probes (Eugene, OR). Hep G-2 hepatoma cells were obtained from American Type Culture Collection and were grown in DMEM containing 10% FBS. CHO D^- cells were obtained from Dr. G. Trail (Amgen) and were grown in RPMI 1640, containing 10% FBS and 1% penicillin/streptomycin/glutamine. D-PBS was obtained from Gibco, BRL (Gaithersburg, MD).

2.2. Liposome preparation

Sonicated vesicles were prepared by drying appropriate amounts of the desired lipids from their chloroform stocks to produce dry lipid films. A mol ratio of 3:1 was used for DOPE/DOTAP and DOPC/DOTAP liposomes; a mole ratio of 2:3 was used for DOPE/DC-CHOL and DOPC/DC-CHOL liposomes. The lipid films were desiccated for 1 h under high vacuum using a Savant speedvac desiccator. The lipid was then hydrated in distilled water on ice for ≥ 1 h. The hydrated lipid samples were sonicated for 2–3 min in a bath type sonicator (Laboratory Supplies, Hicksville, NY) to form optically clear dispersions. The diameter of the liposomes was measured using a Malvern 4700c multiangle light scattering instrument. The mean diameter of DOPE/DOTAP (3:1, molar) liposomes prepared by this method was 65 ± 5 nm (polydispersity = 0.18); the mean diameter of DOPE/DC-CHOL (2:3, molar) was 78 ± 4 nm (polydispersity = 0.10).

2.3. Interliposome fusion

Fluorescence measurements were made using a PTI Alphascan instrument (PTI, South Brunswick, NJ) equipped with two emission channels and a water-jacketed four cuvette turret-type holder connected to a circulating water bath. The samples were excited at 343 nm and emission at 395 nm (pyrene monomer) and 483 nm (pyrene excimer) recorded. The excitation and emission slit widths were 3 nm. For each measurement, 40 nmoles of cationic liposomes (containing 5 mol% Pyr-PE or Pyr-PC, as indicated) were added to a fluorescence cuvette containing 3 ml of distilled water buffered to different pH values using mixtures of 5 mM Hepes (pH 7.0) and 5 mM sodium acetate (pH 4.5) to achieve final pH values between 7.0 and 4.5. In some experiments, small aliquots of 3 M NaCl were added to the samples to achieve final NaCl concentrations between 0–150 mM. The initial pyrene excimer fluorescence was recorded. To initiate fusion, 120 nmol of unlabeled DOPE/DOPG or DOPC/DOPG liposomes were added and the samples mixed by inverting the cuvette. The excimer fluorescence was observed to decrease rapidly for the first minute after mixing. No further decreases were observed after 1 min incubation time. After 2 min, the pyrene excimer fluorescence was read. Triton X-100 was then added to a final concentration of 0.5% to completely mix the lipids. The extent of lipid mixing was calculated using:

$$\% \text{ maximal lipid mixing} = 100 \times \frac{(F_0 - F)}{(F_0 - F_{Tx})},$$

where F_0 and F are the excimer fluorescence intensities in the absence and presence, respectively, of anionic liposomes and F_{Tx} is the excimer fluorescence in the presence of Triton X-100.

In some experiments, cationic liposomes were prepared to contain 1 mol% N-NBD-PE and 0.5 mol% N-Rh-PE. Lipid mixing with DOPE/DOPG or DOPC/DOPG liposomes was evaluated as described by Connor et al. [13].

2.4. Liposome-cell fusion

In order to evaluate lipid mixing between cationic liposomes and cells, a cell suspension was prepared by treating 4–6-day-old monolayers grown in 60 mm dishes with trypsin, EDTA (Gibco, Gaithersburg, MD). Trypsinized cells were counted using a hemacytometer and diluted to 2e6 cells/ml in ice-cold serum-free media. Cells were kept on ice until use. D-PBS (3 ml) was equilibrated to the desired temperature in a fluorescence cuvette. 40 nmol of either DOPE/DOTAP (3:1 mol/mol), DOPC/DOTAP (3:1 mol/mol), DOPE/DC-CHOL (2:3 mol/mol) or DOPC/DC-CHOL (2:3 mol/mol) liposomes (prepared to contain 5 mol% Pyr-PE or 5% Pyr-PC, as indicated) were allowed to bind to 200 000 cells in 1 ml of D-PBS for 15 min on ice. The cells were then pelleted by centrifugation (4500 rpm) at 4° C. The cells were resuspended in 1 ml ice-cold D-PBS and centrifuged a second time. This second pellet was resuspended in 100 μl ice-cold D-PBS. The resuspended cells with bound liposomes were then pipetted into a cuvette containing 3 ml of D-PBS at the desired temperature in the sample chamber of the fluorometer. In the absence of cells, no changes in pyrene excimer fluorescence were observed for up to 1 h of liposome incubation in D-PBS at 37° C (data not shown). The samples were stirred throughout the experiment. The initial excimer fluorescence (F_0) recorded upon cell addition. Excimer fluorescence (F) was monitored over a 60 min period. At the end of the incubation period, Triton X-100 was added to a final concentration of 1.0% and F_{Tx} recorded. The extent of fusion was calculated as described above. Inhibitors of

endocytosis, when present, were incubated with cells for 1 h (at 37° C in serum-free RPMI) prior to liposome binding and were present throughout the experiment.

