Aerosol Delivery of Lipid:DNA Complexes to Lungs of Rhesus Monkeys

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Purpose. The potential use of aerosol delivery for non-viral gene therapy was tested by nebulization of lipid:DNA complexes to the lungs of rhesus monkeys.

Methods. Four female rhesus monkeys were dosed with lipid:DNA formulations via aerosol inhalation, where the DNA coded for the human Cystic Fibrosis Transmembrane Conductance Regulator (hCFTR) protein. Delivery of DNA was determined in lung samples by polymerase chain reaction (PCR) by qualitative and quantitative methods. Transgene specific messenger RNA was measured by reverse transcriptase PCR (RT-PCR) and protein expression and localization were evaluated by immunohistochemistry (IHC).

Results. Approximately four mg of DNA, complexed with cationic lipid 1,2-dimyristoyl-sn-glycero-3-ethylphosphatidylcholine (EDMPC) and cholesterol were delivered to the lungs of animals by air jet nebulizer. Three days after dosing, tissue samples from the lung were collected and shown to have vector specific DNA, RNA and the presence of CFTR protein. Specifically, the hCFTR protein was distributed widely, although non-uniformly, throughout airway epithelium being located on the apical surface of epithelial cells. Importantly, no adverse clinical effects were observed and the lungs showed no histological abnormalities or signs of acute inflammation.

Conclusions. This study shows that lipid:DNA formulations based on EDMPC and cholesterol can be administered to primates by nebulization resulting in measurable expression of the hCFTR protein. The absence of inflammation is also encouraging and such systems may have utility for delivery of genes to the lungs for the treatment of a variety of pulmonary diseases including cystic fibrosis.

KEY WORDS: gene therapy; primates; lipid:DNA; aerosol; drug delivery; CFTR.

INTRODUCTION

For gene therapy to become a reality, safe and effective methods must be found to deliver DNA efficiently to target cells of interest. One attractive approach is aerosol delivery because it is non-invasive and has the potential for delivering high concentrations of the therapeutic DNA to a contained area of the body. Since studies have shown the delivery of recombinant adenovirus by aerosol to induce inflammation (1), non-viral methods of delivery could provide an attractive alternative. The use of cationic lipids has been explored for gene delivery to the nasal epithelium and lungs (2–4). The human cystic fibrosis transmembrane conductance regulator (hCFTR) gene (5) has been delivered using lipid:DNA complexes in animal studies (3–8) and has proceeded into clinical trials (9). Efficiency of delivery, however, is still a concern with most non-viral techniques. Recent efforts have focused on the development of more efficacious lipid:DNA delivery systems (10). Lee *et al.* (11) recently reported an extensive study which tested numerous novel lipids; however, all these lipids showed signs of toxicity both *in vitro* and *in vivo*.

Water alone has been used as a vehicle in intratracheal gene transfer (11,12), although it may to lead to osmotic cellular shock (13). Studies have shown that cationic lipid:DNA complexes can afford protection to nebulization, while DNA in water was degraded by this procedure (14).

We have shown previously that the cationic lipid, EDMPC (1,2-dimyristoyl-sn-glycero-3-ethylphosphatidylcholine, chloride salt), had an enhanced ability to deliver DNA to pulmonary tissues following direct instillation into rodent lungs (15), resulting in an order of magnitude higher in expression than DNA in water alone. The level of expression seen with EDMPC is comparable to a low level of adenovirus infection (13) and similar to that seen by Lee et al. (11). However unlike the lipids described in this latter study, lungs treated with EDMPC:DNA complexes showed a visible lack of inflammation upon examination at high power implying a lack of toxicity (15). To address the question of whether this lipid:DNA complex has potential in aerosol delivery to the lung, we undertook a study in rhesus monkeys.

MATERIALS AND METHODS

Expression Vectors and *In Vitro* Validation of DNA Preparations

The expression vector pMB113 contains hCFTR DNA sequences (5) under control of the hCMV promoter (16,17) with a preproinsulin intron (18) 5′ of the cDNA for CFTR and the SV40 early poly A site used for transcription termination (19). This vector also contains the tetracycline resistance gene and a prokaryotic plasmid origin of replication. Expression vectors used as controls contained either the β-galactosidase gene (19) pMB10, or the CAT gene (20) p4119, Gorman *et al.* (15).

