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Research Article

Delivery of Plasmid DNA into Mammalian Cell Lines Using pH-Sensitive Liposomes: Comparison with Cationic Liposomes

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We compare the transfection efficiency of plasmid DNA encoding either luciferase or β-galactosidase encapsulated in pH-sensitive liposomes or non-pH-sensitive liposomes or DNA complexed with cationic liposomes composed of dioleoyloxypropyl-trimethylammonium:dioleoylphosphatidylethanolamine (1:1, w/w) (Lipofectin) and delivered into various mammalian cell lines. Cationic liposomes mediate the highest transient transfection level in all cell-lines examined. pH-sensitive liposomes, composed of cholestryl hemisuccinate and dioleoylphosphatidylethanolamine at a 2:1 molar ratio, mediate gene transfer with efficiencies that are 1 to 30% of that obtained with cationic liposomes, while non-pH-sensitive liposome compositions do not induce any detectable transfection. Cationic liposomes mediate a more rapid uptake of plasmid DNA, to about an eightfold greater level than that obtained with pH-sensitive liposomes. The higher uptake of DNA mediated by Lipofectin accounts for part of its high transfection efficiency. Treatment of cells with chloroquine, ammonium chloride, or monensin decreases (threefold) transfection using pH-sensitive liposomes and either has no effect on or enhances cationic liposome-mediated transfection. Therefore plasma membrane fusion is not the only mechanism available to cationic liposomes; in certain cell lines DNA delivery via endocytosis is a possible parallel pathway and could augment the superior transfection efficiency observed with cationic liposomes.

KEY WORDS: β-galactosidase; cell culture; gene therapy; Lipofectin; liposome; luciferase; pH-sensitive; plasmid.

INTRODUCTION

The introduction of genes into mammalian cells is a powerful method for understanding cell function and is becoming a useful modality for treating genetic diseases (1). Many viral and nonviral techniques are available to deliver genes into cells. Virus-transfection techniques use either retroviruses (2) or adenoviruses (3). However, the risks of viral-based vectors (4) and the conceptual advantages in the use of plasmid DNA constructs for gene therapy have led to the parallel development of various physical and chemical methods for gene transfer (see Ref. 5 for a discussion). Nonviral methods include lipid-based techniques, such as the Lipofectin technique (6) or liposomes (7), polylysine conjugates (8,9), and direct injection of DNA into tissue (10).

Most of the non-virus-mediated techniques have a low transfection efficiency or are unsuitable for *in vivo* use. Nevertheless, two lipid-based techniques, Lipofectin and pH-sensitive liposomes, show interesting properties for gene delivery. pH-sensitive liposomes increase the cytoplasmic delivery of macromolecules compared to non-pH-sensitive formulations of liposomes (11–13). The delivery mechanism involves the destabilization of the liposome bilayer at acidic pH ($5 < pH < 6.3$) in the late endosome and destabilization and fusion of the vesicle membrane with the endosome (13,30). The Lipofectin technique uses a synthetic cationic lipid (dioleoyloxypropyl-trimethylammonium; DOTMA) and mediates high *in vitro* transfection efficiencies in numerous cell lines (6). The mechanism of DNA entry suggests a fusion of the cationic liposomes with the plasma cell membrane and delivery of the DNA into the cellular cytoplasm (6,7,14). However, despite the widespread use of Lipofectin for DNA delivery, factors that control gene transfer by this reagent and the intracellular fate of the delivered DNA are still poorly understood.

In this paper, we describe the delivery of plasmid expression vectors in different mammalian cell lines using either cationic liposomes (i.e., Lipofectin) or pH-sensitive liposomes. We assess the efficiency of pH-sensitive liposomes for *in vitro* transfection and compare their efficiency to that of cationic liposomes as well as that of non-pH-sensitive liposomes. We find cationic liposomes are superior to pH-sensitive liposomes for transfection for two reasons: (i) they bring about a higher cell-associated level of high molecular weight plasmid DNA and (ii) they use a different, or parallel, pathway of DNA delivery into the cell from that available to the pH-sensitive liposomes.

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MATERIALS AND METHODS

Expression Vectors. Plasmids pSV2 luc (15), pRSV luc (16), pCluc4 (16), and pCMV-βGal (17) were generous gifts from Dr. A. Brasier (Harvard Medical School), Dr. M. German (University of California, San Francisco; UCSF), Dr. E. Wagner (Institute of Molecular Pathology, Vienna, Austria), and Dr. G. McGregor (Howard Hughes Medical Institute, Houston, TX), respectively. Plasmids were grown in *Escherichia coli,* extracted by the alkali lysis technique, and purified by centrifugation in equilibrium CsCl gradients (18). Structure and purity were checked by electrophoresis on a 0.7% agarose gel followed by ethidium bromide staining to detect DNA. DNA concentration was determined by absorbance at 260 nm. Plasmids were radiolabeled with ^{32}P-dCTP (Amersham Corp., Arlington Heights, IL) using a nick-translation kit (Bethesda Research Laboratories Inc., Gaithersburg, MD). Separation of the plasmid from nonincorporated nucleotides was carried out on a G50 Sephadex (Sigma, St Louis, MO) column.