2.5. Liposome uptake

Cationic liposomes were prepared to contain a trace amount of [^{3}H]cholesterylhexadecyl ether as a marker for lipid uptake. Cells were grown in 24-well plates to ~ 80% confluency and then washed in serum-free media. Liposomes (40 μM) were incubated with cells for 15 min on ice to allow binding but not internalization. Incubations were also done for 30 min at 37° C to allow both binding and internalization. All incubations were performed in serum free media. Inhibitors of endocytosis, where indicated, were present for 1 h prior to liposome addition and throughout the liposome incubation period. Cells were washed five times in serum-free media and then dissolved overnight using 0.1 M NaOH. The protein content of each well was determined by BCA assay (Pierce, Rockford IL). Data are expressed as nmol lipid uptake per mg cellular protein. All determinations were done in triplicate. Binding and uptake of liposomes by CHO cells was performed in a similar fashion.

2.6. Fluorescence microscopy

CHO D^- cells were grown on 18 mm round glass coverslips (No. 1 thickness) in RPMI 1640, containing 10% FBS. Liposomes composed of DOPE/DC-CHOL (2:3 mol/mol) were prepared to contain 5 mol% Fluorescein-labeled DPPE (FPE) (Molecular Probes, Eugene, OR). CHO cells were washed in serum free media prior to liposome addition. In some studies, ATP-depleting media (RPMI + 20 mM NaN_3/50 mM 2-DG) or hypertonic media (RPMI + 0.45 M sucrose) were incubated with cells for 1 h at 37° C prior to liposome addition and throughout the liposome incubation period. FPE-labeled liposomes at a final lipid concentration of 50 μM were incubated with cells for 30 min at 37° C in serum free RMPI. The cells were then washed five times with serum free media and fixed for 20 min (room temperature) in 1% paraformaldehyde prior to observation on a Nikon Microphot FX fluorescence microscope, using a 60 × oil immersion lens.

3. Results

The assay used in these studies is based on a method developed for following the fusion of viruses with cells [14,15] and monitors the decrease in excimer fluorescence that results from the dilution of lipids in a labeled fusogenic membrane into an unlabeled target membrane. The assay has been demonstrated to be insensitive to lipid transfer [14,15] and other artifacts associated with other lipid mixing assays [15]. Since fusion of cationic liposomes with anionic liposomes has been demonstrated using other lipid mixing assays [11,12], it was first determined if the pyrene excimer assay would faithfully report interliposome fusion. As shown in Fig. 1, addition of DOPE/DOPG (1:1) liposomes to DOPE/DOTAP (3:1) liposomes containing 5 mol% Pyr-PE resulted in a decrease in pyrene excimer fluorescence, a result consistent with the occurrence of liposome fusion under these conditions. Addition of Triton X-100 to fully mix the lipids completely abolished excimer fluorescence (Fig. 1). Using 5 mol% Pyr-PE in unfused DOPE/DOTAP (3:1) liposomes led to an excimer/monomer ratio of 0.36. Similar data were obtained using DOPE/DC-CHOL liposomes containing Pyr-PC (data not shown). In agreement with a previous study [15], a linear increase in excimer/monomer ratio with increasing mol% of Pyr-phospholipids was observed (data not shown). Addition of negatively charged liposomes to pyrene labeled DOPE/DOTAP liposomes led to a decrease in the excimer to monomer ratio. In the assay, either absolute excimer fluorescence or E/M ratio can be followed. In our hands both methods yielded similar results. For simplicity, only the data obtained using measurements of excimer fluorescence is reported here. In order to avoid any potential alteration of liposome behavior at higher amounts of Pyr-phospholipid, all subsequent studies were performed using 5 mol% Pyr-PE or Pyr-PC.

The pH dependence of the fusion of cationic liposomes with anionic liposomes was studied. The data in Fig. 2 demonstrate that the fusion of DOPE/DOTAP (3:1 molar) with DOPE/DOPG (1:1 molar) is insensitive to pH in the range of 4.5–7. The fusion of DOPE/DOTAP (3:1) with DOPC/DOPG (1:1) was virtually indistinguishable from the fusion observed using DOPE/DOPG liposomes as target membranes (Fig. 2) a finding consistent with the work of others [11,12]. No fusion between DOPC/DOTAP (3:1) and DOPC/DOPG (1:1) membranes was observed (Fig. 2), demonstrating the important role played by DOPE in the fusion process. The excimer fluorescence for pyrene-labeled DOPC/DOTAP liposomes did not change over the pH range examined suggesting that the signal monitored was not pH-dependent. The fact that DOPC/DOTAP/Pyr-PE liposomes showed no apparent fusion with DOPC/DOPG liposomes also suggests that interliposome transfer of Pyr-PE was insignificant. We did observe some lipid mixing (up to 15% maximal mixing) when DOPC/DOTAP/Pyr-PE liposomes were combined with a 3-fold molar excess of DOPE/DOPG liposomes (data not shown). This indicates that only one of the liposome populations needs to contain DOPE for fusion to occur, in this case the fusion is mediated by the presence of DOPE in the DOPE/DOPG liposomes rather than in the cationic liposome population. Similar data were obtained using DOPC/DC-CHOL/Pyr-PC liposomes (data not shown).