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ABBREVIATIONS: hCFTR, human Cystic Fibrosis Transmembrane Conductance Regulator; CAT, Chloramphenicol Acteyltransferase; β-gal, β-galactosidase; ΔF508, mutant hCFTR protein, missing phenylalanine at position 508; EDMPC, 1,2-dimyristoyl-sn-glycero-3-ethylphosphatidylcholine; HCMV, Human cytomegalovirus; cDNA, complementary DNA; poly A, poly adenylation site; TetR, Tetracycline resistance gene; RT-PCR, Reverse Transcriptase-Polymer Chain Reaction; PCR, Polymer Chain Reaction; mRNA, messenger RNA; PBS, phosphate buffered saline; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium doedecyl sulfate poly-acrylamide gel electrophoresis; BSA, bovine serum albumin; DAB, diaminobenzidine; bp, base pair; SV 40, Simian Virus 40; IV, Intravenous; IT, Intratracheal; HPLC, High Performance Liquid Chromatography; LAL, Limulus Amoebocyte Lysis Assay; TBE, Tris borate buffer; OCT, Embedding medium for frozen tissue specimens, Tissue Tek, Miles, Elkhart, IN.

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Expression of the hCFTR cDNA was monitored by the *in vitro* cell based chloride ion efflux assay in 293 cells (21,22).

Preparation and Validation of Lipid:DNA Formulation

Liposomes consisting of a 1:1 mole ratio of EDMPC and cholesterol were prepared by sonication (15) resulting in a 20 mM concentration of each lipid in 5% w/v dextrose, stored at 2–8°C until use. The liposomes were complexed with the DNA expression plasmid (in 10mM Tris pH 8.0) at the ratios and DNA concentrations described in Table I. A 15 ml aliquot of each formulation was used for dosing and samples retained for activity testing by intralobal instillation (11,15).

Particle size measurements were made by photon correlation spectroscopy (Nicomp model C370, Nicomp, Santa Barbara, CA).

Dosing of Animals

Four female rhesus monkeys (Macaca mulatta) were pretreated with Ivermectin approximately 6 weeks before the start of the experiment. The monkeys were colony-born at University of California, Davis (UCD) California Regional Primate Research Center (CRPRC). Health screening included full physical examination. Animals were individually housed during the study and lightly anesthetized before drug administration and before necropsy. Animals were handled according to CRPC protocols and in accord with the NIH guidelines for the care and use of research animals.

The MiniHEART™ low flow continuous nebulizer (Vortran Medical Technology Inc., Sacramento, CA) was used for aerosolization and delivery of the dosing formulations to the primates. This nebulizer was operated within a flow rate range of 1–2.5 L/min, (30–40 psig) which is comparable with the breathing requirement of the primates, generating a mass median aerosol droplet size of 2–2.5 μM.

The four animals selected were divided into two groups matched on the basis of age and weight and assigned to treatment with either a control complex or test complex (Table II). For dosing, 12 ml complex was added to the nebulizer, and aerosol was generated until approximately 2 ml remained. Animals were treated with complexes while sedated (Telazol, 8 mg/kg IM and ketamine, 1–2 mg/kg IM or IV) and breathing spontaneously through a standard veterinary anesthesia mask, with the nares plugged. Aerosol was provided at ambient pressure in a closed system using concurrent flow spirometry (23) with full instrumentation. After treatment, each nebulizer was weighed and the flow rate of dry gas through the nebulizer was measured. These parameters were used to calculate the nebulizer output during treatment (ventilation fraction), and in the determination of the amount of material inhaled by and retained by each animal. For aerosol particles of 2–2.5 μ mass median aerodynamic diameter, the dose of complex retained by each animal was subsequently calculated as 65% of the dose inhaled (Table II).

Table I. Test and Control Formulations

	Plasmid	Lipid:DNA* ratio	DNA concentration (mg/ml)
Control	pMB4119 (CAT)	1:3	0.625
Control	pMB10 (β-gal)	1:3	0.625
Test	pMB113 (hCFTR)	1:2	1.000
Test	pMB113 (hCFTR)	1:3	0.625

* Lipid:DNA ratio = μmole cationic lipid:mg DNA, Total lipid = EDMPC/Cholesterol 1:1 molar ratio.

Table II. Inhalation Data

	Control		Test	
Plasmid Animal number	p4119 One	pMB10 Two	pMB113 Three	pMB113 Four
Day Dosed	1	2	2	1
Lipid:DNA Ratio	1:3	1:3	1:2	1:3
mg DNA / ml complex	0.625	0.625	1.0	0.625
Wt at Dosing (kg)	6.6	5.0	6.2	5.6
Time of Dosing (min)	83	133	92	133
Ventilation Fraction	1.06	0.57	0.97	0.51
Inhaled Volume (L)	135.0	94.6	130.7	92.6
Inhaled Liquid (ml)	6.4	4.0	6.2	3.7
Lung Deposition * (ml)	4.2	2.6	4.0	2.4
Dosage ml/kg	0.63	0.52	0.65	0.43
Dosage mg DNA/kg	0.39	0.33	0.65	0.27
Dosage DNA (mg)	2.6	1.6	4.0	1.5

* Assumes a lung deposition = 65% of volume inhaled.