Preparation of Liposomes. Dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), and bovine brain phosphatidylserine (PS) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesterol hemisuccinate morpholine salt (CHEMS) and cholesterol (Chol) were purchased from Sigma. Liposomes were prepared by the method of reverse-phase evaporation (19). pH-sensitive liposomes were composed of DOPE:CHEMS at molar ratios of 2:1, while control liposomes were made of DOPC:CHEMS and PS:Chol at molar ratios of 2:1 and 1:1, respectively. Typical preparations contained 12 μmol of lipids/80 μg of plasmid and traces of ^{32}P-pSV2. The lipidic film was dissolved in 0.6 ml of 30 m*M* Tris–HCl, pH 8.5, buffer-washed ether and DNA was introduced in a volume of 0.2 ml of 30 m*M* Tris–HCl, pH 8.5, sterile buffer. An emulsion was formed by sonication for 15 sec in a bath sonicator (Laboratory Supplies Company Inc., Nicksville, NY). Nonencapsulated DNA was removed by floating the liposomes through a discontinuous Ficoll gradient, with 0, 10, and 12.5% Ficoll concentration (w/v) steps. Phospholipids were assayed by the method of Bartlett (20) and ^{32}P-pSV2 was measured using a scintillation counter (Beckman LS 3801, Beckman Instruments, Irvine, CA). Liposome diameter was determined by dynamic light scattering (Malvern Instruments, Southborough, MA). Liposomes were kept at 4°C and used within 5 days after preparation.

Lipofectin Reagent. Dioleoyloxypropyl-trimethylammonium (DOTMA) was a gift from Dr. J. Senior (Syntex Corp. Palo Alto, CA) and Dr. R. Debs (UCSF). DOTMA was 95% pure (Dr. Judy Senior, personal communication) and exhibited a single spot on thin-layer chromatography (TLC) at high loading. Lipids (DOTMA:DOPE, 1:1, w/w) were dried at room temperature under nitrogen. The film was then rehydrated with sterile water to a final concentration of 1 mg/ml. The lipid mixture (10 μg of DOTMA:DOPE mixture, i.e., 14 nmol of lipids) was diluted with sterile water and gently mixed with an appropriate amount of DNA (also diluted in sterile water to a final volume of 50 μl) in polystyrene tubes. The mixture was allowed to stand at room temperature for 15 min, and then 100 μl of the complex was added to 2 ml of DME H-21 medium per 60-mm culture dish.

Cells and Transfection Protocol. Adherent and suspension cells were transfected. CV-1 (monkey fibroblasts), p388D1 (mouse macrophages), HepG2 (Human hepatocytes), and HeLa (human cervix cells) were provided by the UCSF cell culture facility. KD83 cells (mouse plasmacytoma cells) were a generous gift from Dr. F. Lee of DNAX (Palo Alto, CA). CV-1 and HeLa were grown in DME H-21 medium, p388D1 in RPMI 1640, HepG2 in MEM Eagle, and KD83 in RPMI 1640 supplemented with interleukin-6. Cells were plated at a density of 0.5–1 × 10^6 cells per 60-mm dish and incubated for 16 to 20 hr at 37°C under 5% CO_2 in appropriate medium containing 10% fetal calf serum (FCS). Prior to incubation with liposomes, cells were washed once with 2 ml of FCS-free DME H-21 medium. The transfection system was then added as a dilution in 2 ml of the same medium. In some experiments, transfection took place in 10% FCS containing DME H-21. After 5 hr, medium was removed and replaced with 3 ml of appropriate medium with 10% FCS. Luciferase activity was measured after 48 hr as described previously (16). Briefly, cells were washed twice with ice-cold phosphate-buffered saline (0.2 g/L KH_2PO_4, 2.16 g/L Na_2HPO_4, 0.2 g/L KCl, 8.0 g/L NaCl) without Ca^{2+} and Mg^{2+} (PBS), treated with 400 μl of 25 m*M* glycylglycine, pH 7.8, lysis buffer (containing 1% Triton X-100), and scraped. After centrifugation, 100 μl of supernatant was mixed with an optimal amount of 50 m*M* ATP. One hundred microliters of 1 m*M* D-luciferin (Sigma) was then injected and the emitted light signal was integrated during the first 10 sec using a bioluminometer (Bioluminescence Analytical Laboratories Inc., San Diego, CA). Proteins in the supernatant were assayed using a modified technique of Bradford (Bio-Rad kit). Results are expressed as 10^5 light units/mg of cell protein. Luciferase background (150 to 200 light units) was substracted from each measure.