We also compared the pH-dependency of fusion using a different lipid mixing assay which is based on energy

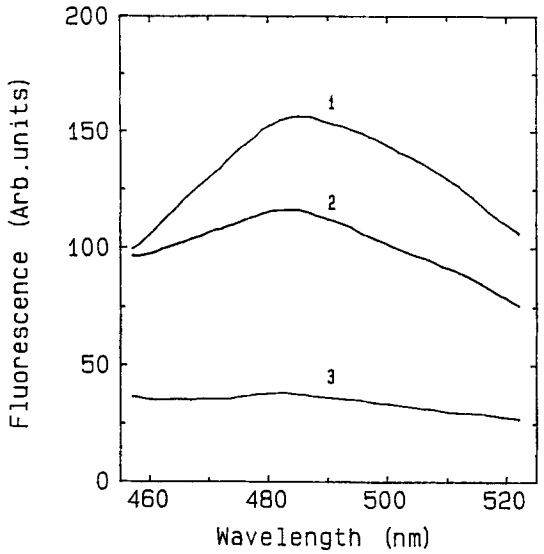

Fig. 1. Effect of anionic liposomes on excimer emission from pyrene-labeled cationic liposomes. Pyrene excimer emission spectra (excitation wavelength = 343 nm) for 40 nmol of DOPE/DOTAP/Pyr-PE (3:1:0.21, molar) liposomes in the absence (curve 1) or presence (curve 2) of 120 nmol of DOPE:DOPG (1:1, molar) liposomes and after addition of Triton X-100 to 0.5% (curve 3).

transfer between N-NBD-PE and N-Rh-PE was examined [14,16]. No pH dependency of fusion was observed using the latter assay, confirming our results using the pyrene excimer assay (data not shown). Similar data were obtained using DOPE/DC-CHOL liposomes (data not shown). The effect of NaCl on the fusion of DOPE/DOTAP (3:1) with DOPC/DOPG (1:1) was also studied. In contrast to the observations of Stamatatos et al. [11], NaCl had no effect on the extent of fusion of DOPE/DOTAP with DOPC/DOPG liposomes (Fig. 2, inset). In this respect, or data is more similar to that reported by Düzgünes et al. [12], who observed that while multivalent anions promoted fusion, sodium acetate, a monovalent anion had no effect on the fusion of DOPE/DOTMA liposomes. The effect on NaCl on liposome fusion using the N-NBD-PE/N-Rh-PE resonance energy transfer assay was also examined. DOPE/DOTAP liposomes were labeled with 1 mol% N-NBD-PE and 0.5 mol% N-Rh-PE and fusion with unlabeled DOPC/DOPG liposomes examined as described above. Using this assay, lipid mixing was not affected at NaCl concentrations up to 150 mM (Fig. 2, inset). Similar data were obtained using DOPE/DC-CHOL liposomes (data not shown).

