Target Specific Optimization of Cationic Lipid-Based Systems for Pulmonary Gene Therapy

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Purpose. Cationic lipids are capable of transferring foreign genes to the pulmonary epithelium *in vivo*. It is becoming increasingly clear that factors other than lipid molecular structure also influence efficiency of delivery using cationic lipid systems. This study is aimed at evaluating the effect of formulation variables such as cationic lipid structure, cationic lipid/DNA ratio, particle size, co-lipid content and plasmid topology on transgene expression in the lung.

Methods. The effect of varying the surface and colloidal properties of cationic lipid-based gene delivery systems was assessed by intratracheal instillation into rats. An expression plasmid encoding chloramphenicol acetyl transferase (CAT) was used to measure transgene expression.

Results. Cationic lipid structure, cationic lipid/DNA ratio, particle size, co-lipid content and topology of the plasmid, were found to significantly affect transgene expression. Complexation with lipids was found to have a protective effect on DNA integrity in bronchoalveolar lavage fluid (BALF). DNA complexed with lipid showed enhanced persistence in rat lungs as measured by quantitative polymerase chain reaction.

Conclusions. Fluorescence microscopy analysis indicated that the instilled formulation reaches the lower airways and alveolar region. Data also suggests cationic lipid-mediated gene expression is primarily localized in the lung parenchyma and not infiltrating cells isolated from the BALF.

KEY WORDS: gene transfer; airways; cationic lipids; surface charge; co-lipid content; topology.

INTRODUCTION

The unique accessibility of the lung epithelium by instillation or inhalation makes the lung a suitable target for gene therapy. Diseases of the lung have to date represented a large fraction of the approved gene therapy protocols, despite the variety of potential disease states approachable by gene therapy (1). It is generally accepted that adenoviral vectors are quite effective *in vivo*. However, safety issues and immunological responses are major concerns for repeated administration of viral vectors (2). Viral-mediated gene delivery approaches currently investigated in clinical trials for the treatment of cystic fibrosis have resulted in immunological and inflammatory side-effects, indicating a need for safer methods (3,4). Non-viral approaches in which plasmid is complexed with cationic lipids have been used to deliver genes to the lungs (5–8). These plasmid/lipid complexes offer several advantages including ease of production, low immunogenicity, cost effectiveness and reproducible bioavailability. However, efficiency of gene transfer with non-viral approaches is relatively low. In addition, we and others have found that instillation of cationic lipid/DNA complexes into the mammalian lung caused an acute, dose-dependent inflammatory response (9,10). Significant improvement in the potency of cationic lipid/DNA formulations may be needed to reduce doses in order to minimize toxicity associated with cationic lipids.

Colloidal and surface properties of plasmid/lipid complexes control their distribution, access, intracellular uptake and nuclear translocation (11). The studies reported herein focus on the optimization of the colloidal and surface properties of non-viral gene delivery systems for maximizing transgene expression following intratracheal instillation in rats. The results from our studies demonstrate that the critical characteristics of plasmid/lipid complexes that affected the efficiency of gene transfer are particle size, surface charge, co-lipid content, plasmid physical form and stability in physiological media.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 130–150 g were housed in the vivarium at GENEMEDICINE, INC., and maintained on ad libitum rodent feed and water at 23°C, humidity 40% and a 12hr/12hr light cycle. The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Gene Expression System

A plasmid containing the bacterial reporter gene, chloramphenicol acetyl transferase (CAT), driven by cytomegalovirus (CMV) promoter/enhancer produced at GENEMEDICINE, INC. (P-CMV-CAT), was used to measure transgene expression.

Preparation of Liposomes

Ethyl dioleyl phosphatidyl ethanolamine (EDOPC), ethyl palmityl myristyl phosphatidyl choline (EPMPC), dioleylphosphatidylethanolamine (DOPE) and cholesterol (Chol) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Myristyl myristoyl carnitine ester (DL-MMCE), oleyl oleoyl carnitine ester (DL-OOCE) were synthesized at GENEMEDICINE, INC. using procedures developed earlier (12). For fluorescence microscopy studies, N- (Texas Red® sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red® DHPE) was purchased from Molecular Probes (Eugene, OR). Liposomes were prepared by drying down lipids as a thin film in a 50 ml round bottom flask using a rotary evaporator. The film was then exposed to high vacuum for at least 1 hour and then hydrated in sterile water. Unilamellar vesicles (SUVs) were prepared by extrusion through 100, 400, and 800 nm pore size polycarbonate filters.

Preparation of Plasmid/Lipid Complexes

p-CMV-CAT/DOTMA:Chol and p-CMV-CAT/DOTMA: DOPE complexes at different charge ratios were formulated in

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10% (w/v) lactose by mixing the plasmid with liposomes under controlled conditions. The plasmid concentration in the formulation was 125 μg/mL. Plasmid/lipid complexes of different size and surface charges were prepared by altering the stoichiometry of plasmid and lipids. To ensure reproducibility, the formulations tested were characterized systematically for particle size, complexation efficiency and zeta potential. Particle size analysis was performed using Dynamic Light Scattering [Coulter (Model N4MD) submicron particle sizer]. Complexation efficiency was determined using agarose gel electrophoresis. Zeta potential measurements were performed using Doppler Electrophoretic Light Scattering [Coulter DELSA 440].