Cytochemical Stains. To detect β-galactosidase activity, cells were incubated for 48 hr after transfection with pCMV-βgal plasmid, rinsed with PBS, fixed for 5 min with 4% formaldehyde in PBS, and then stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Promega, Madison, WI) (21). The blue β-galactosidase-expressing cells were visualized under the microscope, 8 hr after staining. The number of blue-colored cells in a culture was counted and the percentage of the total cell population determined.

Cell-Association Studies. An aliquot of each solution (plasmid alone or different liposome compositions), corresponding to 4 μg of pSV2 plasmid, was added to the cells as described in Cells and Transfection Protocol (above). After 2 hr, medium was removed and cells were rinsed 5 times with 2 ml of ice-cold PBS. Cells were digested in 1 ml of 0.5 *N* NaOH and scraped. An aliquot, 900 μl, of the solution was assayed for ^{32}PSV2 with the beta scintillation counter. An aliquot, 50 μl, was assayed for proteins by the method of Bradford (Bio-Rad kit). Results are expressed as the percentage of the added dose which is cell associated per milligram of cell protein.

Extraction of DNA from CV-1 Cells After Transfection. At various times after transfection with the pSV2 plasmid, medium was removed, and cells were rinsed five times with 2 ml of ice-cold PBS and trypsinized. Cells were scraped, washed twice, and resuspended in 1 ml of PBS. At this stage, 100 μl of sample was taken and counted for radioactivity

(total cell-associated DNA). Cells were then recovered by centrifugation and digested overnight at 50°C in 0.3 ml of digestion buffer (100 m*M* NaCl, 10 m*M* Tris–HCl, 25 m*M* EDTA, 0.5% sodium dodecyl sulfate) with proteinase K (100 μg/ml), as described (22). Proteins were extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol, 25: 24:1. High molecular weight DNA was precipitated at −70°C with 1 ml of ethanol and 0.3 ml of 4 *M* ammonium acetate. This procedure precipitates large DNA fragments (above about 50 base pairs) (18). DNA was recovered by centrifugation and radioactivity was counted in the pellet (cell-associated high molecular weight DNA). The DNA in the pellet was also analyzed by 0.7% agarose gel electrophoresis, followed by Southern blot transfer and hybridization using nick-translated pSV2 as a probe (23).

Effects of Lysomotrophic Agents on Expression Level. Chloroquine, 100 μ*M* (Sigma), monensin, 10 μ*M* (Calbiochem, La Jolla, CA), or NH_4Cl, 100 m*M*, was added to CV-1 cells simultaneously with the transfection system. Medium was then removed after 5 hr and luciferase activity was measured at 48 hr as described in Cells and Transfection Protocol (above).

RESULTS

Luciferase Gene Promoter Activity. Prior to comparing the systems, we measured the various plasmids for luciferase expression using Lipofectin-mediated transfection in different cell lines. The CMV promoter (pCluc4 plasmid) led to the highest luciferase expression in HeLa, HepG2, and p388D1 cells, while the SV40 promoter (pSV2 plasmid) was the most potent in CV-1 cells (data not shown). Therefore, pSV2 plasmid was used in CV-1 cells and pCluc4 in other cell lines for the experiments described below.

Liposome Characterization. Plasmid encapsulation efficiency was determined after Ficoll gradient separation and was about 24 ± 3% of the total DNA added for each formulation. Liposome diameter was 372 ± 38, 295 ± 65, 464 ± 20, and 117 ± 5 nm for DOPE:CHEMS, DOPC:CHEMS, PS:Chol, and DOTMA:DOPE liposomes, respectively (results are the mean ± SD of three determinations). The diameter of the DOTMA:DOPE liposomes increased appreciably after they were mixed with DNA. The DNA-DOTMA:DOPE complex has an apparent diameter greater than 500 nm.

Transfection and Cell-Association Studies in CV-1 Cells with Lipofectin or Liposomes. We first compared pH-sensitive liposomes and Lipofectin in terms of luciferase activity expressed at 48 hr as a function of the amount of plasmid added per 60-mm cell dish. A constant amount of DOTMA:DOPE of 10 μg (14 nmol of lipids) was added per dish, while the liposome concentration varied from 0.17 m*M* (0.5 μg plasmid) to 1.4 m*M* (4 μg plasmid). The results are illustrated in Fig. 1. Intermeasurement variation for each value was equal to or less than 55% for cationic liposomes and 28% for pH-sensitive liposomes. Despite this large variation, the DOTMA:DOPE liposomes led to significantly higher luciferase expression than the pH-sensitive liposomes at all DNA levels studied. Lipofectin-treated cells showed a sharp increase in luciferase expression between 0.5 and 1 μg of plasmid, followed by a slight decrease between 1 and 5 μg.