Using liposomes prepared to contain trace amounts of

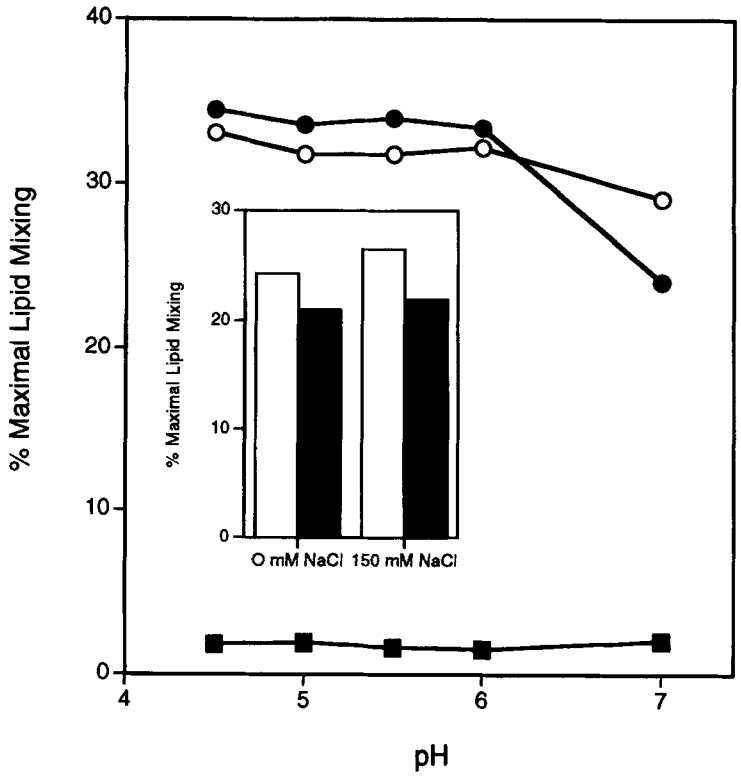

Fig. 2. Interliposome lipid mixing is pH-independent. Lipid mixing at different final pH was assayed as described in Materials and methods for mixtures of either DOPE/DOTAP/Pyr-PE (3:1:0.21) liposomes (○, ●) or DOPC/DOTAP/Pyr-PE (3:1:0.21) (■) liposomes with DOPE/DOPG (1:1) (○) or DOPC/DOPG (1:1) (●, ■) liposomes. (Inset) Lipid mixing between DOPE/DOTAP and DOPC/DOPG liposomes was assayed at pH 7.0 in the presence or absence of 150 mM NaCl. Lipid mixing was assayed using either the pyrene excimer assay (open bars) or the NBD-PE/Rh-PE energy transfer assay (stippled bars) [13,16].

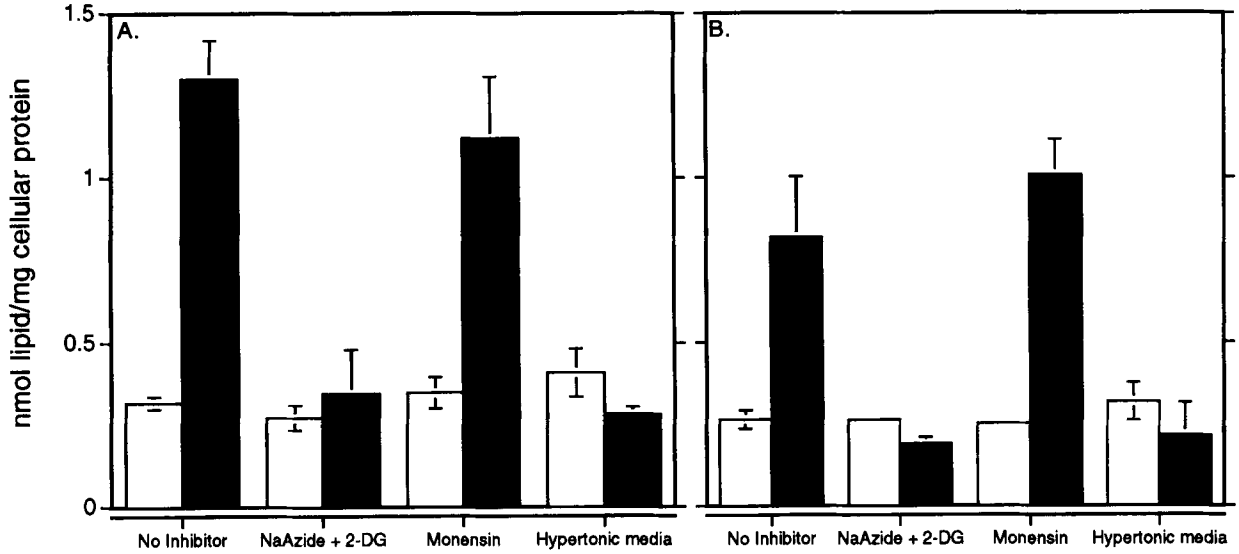

Fig. 3. Effect of inhibitors of endocytosis on binding and uptake of liposomes by Hep G2 cells. The binding and uptake of ^{3}H-labeled DOPE/DOTAP (A) and ^{3}H-labeled DOPC/DOTAP (B) liposomes by Hep G2 cells was evaluated after incubation for 15 min on ice (open bars) or for 30 min at 37° C (filled bars). The liposome concentration added was 40 μM. Inhibitors were present at the concentrations indicated in Fig. 4 for 1 h (at 37° C) prior to addition of liposomes and were present throughout the liposome incubation.

[^{3}H]CE, the cell binding (on ice for 15 min) of DOPE/DOTAP/Pyr-PE and DOPC/DOTAP/Pyr-PE liposomes was compared. No significant differences in binding between DOPE/DOTAP and DOPC/DOTAP liposomes were observed (Fig. 3). Likewise, no major differences in the binding of either liposome composition was seen when cells were pre-treated with either monensin, hypertonic media or sodium azide/2-deoxyglucose (Fig. 3). Uptake of both types of liposomes was significantly increased when the incubation was performed at 37° C, since at this temperature the internalization of surface-bound liposomes is allowed (Fig. 3). While liposome binding (on ice) was not affected, liposome uptake at 37° C was inhibited when cells were pre-incubated in either ATP-depleting or hypertonic conditions (Fig. 3). No major decrease in uptake (at 37° C) for either liposome composition was observed when cells were pre-incubated in the presence of 10^{-5} M monensin (Fig. 3). These data suggest that liposome endocytosis occurs at 37° C and that binding and uptake of DOPE/DOTAP and DOPC/DOTAP liposomes is not significantly different. Furthermore, the data show that inhibitors of endocytosis, such as sodium azide/2-deoxyglucose and hypertonic media, allow binding but not uptake of liposomes at 37° C. Similar binding and uptake results were obtained using CHO D^{-} cells (data not shown).

