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Use of a quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells

Purnima Pinnaduwage, Lori Schmitt and Leaf Huang

Department of Biochemistry, University of Tennessee, Knoxville, TN (U.S.A.)

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Sonicated liposomes composed of dioleoylphosphatidylethanolamine (DOPE) and a quaternary ammonium detergent (dodecyl-, tetradecyl-, or cetyl-trimethylammonium bromide) mediates functional transfer of pSV2 CAT plasmid DNA to mouse L929 fibroblasts. Successful transfection was determined by assaying for chloramphenicol acetyltransferase activity in cell lysates collected 40 h after exposure to the lipid-DNA complexes. Liposomes prepared with the quaternary ammonium detergents were less toxic than the free detergents at the same concentrations and were more efficient in their delivery of the plasmid DNA to the cells. Analysis of the three detergents in combination with the lipid showed that cetyltrimethylammonium bromide was least toxic to the cells. This detergent, at a minimal concentration of 20 mol% in DOPE, allowed for stable liposome preparations and efficient transfection. Optimal efficiency of transfection occurred with 30 μg of DNA. Further increases in the DNA concentration caused a decrease in the transfection efficiency, perhaps due to charge repulsions between the liposomes now saturated with negatively charged DNA and the negatively charged cell surface. The transfection activity of the liposome was limited by its cytotoxicity at high liposome concentrations. These results are compared with that of the Lipofectin, another positively charged liposome preparation which is commercially available. Although the overall transfection activity of the liposome containing the quaternary ammonium detergent is somewhat lower than that of the Lipofectin, it may serve as an inexpensive and convenient alternative.

Introduction

Gene therapy is rapidly becoming a growing field of medicine used to correct genetic disorders [1,2]. In this process, normal exogenous genes are introduced into target cells where their products work with or replace the defective gene products, hence aiding the cell in normal function. One of the major obstacles in gene therapy is finding methods which allow for specific and efficient delivery of the genes. Current methods used for this process include cell fusion [3] and DNA-mediated gene transfer, which includes microinjection [4], electroporation [5], calcium phosphate [6], and DEAE-dextran-mediated transfection [7]. These methods, however, pose problems such as low efficiency, instability of the introduced genes, and toxicity to the target cells.

A more recent method of DNA transfection is through the use of liposomes [8–13]. Of particular interest is the use of cationic liposomes which have the ability to transfer DNA into cells through fusion with the cell membrane. The cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N*, *N*, *N*-trimethylammonium chloride (DOTMA), was used to form positively charged liposomes (Lipofectin) that spontaneously form complexes with DNA or RNA [12,13]. These complexes adsorb to the cell surface, fuse with the plasma membrane and deliver active poly-nucleotides into the cytoplasm. The efficiency of transfection with such liposomes tends to be greater than that of other methods with the same cell lines and can be used for both transient and stable expression. Recently, Loyter et al. [14] reported the use of liposomes bearing another cationic lipid, diisobutylcresoxyethoxyethyldimethylbenzylammonium

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Abbreviations: DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*, *N*, *N*-trimethylammonium chloride; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DTAB, dodecyltrimethylammonium bromide; TTAB, tetradecyltrimethylammonium bromide; CTAB, cetyltrimethylammonium bromide; DCS, donor calf serum; HBS, Hepes-buffered saline (150 mM NaCl, 20 mM Hepes, pH 7.4); CAT, chloramphenicol acetyltransferase; DEBDA[OH$^-$], diisobutylcresoxyethoxyethyldimethylbenzylammonium.

Correspondence: L. Huang, Department of Biochemistry, University of Tennessee, Knoxville, TN 37996-0840, U.S.A.

(DEBDA[OH$^-$]) for functional transfer of RNA into plant protoplasts. They have shown that the efficiency of transfection was slightly higher with empty liposomes complexed with external RNA than with liposomes carrying the encapsulated RNA.

Our lab. in keeping with this idea, has developed positively charged liposomes which consist of different quaternary ammonium detergents. We show here that these liposomes, which can be prepared with easily available and inexpensive lipids by a simple procedure, can efficiently deliver DNA to the mouse L929 cells.