Necropsy and Tissue Collection

Necropsy was performed three days after dosing. Animals were sedated with xylazine and euthanized with an overdose of pentobarbitol sodium. Arterial blood gas sampling, serum chemistry and complete blood counts were obtained from sedated animals immediately prior to necropsy. Identical sets of tissues were harvested from each animal immediately after death. A collection template (Fig. 1) was developed for each lung lobe, providing a systematic sampling and localization pattern for collection of tissues for PCR analysis, histopathology and immunohistochemistry. Sections of the pharynx, trachea, esophagus, mediastinal lymph nodes, liver, spleen and gonads were also harvested and analyzed.

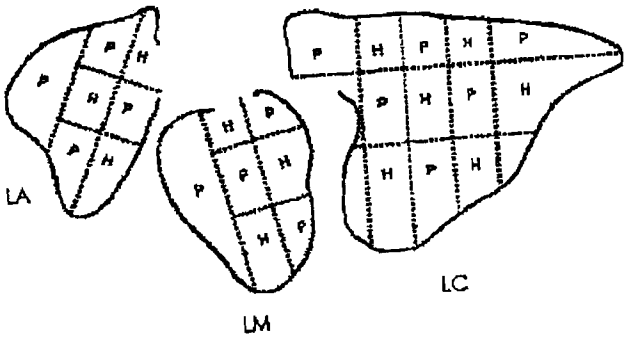


Fig. 1. Template illustrating the lung tissue collection. Pattern shown is for the left anterior (LA), middle (LM) and caudal (LC) lung lobes. Portions of the entire left and right lungs were collected for either histological/immunohistochemical (H) or PCR (P) analysis. The right lung lobes were divided into a similar pattern (not shown).

Tissues intended for PCR analysis were harvested and immediately frozen in liquid nitrogen. Samples for immunohistochemistry were injected with a 50:50 mixture of OCT and saline. Tissues were then individually placed in OCT filled cryomolds (Miles Diagnostics, Elkhart, IN) and frozen in liquid nitrogen chilled isopentane. Histology samples were fixed in 4% w/v paraformaldehyde, embedded in paraffin, sectioned and stained with hemotoxylin and eosin. Histologic sections of lung and other tissues were read by board certified pathologists who were blinded as to sample identities.

Detection of Transgene DNA Following Aerosol Delivery

Plasmid DNA was detected by PCR, where amplification was performed using oligonucleotide primers directed to the Tet(R) gene present in the CFTR plasmid. The primer pair 113FOR (5'GCGCCTATATCGCCGACATCAC3') and 113 REV3 (5'CACCTGTCCTACGAGTTGCATG3') results in a 315 bp band on gel electrophoresis. Following amplification, reaction products were analyzed by TBE agarose gel (1%) electrophoresis.

A quantitative PCR assay was developed to quantitate the amount of DNA delivered. An internal control competitor vector pΔMB10 was constructed. This vector is identical to the pMB10 plasmid except for a deletion between the two PCR primers. These two vectors compete equally for primer annealing and amplification and quantitation is made by end-point dilution on the gel. pMB10 generates a band of 800 bp and pΔMB10 generates a band of 428 bp.

Analysis of Expression of mRNA

Transgene RNA was monitored in one animal treated with the hCFTR transgene and in one animal treated with the CAT transgene. The mRNA was isolated from tissues utilizing guanidium isothiocyanate lysis/denaturation with poly(A)+ selection by oligo dT annealing (Stratagene, San Diego, CA #200347) (24). After isolation, the mRNA was converted to cDNA utilizing the MMLV reverse transcriptase (RT) with either a 3′ reverse primer or a random hexamer.

Samples analyzed for hCFTR transgene mRNA by RT-PCR used specific primers which span the 5′ intron region of pMB113 thus distinguishing between a DNA signal and mRNA. The 3′ reverse primer used for the CFTR transgene was REV5END, 5′ GCT CCT AAT GCC AAA GGA AT 3′, at 1254–1273 bp. A mixture of a ^{32}P labeled forward primer, ^{32}P NECFTRFOR 5′ AGA TCG CCT GGA GAC GCC AT 3′, 772–791 bp in plasmid hCFTR, and the unlabelled primer REV5END was used for subsequent PCR amplification of the cDNA.