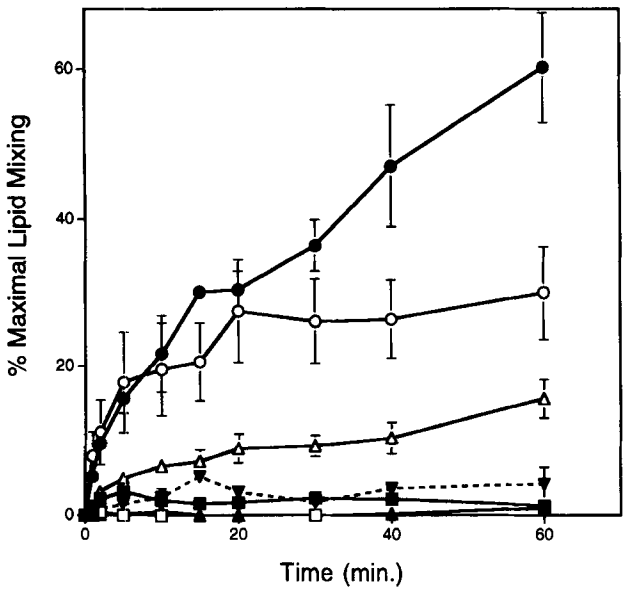

Fig. 4. Fusion of cationic liposomes with Hep G-2 cells requires endocytosis. Lipid mixing was assayed as described in Materials and methods for cationic liposomes composed of either DOPE/DOTAP/Pyr-PE (3:1:0.21 molar) (●, ○, ■, □, ▲, △) or DOPC/DOTAP/Pyr-PE (3:1:0.21 molar) (▼) and trypsinized Hep G-2 cells. Incubations were performed in the absence of inhibitors at 37° C (●, ▼), 15° C (○) and 4° C (■). Lipid mixing was also assayed on cells pretreated for 1 hr with 20 mM NaN_3 + 50 mM 2-DG (□), 0.45 M sucrose (▲), and 10^{-5} M monensin (△). Both pretreatment and lipid mixing were performed at 37° C and inhibitors were present throughout the liposome incubation. The data represent the mean ± S.D. for four separate experiments.

The fusion of cationic liposomes with Hep G-2 cells was then examined. In order to make fluorescence measurements on these cells it was necessary to detach the cells from the culture flask by trypsinization. Trypsinized Hep G-2 cells have been used in previous studies to monitor respiratory syncytial virus fusion [17]. Cell viability, as measured by trypan blue exclusion was greater than 95% after trypsinization and greater than 90% after 1 h in D-PBS at 37° C (data not shown).

Lipid mixing at three different temperatures, 4° C, 15° C and 37° C was studied. At 4° C where endocytosis is

inhibited, no lipid mixing between DOPE/DOTAP/Pyr-PE (3:1:0.21) liposomes and Hep G-2 cells was observed, even after 1 hr. incubation (Fig. 4). If the incubation was carried out at 37° C, lipid mixing, beginning within 1–2 min and continuing until 1 h was seen (Fig. 4). At 15° C, where the transfer of endosome contents to lysosomes is blocked [18,19], lipid mixing did not increase at incubation times greater than 20 min, indicating that the later phase of lipid mixing occurred late in the endocytic pathway, possibly after reaching lysosomes. Incubation of Hep G-2 cells with liposomes composed of DOPC/DOTAP/Pyr-PE (3:1:0.21 molar) led to no detectable lipid mixing at time points shorter than 20 min; only minor amounts of lipid mixing were observed using this lipid composition (Fig. 4). Since the uptake of DOPC/DOTAP and DOPE/DOTAP liposomes by Hep G-2 cells is similar (Fig. 3), these data strongly suggest that liposome-to-cell transfer of Pyr-PE is not responsible for the decreased excimer fluorescence obtained when DOPE/DOTAP liposomes are used.