Materials and Methods

Quaternary ammonium detergents (dodecyl-, tetradecyl- or cetyl-trimethylammonium bromide) were purchased from Sigma Chemical Co. DOPE was obtained from Avanti Polar Lipids. Lipofectin was obtained from Syntex Corp. (It is now available from Bethesda Research Lab.).

Isolation of PSV2 CAT plasmid DNA. Plasmid DNA (PSV2 CAT) confirming resistance to ampicillin was grown in *Escherichia coli*, isolated and purified according to the method of Gorman et al. [15]. However, the final purification process involved the use of PZ523 spun columns [16]. After linearizing the plasmid DNA with restriction endonuclease *Pst*I and/or *Hind*III, the degree of purity was further confirmed by agarose gel electrophoresis.

Cell cultures. Mouse L929 fibroblast cells were maintained as monolayer cultures in McCoy's medium supplemented with 10% donor calf serum at 37°C.

Preparation of liposomes bearing cationic detergents. Small unilamellar vesicles were prepared from DOPE and cationic detergents using a bath sonicator. In routine experiments, 3 μmol of DOPE and cationic detergents (DTAB, TTAB, or CTAB) were mixed in an organic solvent, dried under a stream of N_2 and vacuum desiccated for no less than 2 h. The lipid mixture was then hydrated with phosphate-buffered saline (300 μl, pH 7.4) at room temperature for 3–5 h with occasional mixing. The hydrated lipid mixture was sonicated for two 5-min cycles with an intervening rest period of 2 h at room temperature. Light scattering at 90° was measured in a Perkin-Elmer LS5 spectrofluorometer with excitation and emission at 660 nm and a slitwidth of 5 nm. Liposomes containing 20 mol% CTAB were negatively stained with 0.5% uranyl acetate and viewed in a Hitachi 600 electron microscope operating at 75 kV.

Preparation of liposomes containing [^{3}H]CE and [^{14}C]sucrose. Liposomes were prepared as described above except the lipid mixture contained DOPE/CTAB (20 mol%) and hexadecyl [^{3}H]cholestanyl ether (CE) (final specific activity $1 \cdot 10^{12}$ cpm/mol lipid). Dried lipid mixture was then hydrated with PBS containing 1 μCi [^{14}C]sucrose. After sonication, the non-entrapped [^{14}C]sucrose was removed by chromatography on a Bio-Gel A 0.5 M column equilibrated in PBS. Approximately 3% of the [^{14}C]sucrose was eluted with liposomes in the void volume fractions.

Cell toxicity assay. Monolayer cultures of L929 cell (90–95% confluent) in 100 mm plates were rinsed three times with Hepes-buffered saline (HBS) (150 mM NaCl, 20 mM Hepes, pH 7.4). The cell monolayers were then incubated with liposomes (40 μM) containing varying mol% of cationic lipid in HBS (3 ml). After incubation for 3 h at 37°C, the liposomal suspension was replaced with 10 ml of McCoy's medium supplemented with 10% DCS. The cells were harvested 40 h later. Number of cells was determined using a Coulter cell counter. Viability of the cells was determined by a Trypan blue dye exclusion test.

Transfection of cell cultures using cationic lipids.

(i) Formation of lipid-DNA complex. Small unilamellar liposomes composed of cationic detergent and DOPE or free detergent diluted to 1.5 ml with HBS were mixed with plasmid DNA also diluted to 1.5 ml with HBS as described [12]. The concentrations of lipid and DNA used are specified in the legends of figures. In a typical transfection experiment, 20 μg of DNA in 1.5 ml of HBS was mixed with 75 μg of total lipid (DOPE/CTAB, 4:1 molar ratio) also in 1.5 ml HBS. The addition of DNA caused an increase in turbidity of the liposome suspension indicating the formation of liposome-DNA complex.