In Vivo *Administration*

The animals were anesthetized with 80 mg/kg of ketamine given intra-peritoneally. The animals were divided into treatment groups ($n = 5$). A lavage canula was inserted into the trachea by intubation and the animal received a 400 μl bolus of formulation. The treatment groups were instilled with 50 μg of uncondensed or formulated plasmid.

Tissue Harvest and Extraction

Bronchoalveolar lavage fluid (BALF) and/or lung tissue were collected at various time points following administration. Animals were euthanized with 50 μL ketamine cocktail (73.96 mg/ml ketamine, Fort Dodge; 3.74 mg/ml xylazine, Fermenta Animal Health; 0.73 mg/ml acepromazine; Bayer). For BALF collection, lungs were lavaged with 2 × 1.0 ml aliquots of Hank's Balanced Salt solution, (GIBCO, Grand Island, NY) without Ca^{++} or Mg^{++}. For preparation of lung tissue extracts, excised lung lobes were transferred to 2.0 ml screw-cap polypropylene tubes containing 0.25 gram zirconium beads (Biospec, Bartelsville, OK) and snap frozen in liquid nitrogen for storage at −80°C. Harvested tissue was analyzed using the ELISA assay (Boehringer Mannheim, Cat.No.1363727) for CAT expression.

Quantitative PCR Analysis

The stability and integrity of unformulated plasmid and plasmid/lipid formulations in the epithelial surface fluid were assessed using quantitative PCR and Southern blot analyses. At various time points (0 hr, 15 min, 1 hr, and 24 hr) following instillation, the lung tissue was harvested to quantify DNA retention and plasmid integrity.

Total DNA was first isolated from rat lungs using the SDS/Proteinase K method. Tissues were homogenized in 2 ml of digestion buffer (100mM NaCl, 10mM Tris-HCl, pH8, 25mM EDTA, pH8, 0.5% SDS, and 0.1mg/ml Proteinase K) per 100mg wet tissue and incubated with shaking at 50°C overnight. The samples were then extracted with an equal volume of Tris buffered phenol pH 8, followed by extraction with chloroform:isoamyl alcohol (24:1 v/v) and ethanol precipitated. Final DNA pellet was dissolved in TE (10mM Tris pH 7.5, 1mM EDTA). The DNA concentration was determined by measuring absorbance at 260nm.

Evaluation of plasmid content isolated from tissues was done by the quantitative Polymerase Chain Reaction (PCR) using Taqman PCR reagent kit (Perkin Elmer). This kit employs a fluorogenic probe technology that exploits the 5′ → 3′ exonucleolytic activity of AmpliTaq DNA Polymerase (13,14). All PCR amplifications were performed in the ABI 7700 Sequence Detector (40 cycles). The primers used in the reaction were a sense primer 5′- TGA CCT CCA TAG AAG ACA CCG GGA C-3′, which primes in the CMV 5′ UTR and an antisense primer 5′- GCA AGT CGA CCT ATA ATG CCG - 3′, which primes in the CAT coding region. The probe sequence was 5′-CCA GCC TCC GGA CTC TAG AGG A-3′. The initial copy numbers of the unknown samples were determined using the ABI 7700 sequence detector by comparing them to a standard curve generated from samples of known initial copy numbers.

Fluorescence Microscopy

For the fluorescence microscopy studies, DOTMA:Chol: Texas Red® DHPE (45:45:10, mole %) liposomes were formulated with fluorescently labeled plasmid. To prepare the psoralen-fluorescein labeled p-CMV-CAT, approximately 3 nmol of psoralen-fluorescein was added per 1 mg DNA. The mixture was then subjected to UV irradiation at 365 nm for a minimum of 3 hours. After irradiation, the sample was purified by n-butanol extractions and dialyzed against deionized water. The labeled plasmid was then stored at 4°C.

Animals were instilled with 10 μg labeled plasmid formulated with lipid in 10% lactose. Approximately 30 minutes after instillation, the animals were sacrificed. 1 ml of Tissue-Tek® O.C.T. compound (Sakura Finetek, CA), diluted in phosphate buffered saline was added via the trachea, to inflate the lungs. The lungs were then removed, divided into right lung and left lung, and frozen with O.C.T. in cryomolds, using liquid nitrogen-cooled isopentane. The specimens were stored at −70°C until cryosectioning. For the fluorescence microscopy analysis 10μm cryosections were made and examined with an Olympus BX60 fluorescence microscope. For morphological assessment, serial sections were also stained with hemotoxylin and eosin (H&E).

Transmission Electron Microscopy (TEM)

The preparation and visualization of DNA/lipid complexes utilized for TEM studies were performed as described earlier (15). Briefly, carbon-coated 200 mesh copper grids (Ted Pella, Inc., Redding, CA) were glow discharged for approximately 90 seconds to render them hydrophilic. One drop of the complex of the colloidal suspension was deposited on the grids. Each grid was then stained with a drop of 1% uranyl acetate solution (Ted Pella, Inc., Redding, CA) and excess stain was removed after 30 seconds. The grid was then allowed to dry for at least 20 minutes before TEM examination. Electron micrographs were obtained at magnifications ranging from 28,000–55,000 on a JEOL microscope operating at 60 kV. Particle sizes of the complexes were determined by comparing their outer diameters with grid markings.