Pre-incubation of Hep G-2 cells for 15 min at 37° C with monensin (10^{-5} M) inhibited liposome-cell lipid mixing at 37° C (Fig. 4). Pre-incubation of cells for 1 h (37° C) with a mixture of 20 mM sodium azide and 50 mM 2-deoxyglucose, agents which are known to inhibit endocytosis by depletion of cellular ATP [20–23], decreased liposome-cell lipid mixing to the levels observed at 4° C (Fig. 4). This indicates that endocytosis is required for lipid mixing between cationic liposomes and Hep G-2 cells and that direct fusion of the liposomes with the plasma membrane makes only a minor contribution to the lipid mixing we observe. Incubation of cells under hypertonic conditions (0.45 M sucrose in PBS) leads to the inhibition endocytosis by impairing the formation of coated vesicles from clathrin coated pits [24]. Lipid mixing between DOPE/DOTAP/Pyr-PE (3:1:0.21) liposomes and Hep G-2 cells was completely abolished by performing the experiment in hypertonic PBS (Fig. 4), suggesting that endocytic uptake through the coated pit/coated vesicle pathway is involved in the interaction between cationic liposomes and mammalian cells. When DOPC/DOTAP/Pyr-PE liposomes were used, neither ATP-depleting conditions nor hypertonic media conditions led to any changes in pyrene excimer fluorescence (data not shown).

In a separate set of experiments, Hep G-2 cells were grown on 10 × 22 mm glass coverslips which were held in a fluorescence cuvette using a coverslip holder like that described by Ohkuma and Poole [25]. The kinetics of the decrease in excimer fluorescence and the effects of inhibitors observed using this technique (data not shown) were indistinguishable from the data obtained using trypsinized cells (Fig. 5). One problem with using attached cells is that we found it impossible to calculate the extent of lipid mixing, since addition of Triton to lyse the cells led to an increase in apparent excimer fluorescence (data not shown). This resulted from detachment of cells from the coverslip and an increase in the amount of pyrene label diffusing through light path per unit time as compared to attached cells (data not shown).

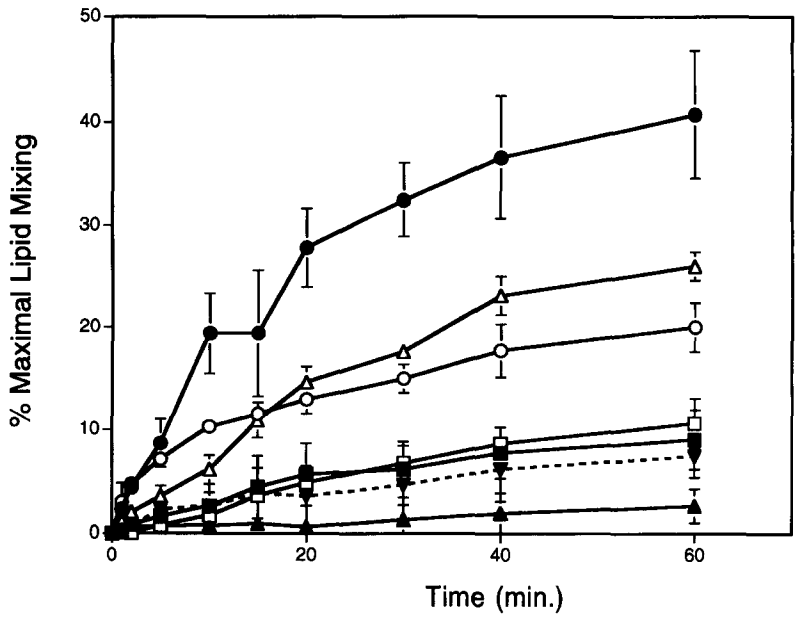

Fig. 5. Endocytosis is required for fusion of cationic liposomes with CHO cells. Fusion between DOPE/DC-CHOL/Pyr-PC (2:3:0.2) (●, ○, △, ■, ▲, □) or DOPC/DC-CHOL (2:3:0.2) (▼) liposomes and CHO cells was assayed as described for Hep G2 cells. Lipid mixing was assayed after incubation in the absence of inhibitors at 37° C (●, ▼), 15° C (○) and 4° C (■) or in the presence of 20 mM NaN_3 + 50 mM 2-DG (▲), 0.45 M sucrose (□) or 10^{-5} M monensin (△). Inhibitors were present during a 1 h pre-incubation (37° C) and throughout the liposome incubation period at 37° C. The data represent the mean ± S.D. for four separate experiments.

The lipid mixing of liposomes composed of DOPE/DC-CHOL/Pyr-PC and DOPC/DC-CHOL/Pyr-PC with CHO cells was also studied. Lipid mixing of DOPE/DC-CHOL liposomes with CHO cells exhibited kinetics similar to those observed using Hep G2 cells and DOPE/DOTAP liposomes (Fig. 5). Again, very little lipid mixing was observed when DOPC-containing liposomes were used. Inhibition of endocytosis by either ATP depletion or hypertonic media inhibited lipid mixing of DOPE/DC-CHOL liposomes with CHO cells (Fig. 5).