(ii) Transfection protocol. L929 cells (90–95% confluent) in 100 mm tissue culture plates were rinsed with HBS and 3 ml of the liposome-DNA suspension was added. Following an initial incubation at 37°C for 3 h, the liposome-DNA solution was replaced with McCoy's medium containing 10% DCS. After a further incubation at 37°C for 40 h, cells were harvested by scraping the plates. Delivery of DNA into cells was monitored by performing a CAT assay [15,17] on the cell extracts as described previously [12,15]. Briefly, the cell extracts in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were frozen at −70°C and thawed at room temperature three times, and centrifuged at $12000 \times g$ for 5–10 min to remove cell debris. The protein concentration of the supernatent was measured according to the method of Lowry et al. [18]. In routine experiments, approximately 1 mg of protein has been used to measure CAT activity. The cell extracts (250 μl) were heated to 60°C for 10 min and 0.4 μCi of [^{14}C]chloramphenicol and 320 μM acetyl-CoA was added. After incubation at 37°C for 2 h, the acetylated products were extracted with ethyl acetate, separated on silica gel thin-layer plates and visualized by autoradiography.

Results and Discussion

Unsaturated phosphatidylethanolamine does not form stable bilayers by itself at physiological tempera-

ture and pH [19]. However, it has been well documented that the bilayer form of unsaturated PE can be stabilized by a variety of stabilizers [20–25] including detergents such as Triton X-100, deoxycholate, octyl glucoside and lysophosphatidylcholine [26]. In the initial part of our project, we studied the stabilization ability of three cationic detergents: DTAB, TTAB, and CTAB on DOPE bilayer liposomes. Unsaturated PE is known to promote membrane fusion [29]. Liposome-cell fusion, whether with plasma membrane or with endosome/lysosome membrane, could be important for DNA delivery. We have thus used DOPE, a non-bilayer forming lipid for this work.

To determine the minimum concentration of cationic detergent needed to stabilize otherwise unstable DOPE bilayers, various amounts of the quaternary ammonium detergents were mixed with DOPE. Liposomes were prepared by sonication. Light scattering is a convenient way to monitor the stabilization of the DOPE liposomes [23,25]. When DOPE is in the hexagonal phase, as is the case of zero mol% detergent, it scatters little light because the lipid aggregates into large clumps and does not appear in the light path. As more stabilizers are added, the lipid suspension becomes more homogeneous, but quite cloudy. When stable, small bilayer vesicles are formed with sufficient amounts of the stabilizer, the light scattering approaches that of the vesicles composed of DOPC which is used for a comparison. Data in Fig. 1 show that DTAB has a greater stabilization effect compared to that of TTAB or CTAB. 10 mol% of DTAB was sufficient to stabilize the bilayer form of DOPE whereas almost 20 mol% of TTAB or CTAB was required. Concentrations of detergent appreciably higher than those required for bilayer stabilization exhibit a low light scattering value, presumably due to the formation of mixed detergent-phospholipid micelles. Light scattering of DOPC liposomes containing 30 mol% of CTAB also showed a lower value due to the formation of mixed micelles.

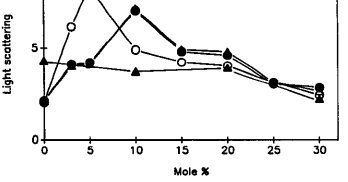

Fig. 1. Stabilization of DOPE bilayers with cationic detergents. 90° light scattering of the sonicated liposomes containing various mol% of DTAB (○), TTAB (△), or CTAB (●, ▲) was measured for DOPE (○, △, ●) or DOPC (▲) lipid.

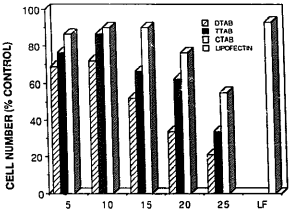

Fig. 2. Effect of DOPE liposomes, bearing various amounts of cationic lipids, on cell toxicity. Cells were incubated with 40 μM lipid composed of DOPE and varying amounts of DTAB (▨), TTAB (■), or CTAB (▨) for 3 h. Lipofectin (LF) was also used at 40 μM lipid for comparison.

Negative-stained electron micrographs revealed a diameter of 3600 Å ± 1100 Å for DOPE : CTAB (20 mol%) liposomes. The trap volume of the liposome was 3.2 μl/μmol lipid as measured by using [^{14}C]sucrose as an aqueous marker. The entrapped [^{14}C]sucrose stayed liposome-associated for the initial 6–8 h and leaked gradually thereafter. Addition of DNA (20 μg) to freshly prepared liposomes (75 μg lipid) caused immediate aggregation of liposomes as seen by turbidity increase. However, no leakage of entrapped [^{14}C]sucrose was observed by gel filtration chromatography (data not shown). Work by Loyter et al. [14] has shown that the delivery efficiency of empty liposomes complexed with external RNA is higher than that of liposomes carrying encapsulated RNA. Since we have been using freshly prepared liposomes complexed with external DNA, the integrity of the liposome does not play a key role.