Statistical Analysis

Data were analyzed by ANOVA followed by post-hoc analysis of groups with the nonparametric Mann-Whitney test using the SPSS Base 7.5 for Windows statistics software package (SPSS Inc., Chicago, IL). Data were considered statistically significant if p values were <0.05.

RESULTS

Optimization of Formulation Variables

Structure of Cationic Lipid

We have evaluated a panel of mono-cationic lipids of various structures, i.e., DOTMA, EDOPC, OOCE, MMCE, EPMPC, for transfection efficiency in the lung (Fig. 1A). This is especially critical in developing formulations for *in vivo* gene therapy because we found that cell culture experimentation is not predictive of efficacy *in vivo*. The molecular structures of the lipid reagents tested are shown in Fig. 1B. A majority of the lipids showed weak transfection activity in absence of a co-lipid, therefore activities were compared for lipids formulated with either DOPE or Chol. The activities are shown normalized to the level of expression attained with instillation of 'naked' plasmid in 10% lactose. The structure of cationic lipids was found to signficantly influence transfection efficiency in the lung upon intratracheal instillation. As shown in Fig. 1, changes in the acyl chain length and degree of saturation, as in case of OOCE and MMCE, were found to influence transgene expression.

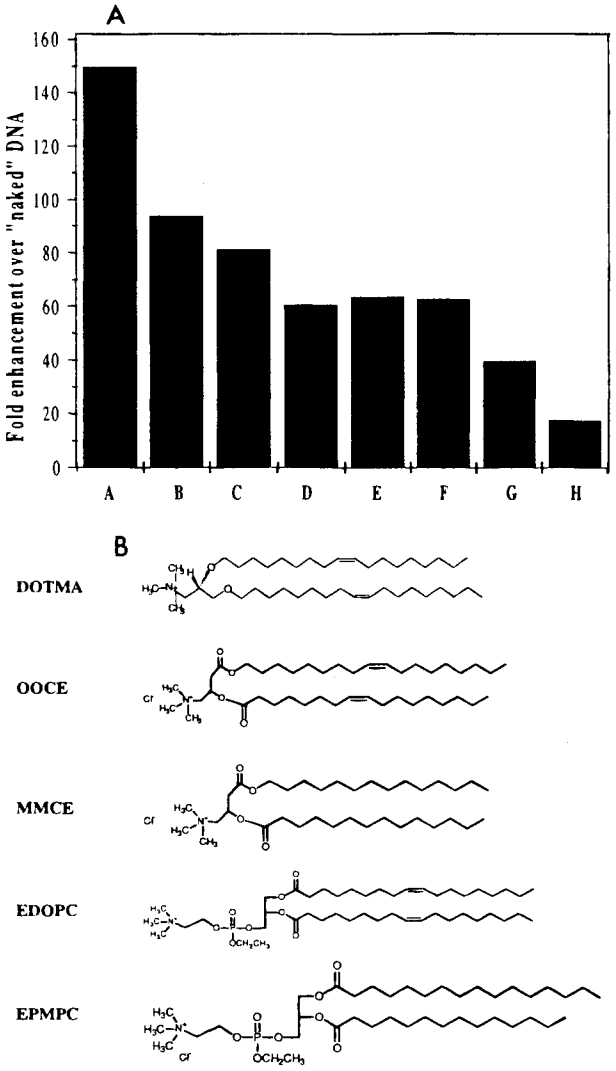

Fig. 1. (A) Comparison of cationic lipids for transfection efficiency in the lung. (B) Molecular structures of cationic lipids. Rats were instilled intratracheally with 400 μl p-CMV-CAT, p-CMV-CAT/lipid complexes, containing 50 μg DNA. The plasmid/lipid formulations were prepared in 10% lactose at charge ratio of 1:3 (−:+). Lungs were harvested at 48 hours post-instillation and analyzed for CAT expression. A: MMCE:DOPE (1:1, mole:mole), B: EDOPC:DOPE (1:1, mole:mole), C: DOTMA:Chol (1:1, mole:mole), D: DOTMA:DOPE (1:1, mole:mole), E: EDOPC:Chol (1:1, mole:mole), F: OOCE:Chol (1:1, mole:mole), G: EPMPC:Chol (1:1, mole:mole), H: OOCE:DOPE (1:1, mole:mole).

Cationic Lipid/DNA Ratio

To determine the optimal ratio of lipid to DNA, 50 μg p-CMV-CAT formulated with DOTMA:Chol (1:1, mole:mole) was instilled intratracheally into rat lungs. The plasmid/lipid complexes were formulated at charge ratios of 1:0.5, 1:0.8, 1:2, and 1:3 (−:+). The zeta-potential was found to increase from a negative to a positive value with increase in the lipid/DNA ratio. Liposomes extruded through 800 nm polycarbonate filters were used for this study, as we have found that larger particles showed better transfection efficiency in the lung.