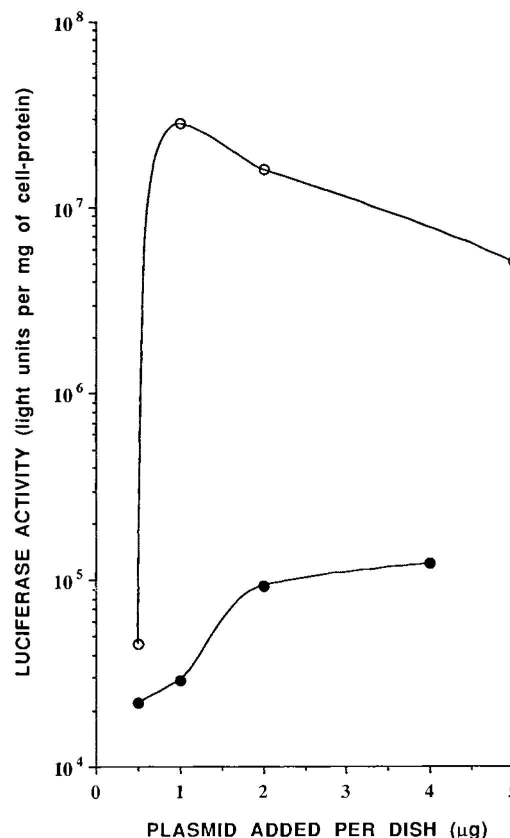

Fig. 1. Effect of DNA amount on transfection efficiency. CV-1 cells are transfected with either pH-sensitive liposomes (●) or cationic liposomes (○) containing various amounts of pSV2 plasmid. Luciferase expression is measured 48 hr after transfection. Each point is the mean of two experiments. In general, luciferase activity per milligram of cell protein agreed to within 50% between experiments.

Therefore, a DNA:Lipofectin ratio between 1:10 and 5:10 is optimal for CV-1 transfection. This result correlates with previous reports for CV-1 Lipofectin-mediated transfection (6). pH-sensitive liposomes were able to mediate gene transfer at the low dose (0.5 μg of plasmid) at a level close to that of cationic liposomes. Transfection using pH-sensitive liposomes approached a plateau at about 2 μg of DNA. Conditions that resulted in the maximal level of luciferase expression when delivered by pH-sensitive liposomes (4 μg DNA) were used for the experiments described below. At this dose, which is a compromise between the optimal transfection for both systems, a small variation of dose did not lead to a wide variation of the transfection level. pH-sensitive liposomes mediated gene transfer with an efficiency about 6% that of DOTMA:DOPE liposomes. Interestingly, empty pH-sensitive liposomes incubated with free plasmid show a very low transfection level, in line with the observation of Wang and Huang (24). The transfection efficiency of pH-sensitive liposomes and cationic liposomes was also compared to that of non-pH-sensitive liposomes. Conventional formulations of liposomes (i.e., DOPC:CHEMS or PS:Chol compositions) were unable to transfect luciferase activity into CV-1 cells (Table I).

In order to learn whether the transfection efficiency could be related to the amount of DNA that becomes cell

Table I. Transfection of CV-1 Cells[a]

System	Luciferase activity[b]	
Plasmid alone	0	(2)
DOTMA:DOPE liposomes	21.5 ± 13.2	(5)
DOPC:CHEMS liposomes	0	(5)
PS:Chol liposomes	0	(4)
DOPE:CHEMS liposomes	1.2 ± 1.2	(5)
Empty DOPE/CHEMS liposomes + PLASMID	0.012 ± 0.008	(3)

[a] Four micrograms of pSV2 plasmid is administered to CV-1 cells either alone, with 10 μg of Lipofectin reagent, in pH-sensitive liposomes, or in control liposomes (final total lipid concentration was between 0.9 and 1.5 m*M* for pH-sensitive and control liposomes).

[b] Luciferase activity at 48 hr expressed as 10^5 light units/mg of cell protein. Light units background was subtracted from each value. Results are the means ± SD of the indicated number of experiments.

associated using the various systems, we measured the fraction of the plasmid which was cell associated at 2 hr postincubation with 4 μg of plasmid per culture dish (Fig. 2). Plasmid alone or plasmid in DOPC:CHEMS liposomes had a very low uptake (<2.5% of the dose/mg of cell protein), and plasmid in DOPE:CHEMS or PS:Chol liposomes showed a similar uptake for both formulations (20 and 13%, respectively, of the dose/mg of cell protein). Plasmid complexed with DOTMA:DOPE liposomes had the highest cell-association rate (40% of the dose/mg of cell protein) (Fig. 2).