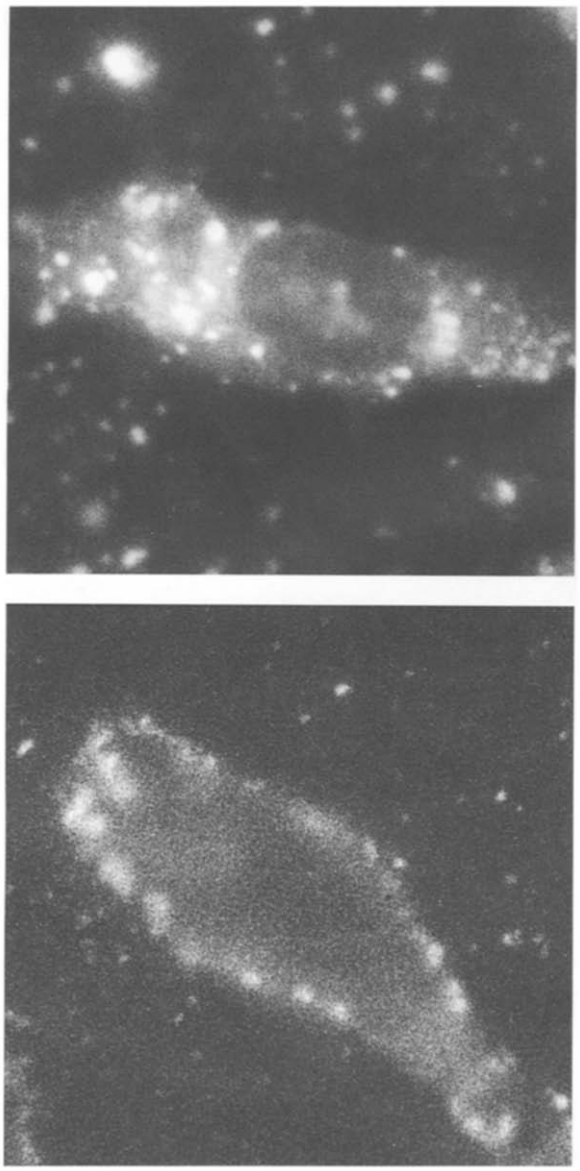

Fig. 6. Cationic liposomes are endocytosed by CHO cells. DOPE/DC-CHOL liposomes, prepared to contain 5 mol% FPE, were incubated with CHO cells grown on coverslips for 30 min at 37° C. CHO cells were pre-incubated (1 h) in the presence (lower panel) or absence (upper panel) of 20 mM NaN_3 + 50 mM 2-DG. In the latter case, ATP-depleting conditions were maintained throughout the liposome incubation period. The cells were then washed, fixed and photographed as described in Materials and methods.

Monensin (37° C) reduced lipid mixing to the level observed at 15° C in the absence of inhibitors (Fig. 5). By contrast, ammonium chloride (10 mM) had no effect on lipid mixing between DOPC/DC-CHOL/Pyr-PC liposomes and CHO cells (data not shown), indicating that endosome acidification was not required for liposome fusion.

CHO cells which have been incubated with FPE-labeled DOPE/DC-CHOL liposomes for 30 min at 37° C exhibit a punctate intracellular fluorescence consistent with localization of the liposomes within the endocytic pathway (Fig. 6, upper panel). At time points longer than 60 min, labeling of other cellular membranes, including the plasma membrane and nuclear membrane was observed for FPE-labeled DOPE/DC-CHOL, but not DOPC/DC-CHOL, liposomes (data not shown). This is consistent with previous observations [1]. By contrast, under conditions of ATP depletion (Fig. 6, lower panel), the liposomes are located exclusively at the cell surface. These data show that while cationic liposomes can still bind to the surface under these conditions, uptake of the liposomes into the endocytic pathway is abolished. Under these conditions, little or no fusion occurs (Fig. 5).

4. Discussion

We have used a lipid mixing assay developed for virus-cell fusion to examine the interaction of cationic liposomes with cells. This assay has been reported to reliably reflect membrane fusion with fewer artifacts than other probe dilution assays [15]. We first examined the fusion of cationic liposomes with negatively charged liposomes. In agreement with previous work [4,11,12] we found that cationic liposomes prepared using DOPE fused with anionic liposomes, while those prepared using DOPC did not. These data showed the importance of DOPE in the fusion process. The fact that no decrease in excimer fluorescence was seen for DOPC-containing cationic liposomes in the presence of DOPC/DOPG liposomes, strongly suggests that fusion rather than interliposome transfer of the pyrene-labeled phospholipid is responsible for the decreases seen when DOPE-containing liposomes are used. Fusion between cationic liposomes and anionic liposomes was not pH-sensitive in the range of pH 4.5–7.0 and was not affected by NaCl up to 150 mM, suggesting that transfer of the liposomes into media with physiological salt concentration does not affect fusion. We confirmed these results using an independent assay based on energy transfer [16].