There was approximately a 30-fold, 21-fold, 3-fold enhancement in expression levels over DNA in 10% lactose, with complexes formulated at 1:3, 1:2, 1:0.8 (−:+) respectively (Fig. 2). A net positive surface charge on the particles was required for optimal transfection. Similar trends have been observed with p-CMV-CAT/DOTMA:DOPE formulations, which showed up to an 83-fold enhancement in expression levels at 1:3 (−:+) (results not shown). This data indicates that surface charge of the plasmid/lipid complex may influence its interactions with epithelial cell surface and subsequent endocytosis *in vivo*.

We have analyzed the complexes prepared at the various charge ratios by transmission electron microscopy. Our analysis revealed that at negatively charged ratios the complexes appear to have projections (consistent with DNA) protruding from the surface of the complex. The extent of DNA compaction was found to be greater at higher charge ratios of cationic lipid to DNA, which could enhance transfection efficiency. Figures 2B and 2C are transmission electron micrographs of the DNA:lipid complexes formulated at 1:0.5 (−:+) and 1:3 (−:+).

Conformation of Plasmid

To determine the conformation of plasmid for optimal transgene expression, plasmid preparations enriched for open circle, or supercoiled forms, were used. We compared expression levels of plasmid enriched for supercoiled content, plasmid enriched for open circle content, plasmid of equal amounts of open circle and supercoiled forms. Following separation using agarose gel electrophoresis, the open and supercoiled content was determined using a fluorimager. The supercoiled content in the enriched supercoiled form was determined to be 90%. The open circle content in the enriched open circle form was determined to be 87% (Fig. 3A).

We found that there was an increase in levels of CAT expression as the fraction of the supercoiled content in the formulated plasmid was increased. Complexes formulated using the enriched supercoiled form of plasmid showed approximately a 9-fold enhancement in transfection efficiency *in vivo* over

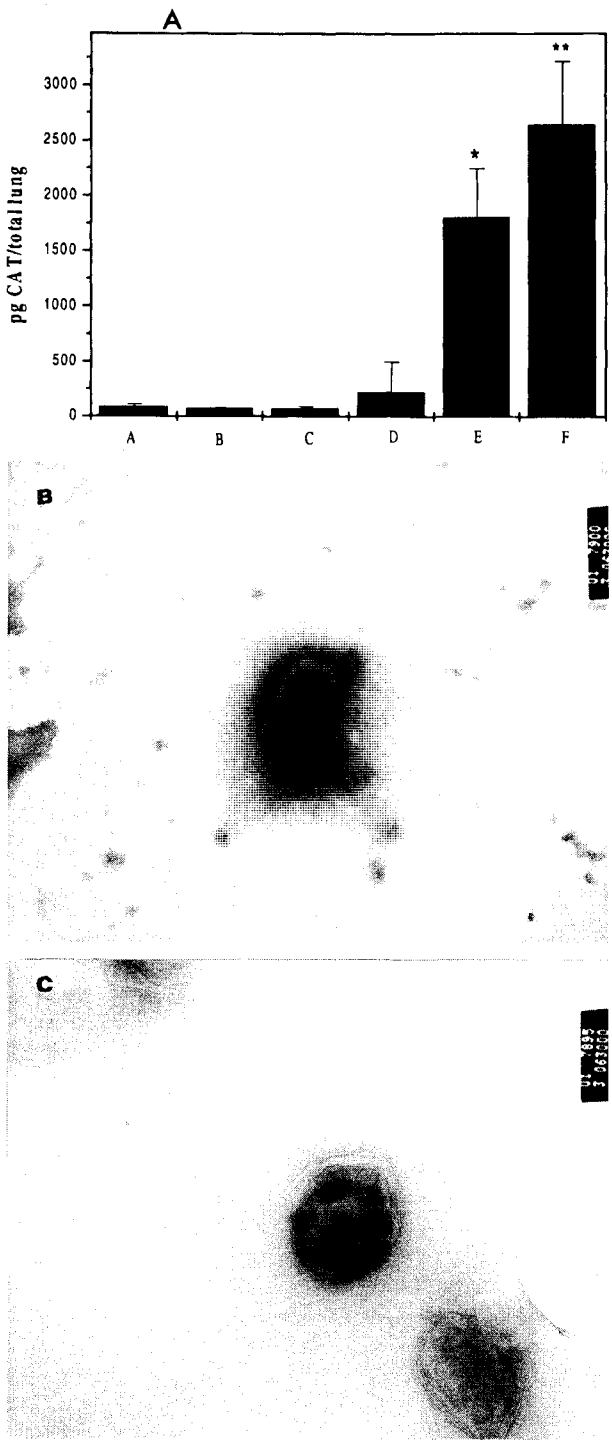

Fig. 2. (A) Effect of surface charge on expression following intratracheal instillation. Transmission electron micrographs of DNA/lipid complexes formulated at (B) 1:0.5 (−:+), (C) 1:3 (−:+). Rats were instilled intratracheally with 400 μl pCMV-CAT, pCMV-CAT/lipid complexes, containing 50 μg DNA. The plasmid/lipid formulations were prepared in 10% lactose at charge ratios of 1:0.5, 1:2 and 1:3 (−:+). The zeta potential of the complexes as determined by Doppler electrophoretic light scattering was −25 mV, +28 mV, and 50 mV respectively. Lungs were harvested at 48 hours post-instillation and analyzed for CAT expression. A: pCMV-CAT in 10% lactose; B: pCMV-CAT in water; C: pCMV-CAT/DOTMA:Chol (1:0.5, −:+); D: pCMV-CAT/DOTMA:Chol (1:0.8,−:+); E: pCMV-CAT/DOTMA:Chol (1:2, −:+); F: pCMV-CAT/DOTMA:Chol (1:3, −:+). The data are expressed as mean ± SD (n = 6). * indicates a $p < 0.01$, ** indicates $p < 0.011$, when comparing treatments to animals receiving pCMV-CAT in 10% lactose.