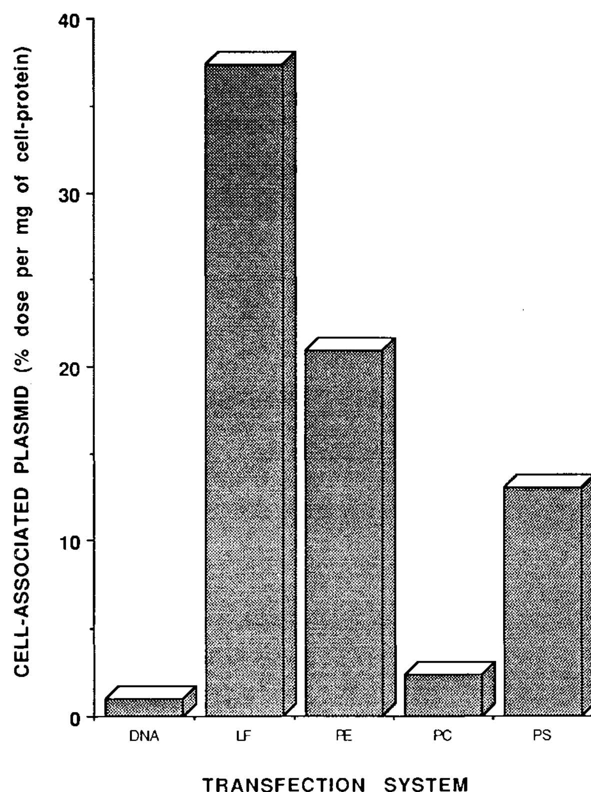

Fig. 2. Cell-associated plasmid 2 hr postincubation using various delivery vehicles. CV-1 cells are incubated with plasmid alone (DNA) or plasmid associated with DOTMA:DOPE liposomes (LF), DOPE:CHEMS liposomes (PE), DOPC:CHEMS liposomes (PC), or PS:Chol liposomes (PS). The fraction of cell-associated radiolabeled plasmid is measured 2 hr postincubation. Each point is the mean of two experiments (deviation between two values was below 20%).

Transfection of Different Mammalian Cell Lines with Cationic Liposomes or pH-Sensitive Liposomes. The transfection efficiency of cationic liposomes and pH-sensitive liposomes was measured in four other mammalian cell lines, HepG2, p388D1, HeLa, and KD83, in the presence or absence of serum (Table II). pH-sensitive liposomes were able to mediate gene transfer in all cell lines except KD83, with efficiencies 3- to 150-fold less than that of DOTMA:DOPE liposomes. The efficiency depended on the cell line and whether or not serum was present in the medium. With both transfection systems, the highest luciferase expression occurred in HepG2 and HeLa cells, while the lowest expression was found in p388D1, CV-1, and KD83 cells. The presence of 10% serum reduced cationic liposome-mediated transfection in CV-1 by fivefold, while pH-sensitive liposome efficiency decreased by less than twofold. In HepG2 cells, there was no visible effect of serum during transfection for either DOTMA:DOPE liposomes or pH-sensitive liposomes.

The transfection experiments were repeated using the pCMV-βgal plasmid with quantitation by counting the cells (except with the KD83 cell line) expressing β-galactosidase activity 48 hr after transfection. Cationic liposomes induced the greatest number of β-galactosidase-expressing cells, and in the best case 5 to 10% of the HeLa cells expressed β-galactosidase activity. Less than 2% of the HeLa cells were stained after pH-sensitive liposome-mediated transfection. The percentages of blue-stained cells after pH-sensitive liposome or DOTMA:DOPE liposome-mediated DNA delivery displayed the same rank order as luciferase activity (data not shown). No stained cells were detected in control dishes (nontreated cells).

Plasmid Uptake and Degradation Kinetics. The kinet-

Table II. Transfection of Different Mammalian Cell Lines with Cationic Liposomes or pH-Sensitive Liposomes[a]

Cell line	Liposomes: Cationic	Liposomes: pH-sensitive	Factor[b]
CV-1	21.5 ± 13.2	1.2 ± 1.2	17
CV-1 + 10% serum	4.0 ± 2.0	0.8 ± 0.1	5
HepG2	827 ± 333	7.5 ± 7.0	110
HepG2 + 10% serum	925 ± 547	6.3 ± 4.0	147
HeLa	196.0 ± 18.0	6.0 ± 2.9	33
p388D1	5.6 ± 1.0	1.9 ± 0.7	3
KD83	0.13 ± 0.03	0	

[a] Cells are treated with 4 μg of plasmid DNA/60-mm culture dish. DNA is complexed with 10 μg of Lipofectin reagent or encapsulated in pH-sensitive liposomes. Luciferase activity at 48 hr is expressed as 10^5 light units/mg of cell protein. Light units background was subtracted from each value. Results are the mean ± SD of three to five experiments.