One of the major drawbacks of using single chain amphiphiles such as detergents for drug delivery is their toxicity to the cells. The degree of toxicity varies with the cell type, cell density, concentration of the detergent, and duration of exposure. Dense cultures (almost confluent) were less susceptible to the toxic effects of the cationic detergents compared to less confluent cultures (data not shown). We have compared the toxic effect of DTAB, TTAB, and CTAB in the presence and absence of phospholipid. Lipofectin, a commercially available liposome preparation containing the cationic lipid, DOTMA, was also included for comparison. As shown in Fig. 2, the cell number decreased with increasing concentration of cationic detergent in liposome. Lipofectin showed much less toxicity to cells compared to the liposomes composed of detergents. Among the three detergents, CTAB showed the least amount of

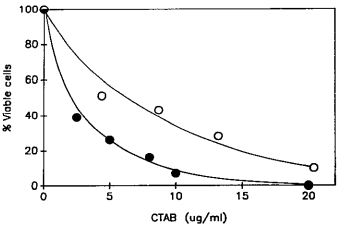

Fig. 3. Effect of different amounts of liposomal or free CTAB on cell viability determined by the Trypan blue exclusion test. Cells were treated with varying amounts of CTAB in the presence (○) or absence (●) of the phospholipid DOPE. After an initial 3-h incubation, the lipid mixture was replaced with McCoy's medium containing 10% DCS. Cell viability was determined 24 h later by Trypan blue exclusion.

toxicity to the cells. Since 20 mol% of CTAB was needed to obtain stable liposomes and these liposomes were the least toxic to the cells, this composition was used for further studies.

The effect of CTAB on cell viability was further tested using Trypan blue exclusion test. 20 μg/ml CTAB in the absence of phospholipids showed a very high toxicity to the cells. However, as shown in Fig. 3, the presence of DOPE greatly improved the cell viability. Thus, the presence of phospholipid reduces the toxicity of the cationic lipid. Similar observations have been reported for another cationic lipid, DEBDA[OH^-], for the plant protoplasts [14].

The data presented in Fig. 4 show that liposomes bearing CTAB and complexed with DNA are able to transfer the DNA into L929 cells as measured by the expression of the CAT enzyme activity. The extent of transfection, however, is dependent on the amount of liposomal lipid added. As shown in Fig. 4, the transfection efficiency increased with increasing amounts of cationic liposomes complexed to DNA. However, an optimum dose was observed at 75 μg total lipid. At high lipid concentrations, there was an increase in cell death due to the presence of high amounts of CTAB. The CAT expression of cells treated with the Lipofectin was greater compared to that of the liposome containing DOPE and CTAB, mainly due to the lack of toxicity of the Lipofectin to the cells.

The data in Fig. 5 indicate that transfection efficiency is also dependent upon the amount of DNA complexed with the liposomes containing DOPE and CTAB. Initially, increasing amounts of DNA resulted in an increased CAT expression. However, further increases resulted in a decrease in the expression of CAT activity (Fig. 5, lanes 3 and 4). This may be due to the

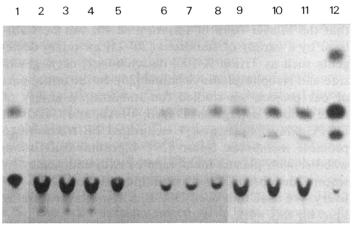

Fig. 4. The effect of lipid concentration on CAT gene expression. Cell monolayers were treated with DOPE liposomes containing 20 mol% CTAB complexed with 20 μg of plasmid DNA. Total lipid amounts varied from 10–100 μg (lanes 4–10). Lane 1 shows the CAT activity of cells treated with Lipofectin (65 μg lipid) complexed with 20 μg of DNA. Lanes 2 and 3: control cells treated with 75 μg lipid in the absence of DNA (lane 2) or complexed with 20 μg of salmon sperm DNA (lane 3). Lane 4: 10 μg lipid; lane 5: 20 μg lipid; lane 6: 30 μg lipid; lane 7: 50 μg lipid; lane 8: 65 μg lipid; lane 9: 75 μg lipid; lane 10: 80 μg lipid; lane 11: 100 μg lipid. Lane 12: one unit of standard CAT enzyme. Protein concentrations used for CAT assay was 0.70 mg for cells treated with Lipofectin; all others contained 1.6 mg protein.