The interaction of Pyr-PE labelled liposomes with mammalian cells was then studied. While excimer fluorescence for DOPE-containing cationic liposomes was observed to decrease in the presence of cells in a time-dependent manner, no decrease in excimer fluorescence was seen for DOPC-containing cationic liposomes under the

same conditions. In most cases, second order lipid transfer rates, which depend on bilayer apposition, are faster for PE versus PC membranes [26]. This is due to the fact that PE-containing membranes are less well hydrated and can be more easily apposed as compared to their PC-containing counterparts [26]. However, the surface properties of cationic liposomes are most likely dominated by the high mol% of the positively charged lipids DOTAP and DC-CHOL. Therefore, we suggest that in cationic liposomes lipid transfer should occur equally well from either PE or PC containing membranes. Since the amount of binding and uptake of the DOPE- and DOPC-containing cationic liposomes was not significantly different, these data suggest that liposome-to-cell transfer of Pyr-PE was not responsible for decreased excimer fluorescence.

Likewise cellular oxygen tension differences were not responsible for the observed fluorescence changes. It is known that pyrene fluorescence is quenched by the presence of oxygen [27–29] and that ATP-depleting agents such as sodium azide and 2-deoxyglucose can alter the oxygen tension of cells. However, excimer fluorescence for DOPC-containing cationic liposomes did not change, either in the presence or absence of ATP-depleting agents. We therefore conclude that DOPE-containing cationic liposomes fuse with mammalian cells while DOPC-containing cationic liposomes do not.

Binding to the cell surface, in the absence of endocytosis, was insufficient for liposome-cell fusion. As we have shown here, ATP depletion and hypertonic media inhibit uptake, but not binding, of the liposomes at 37° C (Figs. 3 and 6). These conditions have been shown previously to inhibit endocytic uptake through clathrin coated pits [20–23]. Inhibition of liposome endocytosis by ATP depletion has also been previously demonstrated [30]. In all cell lines we have examined, inhibition of liposome uptake, either by ATP depletion or hypertonic media, inhibits cationic liposome-cell fusion (Figs. 4 and 5, and unpublished observations). Therefore, we conclude that endocytic uptake is required for the fusion of cationic liposomes with cells.

This conclusion is supported by the temperature dependence of the fusion process. No fusion was detectable at 4° C, where endocytosis is inhibited. Incubation at 15° C, where endosome-lysosome fusion is inhibited [18,19], inhibited fusion at later time points (time $\geq$ 20 min), but not at earlier time points (time $\leq$ 20 min). These data also suggest that trafficking through the endocytic pathway is involved in the fusion described here. The later (time $\geq$ 20 min) component of the lipid mixing may represent either further fusion events, lysosomal degradation of the pyrene label, or further dilution of the pyrene phospholipid from fused liposomes into the cellular membrane pool via normal cellular membrane trafficking. Our fluorescence microscopy data supports the latter conclusion, since at longer time points, FPE appears to label all the cellular membranes including the plasma membrane and the nuclear envelope (data not shown).

The fusion between cationic liposomes and cells occurs rapidly at 37° C. Unlike viral fusion [15] and the intracellular destabilization of pH-sensitive liposomes [31,32], cationic liposome-cell fusion does not involve a lag phase. In the case of pH-sensitive liposomes and acid-triggered viral fusion, a lag phase of 1–2 min is typically observed prior to the occurrence of fusion. This represents the time required to reach an endocytic compartment with sufficient acidity to trigger the fusion event. Cationic liposome fusion appears to be pH-independent (Fig. 2 and data not shown), so entry into an acidified organelle may not be required for fusion. The rapid onset of the fusion and the lack of a lag phase suggest that fusion may be occurring in an early component of the endocytic pathway, perhaps as early as the coated vesicles which pinch off from clathrin coated pits [19,20]. We have no way, at present, of determining the exact endocytic organelle at which fusion between cationic liposomes and cells first occurs. Our data, however, excludes a major role for the plasma membrane in the fusion event. At present, it is unclear why endocytosis is required for fusion since the membrane composition of the plasma membrane and vesicles derived from that membrane should be quite similar.

These findings imply that nucleic acid delivery using cationic liposomes will be largely determined by the endocytic capability of the cell in question. In particular, liposome cell fusion and nucleic acid delivery will be low to non-existent in cells which do not endocytose. Our preliminary data with primary smooth muscle cells (SMC) support this conclusion. When cultured in serum free media for 2 days, SMC are growth arrested in the Go phase of the cell cycle and exhibit a marked reduction in their ability to endocytose (T. Burgess and D. Collins, unpublished observations). Under growth-arrested conditions, cationic liposomes bind, but do not fuse, with SMC. However, if the cells are rescued from growth arrest by serum feeding, the cells recover their endocytic capability and cationic liposomes are able to fuse with the cells (T. Burgess and D. Collins, unpublished observations). Nuclear delivery of FITC-labeled oligonucleotides is not observed for growth-arrested SMC, but is seen when serum fed SMC are used (T. Burgess and D. Collins, unpublished observations).

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