[b] The factor is computed as the ratio of luciferase activity per milligram of cell protein induced by cationic liposomes to luciferase activity per milligram of cell protein induced by pH-sensitive liposomes.

ics of total cell-associated plasmid and high molecular weight plasmid (i.e., DNA larger than 50 base pairs) after DOTMA:DOPE or pH-sensitive liposomes delivery are illustrated in Fig. 3. Cationic liposomes led to a rapid DNA–cell association within the first 30 min, followed by a progressive increase until a plateau was attained at about 4.5% of the dose at 12 hr, even though the DNA was removed from the culture medium at 5 hr. High molecular weight DNA showed a similar profile albeit at about a twofold lower level (2% of the dose at 12 hr). pH-sensitive liposome-encapsulated DNA was taken up by the cells more gradually until 5 hr. At this time the added DNA was removed and the cell-associated DNA decreased to about 1% of the dose at 8 and 12 hr, respectively. The kinetics for the high molecular weight DNA was similar, although only 0.45% of the dose was found in this fraction. These data indicate that plasmid delivery via DOTMA:DOPE liposomes is faster and that cell-associated high molecular weight DNA reached a higher level than via pH-sensitive liposomes.

Southern blot analysis of DNA pellets confirmed these results (Fig. 4). Autoradiography indicated the presence of linear and supercoiled plasmid in the cells at 0.5, 5, and 12 hr postincubation after DOTMA:DOPE and DOPE:CHEMS liposome delivery. The signal from DNA after cationic liposome delivery was much stronger than the signal after pH-sensitive liposome delivery. In the case of the cationic liposomes, the signal corresponding to the supercoiled form was

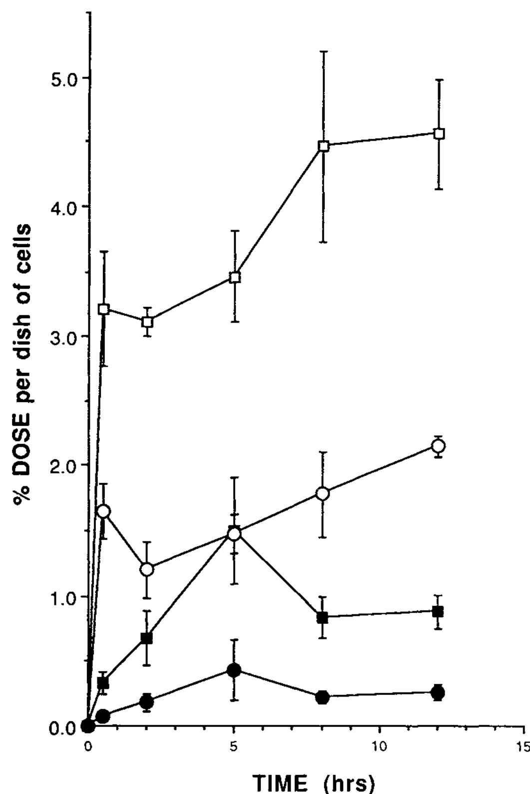

Fig. 3. Comparison of plasmid uptake kinetics using cationic liposomes or pH-sensitive liposomes. Various times after incubation of CV-1 cells with pSV2 delivered by pH-sensitive liposomes (■, ●) or DOTMA:DOPE liposomes (□, ○), the total (■, □) and high molecular weight (●, ○) fractions of radiolabeled plasmid are measured. Each point is the mean ± SD of three experiments.

almost equivalent at each time, while for pH-sensitive liposomes, this signal was the strongest at 5 hr and was weak at 0.5 and 12 hr, similar to that for the radiolabeled plasmid kinetics.

Effect of Lysomotrophic Agents. The effect of three lysomotrophic agents, chloroquine, monensin, and ammonium chloride, on the luciferase expression after Lipofectin- or liposome-mediated transfection has been investigated in CV-1 cells. These compounds are known, beside their other effects, to raise the pH of endosomes and lysosomes and to inhibit endosomal/lysosomal fusion (25). The lysomotrophic agents greatly enhanced (by a factor of 6 to 25) cationic liposomes efficiency and decreased (by a factor of 3 to 13) the pH-sensitive liposomes efficiency (Table III). The lysomotrophic agent-induced increase in transfection efficiency did not occur in all cell lines tested: chloroquine treatment of HepG2 and p388D1 cells did not significantly enhance transfection via cationic liposomes (data not shown).