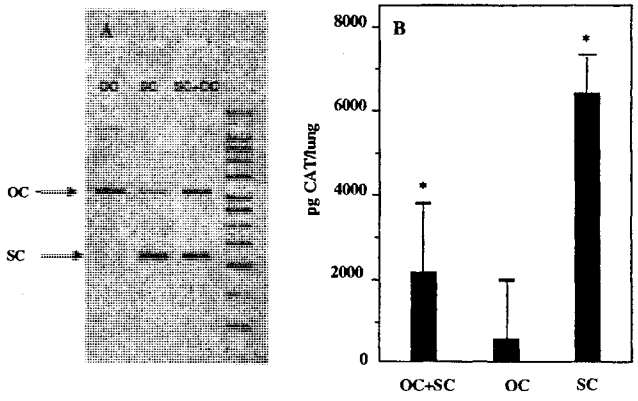

Fig. 3. (A) Agarose gel electrophoresis of the DNA samples, unfractionated (U), enriched for open circle form (O), enriched for supercoiled form (SC), 50 + 50 mixture of supercoiled forms and open circle (SC + OC). Arrows indicate positions of OC and SC bands. (B) Effect of plasmid physical form on *in vivo* expression following intratracheal instillation. Rats were instilled intratracheally with 50 μg/400 μl pCMV-CAT/lipid complexes formulated using plasmid enriched for OC, OC + SC, or SC forms. The complexes were formulated at a charge ratio 1:3 (−:+), using liposomes extruded through 800 nm polycarbonate filters. Lungs were harvested at 48 hours post-instillation and analyzed for CAT expression. The data are expressed as mean ± SD (n = 6). * indicates statistical difference ($p < 0.006$) from OC.

the open circle form. Mixed populations (open circle + supercoiled) showed intermediate levels of expression (Fig. 3B).

Co-Lipid Content

In addition to optimizing the ratio of cationic lipid/DNA, the optimal ratio of cationic lipid DOTMA to neutral co-lipids DOPE and cholesterol was also determined. Routinely, molar ratios of cationic lipid to co-lipid ranging from 2:1 to 1:2 were evaluated. DOTMA in the absence of a co-lipid showed weak transfection activity (data not shown) and presence of a co-lipid was found to be required for transfection. As shown in Fig. 4, altering the co-lipid content was found to significantly affect transgene expression.

Enhanced Protection and Persistence of Formulated Plasmid in the Lung

The stability of formulated versus uncondensed DNA was evaluated in BALF. Uncondensed DNA and DNA formulated in DOTMA:Chol at 1:3 (−:+) were incubated in freshly isolated rat BALF at 37°C. The integrity of DNA as measured by percentage of supercoiled DNA retained, was evaluated using agarose gel electrophoresis at the specified time intervals. As shown in Fig. 5, a significant drop in % supercoiled content of the uncondensed DNA was observed upon incubation with BALF. Complexation with cationic lipid was found to protect DNA integrity.

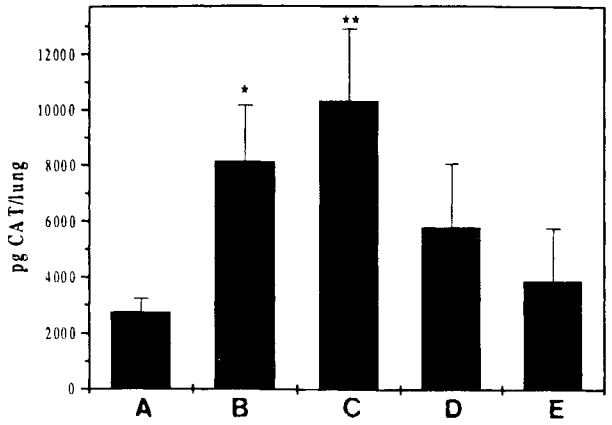

Fig. 4. Effect of co-lipid content on expression following intratracheal instillation. Rats were instilled intratracheally with 50 μg DNA/400 μl pCMV-CAT/lipid complexes using (A) DOTMA:Chol (2:1, mole:mole), (B) DOTMA:Chol (1:1, mole:mole), (C) DOTMA:DOPE (2:1, mole:mole); (D) DOTMA:DOPE (1:1, mole:mole), (E) DOTMA:DOPE (1:2, mole:mole). The complexes were formulated at a charge ratio 1:3 (−:+) using liposomes extruded through 400 nm polycarbonate filters. The lungs were harvested at 48 hours post-instillation and analyzed for CAT expression. The data are expressed as mean ± SD (n = 6). * indicates statistical difference ($p < 0.01$) from DOTMA:Chol (2:1, mole:mole), ** indicates statistical difference ($p < 0.014$) from DOTMA:DOPE (1:1, mole:mole).