fact that the cationic liposomes are now saturated with DNA, hence resulting in a net negative charge on the complex. The cell surface bears a net negative charge due mainly to the presence of negatively charged glycosphingolipids and glycoproteins, resulting in a repulsion between the DNA-liposome complex and the cell

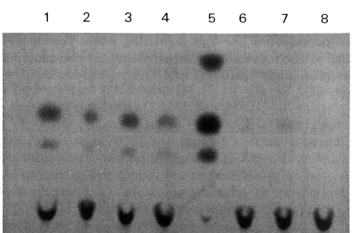

Fig. 5. Effect of increasing amounts of DNA on CAT gene expression. Cell monolayers were treated with 2.5 μg/ml CTAB in liposome (DOPE/CTAB, 4:1 molar ratio) or free CTAB complexed with various amounts of DNA. Lane 1: cells treated with Lipofectin; lanes 2–4: cells treated with CTAB in liposomes; lane 5: one unit of standard CAT enzyme; lanes 6–8: cells treated with free CTAB. Lanes 1, 2 and 6: 20 μg DNA, lanes 3 and 7: 30 μg DNA, lanes 4 and 8: 50 μg DNA. Concentration of protein used for CAT assay was 1.6 mg except for lane 1 which was 0.78 mg.

surface. It can also be seen in Fig. 5 that free CTAB was inefficient for DNA transfection of mouse L929 cells. This may be due to a high degree of cell death which occurs in the absence of the phospholipid. Similar observations have been reported previously [14] using DEBDA [OH^-] to deliver RNA into plant protoplasts.

We have also examined the transfection efficiency as a function of the incubation time. The major obstacle here was that incubation times longer than 3 h resulted in very high level of cell death, and thus decreased the efficiency of transfection. Lower incubation times, i.e. 1 h showed less toxicity, however, the extent of transfection was also lower (data not shown). Although the transfection efficiency was directly proportional to the time of incubation, the viability of the cell monolayers was inversely proportional to the time of incubation with the cationic liposome. One must take into consideration the loss of the cell viability when the incubation conditions are optimized for the efficiency of transfection.

We have shown here that liposomes composed of a quaternary ammonium detergent, CTAB, can be used for the DNA transfection of mouse L929 cells. Despite the fact that toxicity is a major limiting factor in the system, we have been able to determine the minimal amount of lipid required to obtain the maximal amount of transfection in a time frame comparable to other transfection protocols. Although the overall transfection efficiency of our liposome composition is somewhat lower than that of a less toxic liposome composition, i.e. the Lipofectin, the inexpensiveness and the convenience of the present liposome composition may justify its use as an alternative transfection reagent for the animal cells.

While numerous studies have examined the properties of vehicles formed from synthetic cationic amphiphiles [27–30], only a few reports [12,14] have examined the use of these liposomes as a delivery vehicle of DNA or RNA to cells in culture. The mechanism of the cationic liposome-mediated DNA transfection is not known, although it has been suggested [12] that liposome-cell fusion might be responsible for the delivery of DNA into the cells. This is not likely because the DNA is bound to the outer surface of the liposome. A fusion event between the liposome and the plasma membrane will not cause the translocation of DNA across the plasma membrane. Furthermore, the data in Fig. 5 show that free detergent complexed with DNA can also mediate the transfection albeit the activity is weak. It is not likely that free detergent micelles will fuse with the intact cell membrane. A more likely mechanism for the cationic liposome-mediated DNA delivery is via the cellular endocytosis pathway. The liposome-DNA complex might be internalized into the endosome/lysosome compartments where the rupture or destabilization of the organelle membrane induced by the cationic lipids might take place, resulting in the release of the DNA into the cellular cytoplasm. A similar mechanism has been suggested by us for the DNA transfection activity of the negatively charged DOPE liposomes [11]. Much more work is needed to test this hypothesis.

Acknowledgments

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