DISCUSSION

Progress in gene therapy depends to a large degree on the development of delivery systems capable of efficiently introducing DNA into the target cell nucleus. As the details of how viruses efficiently infect cells are elucidated, they provide an excellent model for creating artificial DNA delivery systems. Viruses can attach to the target cell and fuse with cellular membranes to deliver their nucleocapsid into the cytoplasm, and finally, viral DNA can be easily transferred into the cell nucleus.

Numerous gene transfer attempts have been made using DNA encapsulated in liposomes (7,26), but these efforts have resulted in low and poorly reproducible levels of DNA transfer. In a number of these attempts osmotic shock using glycerol is necessary to observe significant gene transfer (7,26). The reason for this is that liposomes prepared from most lipids do not fuse to any appreciable extent with mammalian cells (27,28). This fact means that DNA encapsulated in non-pH-sensitive liposome compositions is delivered to the lysosome where the DNA is degraded. To overcome this problem, virosomes (29) and pH-sensitive liposomes (11–13) have been developed. pH-sensitive liposome compositions are designed to fuse with membranes at an acidic pH and, when internalized via an endosomal route, can fuse with the endosomal membrane and transfer molecules into the cytoplasm (30). Huang and co-workers have used the pH-sensitive composition oleic acid:DOPE:cholesterol (2:4:4) to deliver DNA both in culture and after administration into the intraperitoneal cavity of mice (31,32). In these studies, transfection was reported only when a cell surface specific antibody was attached to the pH-sensitive liposome. In addition, the level of transfection was not compared to a reproducible positive control so that the overall efficiency of the process could not be evaluated.

We have shown here that pH-sensitive liposomes composed of DOPE:CHEMS (2:1) are able to transfect different adherent mammalian cell lines without any targeting or enhancing treatment. However, DOPC:CHEMS and PS:Chol formulations of liposomes did not mediate transfection of the luciferase gene in the cell lines used here. This result can be explained by a lower uptake of the non pH-sensitive lipo-

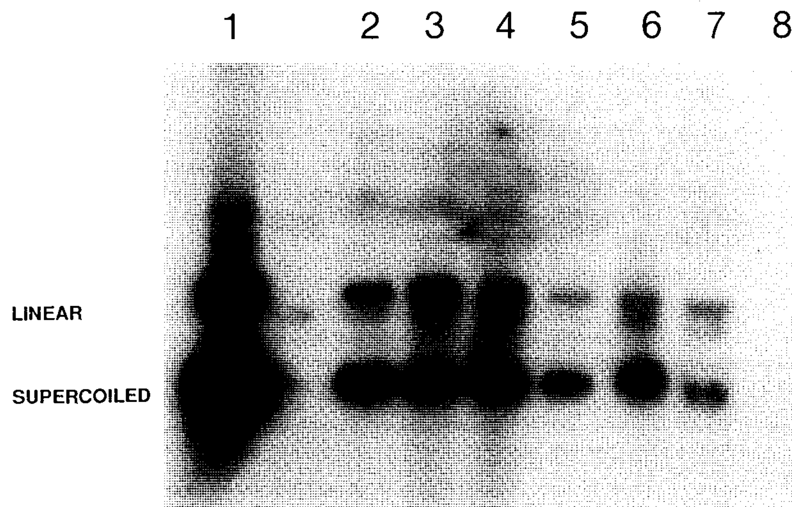

Fig. 4. Southern blot analysis of cell-associated high molecular weight DNA. The high molecular weight fraction of total DNA is extracted at various times postincubation with pSV2 plasmid delivered by either cationic liposomes or pH-sensitive liposomes. DNA is run on a 0.7% agarose gel and hybridized with ^{32}P-pSV2 as a probe. Lane 1: pSV2 plasmid. Lanes 2–4: transfection with cationic liposomes 0.5, 5, and 12 hr postincubation, respectively. Lanes 5–7: transfection with pH-sensitive liposomes 0.5, 5, and 12 hr postincubation, respectively. Lane 8: nontreated cells.

somes by the cell and their inability to transfer the DNA efficiently into the cytoplasm of the cell. Although they have a negative charge density similar to that of the pH-sensitive liposomes, DOPC:CHEMS liposomes have been shown to have a much lower cell association (13). PS:Chol liposomes have an uptake similar to that of DOPE:CHEMS liposomes, but they are not pH-sensitive. They cannot fuse with the endosomal membrane; thus delivered DNA cannot escape degradation in the lysosome.