We also compared the persistence of complexed versus unformulated plasmid *in vivo* following intratracheal instillation. Lung tissue was harvested at various time points following instillation of uncondensed DNA and plasmid/DOTMA:Chol (1:3, −:+) and analyzed for DNA levels using PCR. As shown in Fig. 6, complexation with the lipid significantly enhanced the persistence of the DNA over a period of 24 hours. The mass of DNA retained in the lungs was upto ~1000-fold higher upon complexation with cationic lipids in comparison to the

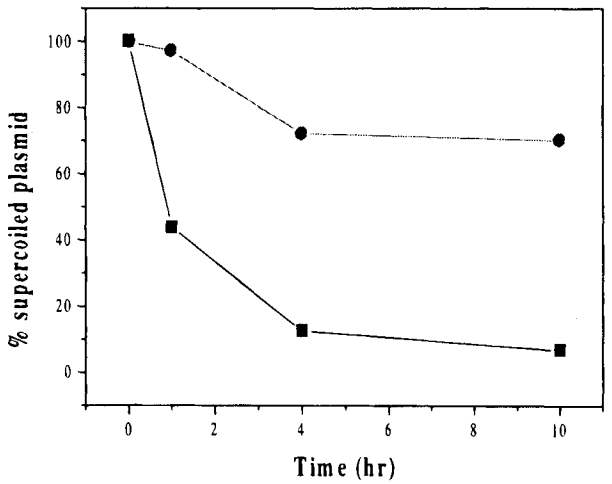

Fig. 5. Stability of plasmid/DOTMA:Chol complexes in BALF at 25°C. 50 μg of (a) ■ uncondensed DNA, or (b) ● DNA complexed with DOTMA:Chol at 1:3 (−: +), were incubated in freshly isolated BALF. At the specified time intervals samples were subjected to agarose gel electrophoresis and supercoiled content was determined using fluorimager.

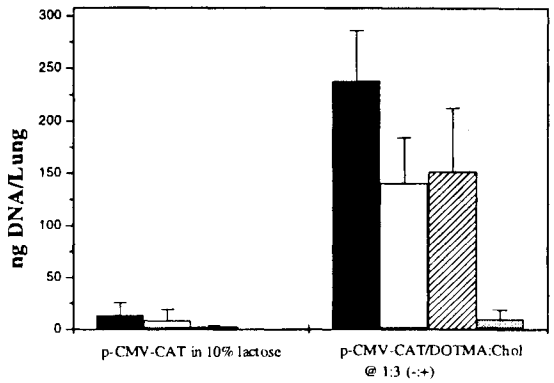

Fig. 6. Evaluation of persistence of DNA using PCR. Rats were instilled intratracheally with 400 μl of 50 μg DNA in 10% lactose or DNA/DOTMA:Chol @ 1:3 (−:+) in 10% lactose. At the specified time intervals the lungs were harvested and analyzed by PCR. (a) 0 min—closed bars; (b) 15 minutes-open bars; (c) 1 hour—hatched bars; (d) 24 hours—gray bars. The data are expressed as mean ± SD (n = 6).

unformulated plasmids, suggesting that the particulate carriers help in protecting the DNA from enzymes present in the epithelial lining fluid. Southern blot analysis suggested that the size of the DNA recovered was comparable to non-instilled control plasmid (data not shown).

Distribution

The distribution of the DNA and lipid components of the formulation was evaluated by fluorescence microscopy following instillation of fluorescently labeled DNA and lipid. Our data suggests that a majority of lower airway epithelial cells and alveolar lining cells showed intense red and green fluorescence. The signal from the labeled DNA was seen to co-localize with the labeled lipid, indicating that most of the DNA remains associated with the lipid (Fig. 7). A gross analysis of different sections suggests that the complex was not delivered uniformly to all the lobes by the instillation procedure.

We also evaluated the distribution of gene expression in the rat lung following instillation of DNA/DOTMA:Chol complexes. The objective was to determine types of cells (epithelial v/s infiltrating cells) showing expression following instillation of the prototype DNA/DOTMA:Chol formulation. Lung lavages were performed in animals 24 hours following treatment, and expression levels measured in the cells in the lavage and tissue fraction. To evaluate the effect of surface charge on distribution, complexes formulated at 1:0.5 (−:+) and 1:3 (−:+) were instilled intratracheally. Consistent with our previous results, expression levels with the 1:0.5 (−:+) complexes were found to be much lower than 1:3 (−:+). As seen in Fig. 8A and 8B, expression levels in the BALF (cell pellet) were found to be significantly lower compared to lung tissue. This suggests that most of the expression following instillation comes from lung parenchyma and not infiltrating cells in the BALF.