Compared to cationic liposomes, pH-sensitive liposomes are 3- to 150-fold less efficient at transfection. The exact number depends on the cell line and the transfection conditions. One of the reasons for the superior transfection efficiency of DOTMA:DOPE liposomes is that the Lipofectin–DNA complex becomes rapidly and extensively (3% of the dose in 0.5 hr) cell associated, while DNA delivered in pH-sensitive liposomes undergoes a more gradual and lower uptake. The fact that uptake increases for DOTMA:DOPE liposomes after medium removal suggests that DNA complexed with the cationic lipid is tightly bound to the cell membrane. The gentle washing that occurs during removal of the transfection system at 5 hr does not displace the Lipofectin-delivered DNA from the cell surface. The cationic liposomes–DNA complex is continuously internalized into the cell from the surface. Compared to DOTMA/DOPE-DNA complexes, the binding of pH-sensitive liposomes to the cell is not as strong and cell-associated DNA decreases as soon as the medium is removed. These data also suggest that Lipofectin-delivered DNA is degraded less readily than the liposomal plasmid. Therefore, cationic liposomes deliver about eight times more high molecular weight plasmid into the cells than pH-sensitive liposomes. If we assume that the high molecular weight pellet is intact and that there are about 2×10^6 cells, approximately 8500 and 1000 plasmids interact with each cell at 12 hr for cationic and pH-sensitive liposomes, respectively. On the other hand, transfection of CV-1 cells is 15- to 100-fold higher for DOTMA:DOPE liposomes than for pH-sensitive liposomes.

In order to investigate further the mechanisms involved in DNA delivery, we treated the cells with lysomotrophic

Table III. Effect of Lysomotrophic Agents on Transfection Level of Cationic Liposomes and pH-Sensitive Liposomes in CV-1 Cells[a]

	Cationic liposomes	Factor[b]	pH-sensitive liposomes	Factor[b]
No treatment	21.5 ± 13.2 (5)		1.2 ± 1.2 (5)	
Chloroquine (100 μ*M*)	544 ± 251 (3)	25	0.09 ± 0.04 (3)	0.075
NH_4Cl (20 m*M*)	168 ± 43 (3)	8	0.4 ± 0.3 (3)	0.33
Monensin (10 μ*M*)	123 ± 82 (3)	6	0.4 ± 0.3 (3)	0.33

[a] Four micrograms of pSV2 plasmid is administered per 60-mm culture dish. DNA is complexed with 10 μg of Lipofectin reagent or encapsulated in pH-sensitive liposomes. Luciferase activity at 48 hr is expressed as 10^5 light units/mg of cell protein. Light units background was subtracted from each value. Results are the mean ± SD of the indicated number of experiments.

[b] The factor is computed as the ratio of luciferase specific activity without treatment to luciferase specific activity with treatment.

and intracellular trafficking perturbating agents. The presence of these compounds during transfection of CV-1 increases DOTMA:DOPE liposome efficiency, while it decreases that of pH-sensitive liposomes. This result supports a mechanism of membrane destabilization in the acidic cellular compartments for the pH-sensitive liposomes. When this acidic environment is perturbed, DNA delivery via pH-sensitive liposomes is lowered. Conversely, an increase in the endosomal/lysosomal pH increases plasmid delivery via DOTMA:DOPE liposomes, which may protect the DNA from degradation in the lysosome. Previous workers have reported that fusion is the major mechanism for Lipofectin–DNA delivery (6,7,14). We show here that, at least in CV-1 cells, endocytosis is also a possible, and perhaps the major, pathway for DNA delivery via DOTMA:DOPE liposomes. The relative importance of both phenomena, i.e., fusion and endocytosis, is a function of the cell line, since chloroquine did not increase transfection from DOTMA:DOPE liposomes in HepG2 or p388D1 cell lines.

Although improved cytoplasmic delivery is required for efficient transfection, interaction with nuclear membrane and transport of the DNA into the nucleus are also key steps in the transfection process. Indeed, Capecchi has shown that injecting plasmid into the cytoplasm of cells results in less than 0.1% transfection but plasmid injection into the nucleus transforms 50% of the cells (33). If this need for nuclear delivery is a generally true phenomenon, the high transfection efficiency observed with DOTMA:DOPE liposomes may be due to effects of this reagent on the cell nucleus (34). We are currently investigating means for improving transport of DNA into the nucleus and its effect on transfection.

CONCLUSION

The order of efficiency of three lipid-based delivery systems was cationic liposomes $>$ pH-sensitive liposomes $\gg$ non-pH-sensitive liposomes. The DOTMA:DOPE liposome is the most efficient system because it mediates the greatest level of cell-associated high molecular weight DNA and because it probably uses at least two pathways to introduce DNA into the cell: fusion with the plasma membrane and introduction via the endocytosis pathway.

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