DISCUSSION

Over the past decade, several critical barriers to gene transfer and expression have been identified, i) access to the target cell population, ii) entry of plasmid into cell, iii) endosomal release, iv) nuclear entry. It is becoming increasingly clear that

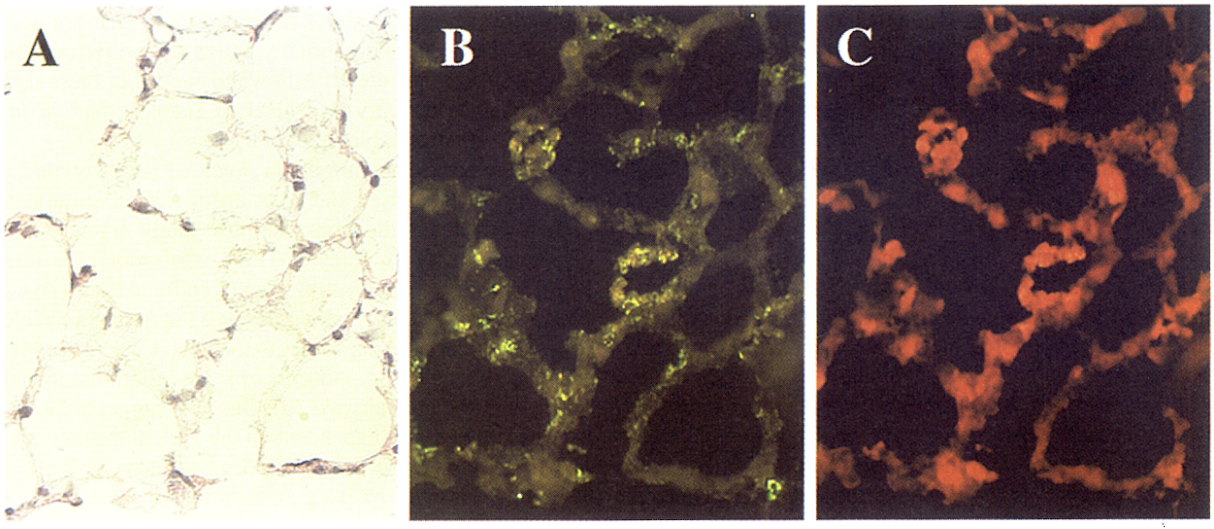

Fig. 7. Flourescence microscopy analysis. Rats were instilled intratracheally with 400 μl pCMV-CAT/lipid complexes containing 50 μg DNA, formulated at a charge ratio 1:3 (−:+). Lungs were harvested at ~ 30 minutes and tissue processed as described under Materials and Methods. (A) H and E staining (B) Localization of labeled DNA (C) Localization of labeled lipid component.

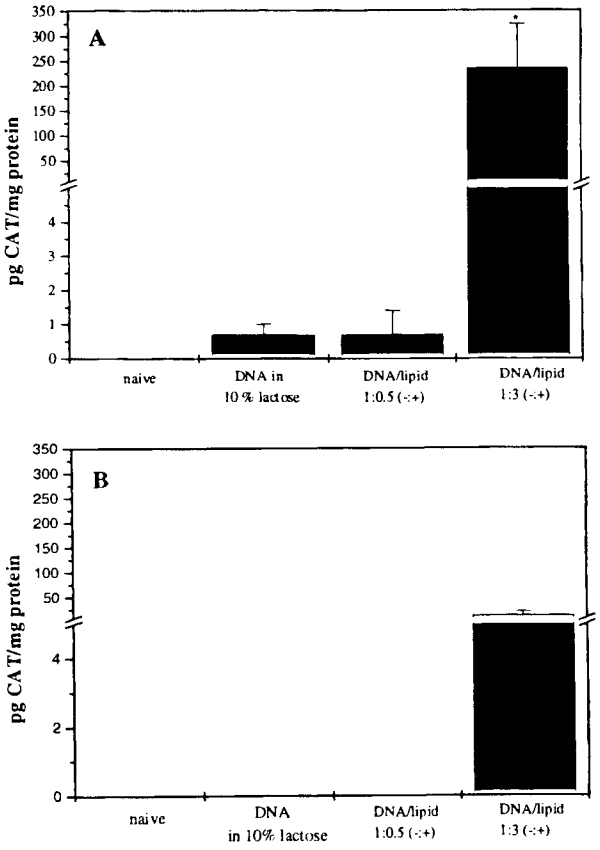

Fig. 8. (A, B): Evaluation of cell types expressing transgene. 10 μg of DNA/DOTMA:Chol complexes formulated at positive (1:3, −:+) and negative (1:0.5, −:+) charge-ratios were instilled intratracheally. Expression levels in lung tissue (A), and cell fraction obtained from lung lavage (B) were measured at 24 hours following treatment. The data are expressed as mean ± SD (n = 6). * indicates a $p < 0.001$, when comparing to cell fraction samples of animals treated with DNA/DOTMA:Chol (1:3, −:+).

formulation variables can influence the ability of plasmid to overcome these barriers. Surface and colloidal properties of the formulated DNA can also greatly affect *in vivo* tissue distribution. For example, the key challenge for effective hepatocyte non-viral gene therapy is to produce colloidally stable particles with a mean diameter below about 100 nm, larger particles are taken up by Kupffer cells, and still larger particles are cleared from circulation by the small capillary bed in the lung (16,17). In this study we have determined surface and colloidal properties for optimal transgene expression of cationic lipid-based formulations in the lung. The prototype formulation used for these studies was a complex of plasmid with DOTMA:Chol (1:1, mole:mole) in 10% lactose.

Our results indicate that formulation variables including cationic lipid structure, surface charge, particle size, physical form of the plasmid and co-lipid content greatly influence transfection efficiency. Cationic lipids facilitate transgene expression through 1) complexation and condensation of DNA molecules via charge interaction, 2) interaction with cell membranes and triggering uptake of the complexed DNA, and 3) delivering DNA across the cellular membrane into the cytoplasm. There are reports suggesting that plasmid/lipid complexes also need to dissociate, probably in the endosomes, to allow the plasmid to translocate to the nucleus of the target cell (18). The structure of cationic lipids can affect the ability of the DNA to overcome these barriers and was found to signficantly influence transfection efficiency *in vivo*. As shown in Fig. 1, changes in the acyl chain length and degree of saturation, as in case of OOCE and MMCE, were found to influence transgene expression.

The surface charge of a DNA/lipid complex is expected to influence its interactions with various biological components, as well as its distribution, access, and entry into target cells. It has been reported that efficiency of endosomal release may also be affected by the charge at which the complex is formulated (19), posing only a moderate barrier for positively charged complexes. Complexes formulated at positively charged ratios were found to show enhanced transfection in the lung (Fig. 2A). The results indicate that surface charge of the plasmid/

lipid complex may influence its interactions with the epithelial cell surface and subsequent endocytosis. This enhanced expression may also be in part due to the protection of the plasmid by the lipid against degradation. Degradation of DNA complexed with lipid was significantly lower than that of free DNA when incubated in BALF at 25°C (Fig. 5). The extent of DNA compaction appears to be greater at higher charge ratios of cationic lipid to DNA (Fig. 2B, C). A microscopic analysis of the complexes revealed revealed that at negatively charged ratios there was seen to be presence of free DNA, and complexes appeared to have projections (consistent with DNA) protruding from the surface of the complex making it more susceptible to degradation.

The diameter of a plasmid/lipid complex is an important parameter that should control not only the deposition of colloidal particulate plasmids after their administration, but also their uptake by target cells (11). We found there was a statistically significant increase in the level of CAT expression as the size of the plasmid/lipid complex was increased from 237 to 901 nm (data not shown).

We evaluated the effect of DNA topology on transgene expression *in vivo*. The normal biological function of DNA occurs only if it is in the proper topological state. In such biological processes as RNA transcription, DNA replication, the recognition of a base sequence requires the local separation of complementary polynucleotide strands. If DNA lacks the proper superhelical tension the above vital processes occur slowly, if at all (20). This would directly influence the efficiency of transgene expression. We found that supercoiled form of the plasmid was most potent in terms of transfection efficiency when formulated with lipids. Expression levels with complexes formulated using the enriched supercoiled form of plasmid showed a significant enhancement in transfection efficiency over the open circle form (Fig. 3).

Neutral co-lipids have been demonstrated to play a significant role in cationic lipid mediated gene transfer through several proposed mechanisms, i.e., increasing DNA uptake into cells, facilitating escape from the endosomes, increasing the ability of the DNA to dissociate from the complex (21,22). Each of these mechanisms may contribute to the net amount of transgene expression. As shown in Fig. 4, co-lipid content in the formulation significantly affects transfection efficiency.

We found that expression levels with the DOTMA:Chol-based formulation were found to peak at 24 and 48 hours following treatment and showed a decline at 72 hours (data not shown). We hypothesize that this may be in part due to degradation of DNA in lung tissue. We see a rapid loss of DNA levels over the 24-hour period following treatment as measured using quantitative PCR (Fig. 6). However, particulate carriers significantly enhanced the residence time of DNA. Our PCR analysis shows that mass of DNA retained in rat lungs was significantly higher (upto ~1000-fold) upon complexation with cationic lipids.

Our data suggests that most of the CAT activity seen in the lung was associated with the lung parenchyma and not with the infiltrating cells in the BALF (Fig. 8A, B). There was seen to be a good correlation between expression levels and amount of plasmid in cell and tissue fractions (results not shown).

In summary, our results suggest that optimization of formulation variables of cationic lipid/DNA complexes can significantly improve *in vivo* efficacy. It is also becoming increasingly apparent that the optimal parameters vary greatly depending on the *in vivo* location, so that formulations that are active in one tissue may not be optimally active by a different route of administration. The identification and control of these determinants of gene delivery is critical to enable effective *in vivo* gene delivery to the biological target.

There are clearly important practical issues related to the transfer of gene-based therapies from the laboratory to the patient. For lung indications, the most likely practical route of administration is by aerosol. There is considerable progress being made towards aerosolization of cationic lipid/DNA complexes (8,23–24). Also, tolerability is a key issue in developing pharmaceutical gene medicines to the lung. Cationic lipids were found to be the primary inducers of a pro-inflammatory cellular infiltrate in the lung airways (9). Our data suggests that pulmonary inflammation is dependent on the dose of the cationic lipid and may be affected by the biodegradability of the cationic lipid (data not shown). Therefore, although there has been an impressive improvement in cationic lipid mediated systems to the lung (17), strategies need to be developed towards more potent, tolerable, cationic lipid-based delivery systems.

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