For analysis of CAT transgene RNA, a nonradioactive RT-PCR was performed. Using the RT step as described above, a random hexamer was used to make cDNA. In the PCR step, an unlabeled forward primer (5′ CATCCACGCTGTTTT-GACCTC 3′) spanning from 782 bp to 802 bp was utilized with an unlabeled reverse primer (5′ GTCTTTCATTGCCA-TACGGAAT 3′) found 215–236 bp 3′ to the translation start site of the CAT gene. The RT-PCR product was visualized by electrophoresis on a 2% agarose TBE gel stained with ethidium bromide. Omission of the MMLV-RT completely eliminated the production of the 159 bp thus verifying that this band was due to the presence of vector specific CFTR RNA. Tissues from non-transfected primates were also tested with both sets of primers.

Preparation of MB-1 Antibody

Peptide C (residues 1468–1480 of hCFTR; KEETE-EEVQDTRL) was coupled to keyhole limpet hemocyanin (KLH; Calbiochem, La Jolla, CA) with glutaraldehyde (Sigma, St. Louis, MO). New Zealand white female rabbits (12 week old; Simonsen, Gilroy, CA) were immunized 100 mg peptide

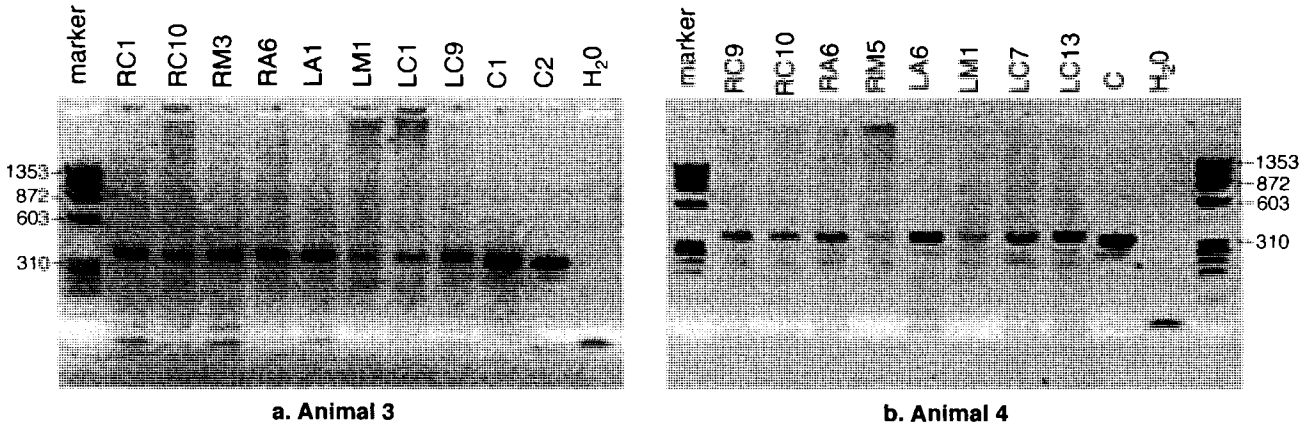


Fig. 2. DNA delivery analyzed by PCR. Aliquots from a variety of lung samples from the hCFTR treated animals 3 and 4 were assayed for the DNA by PCR analysis using the vector specific primers. Following the reaction, the PCR products were size separated by agarose gel electrophoresis using a 1% agarose TBE gel. The expected 315 bp band was present in all samples tested. (a) Samples from animal 3: M, phiX DNA markers; RC1, right caudal section 1; RC10, right caudal section 10; RM3, right medial section 3; RA6, right anterior section 6; LA1, left anterior section 1; LM1 left medial section 1; LC1, left caudal section 1; LC9, left caudal section 9, C1 positive, plasmid DNA; C2 positive control, PCR from 293 cells transfected with hCFTR (pMB113); H_20, water blank, negative control. (b) samples from animal 4: M, phiX DNA markers; RC9, right caudal section 9; RC10, right caudal section 10; RA6, right anterior section 6; RM5, right medial section 5; LA6, left anterior section 6; LM1 left medial section 1; LC7, left caudal section 7; LC13, left caudal section 13, C positive control, PCR from 293 cells transfected with hCFTR (pMB113); H_20, water blank, negative control; M, phiX DNA markers.

conjugate in 0.5 ml phosphate buffered saline (PBS) emulsified with 0.5 ml complete Freund's adjuvant (Sigma, St. Louis, MO). Subsequent injections were performed at 2-week intervals. MB-1 antibody was affinity-purified on a CNBr-Sepharose-peptide C column (Pharmacia). The antibody was characterized in several cell lines known to express the hCFTR gene (25) including HT29, T84 and NIH3T3 and followed by Western blotting.

Detection of Transgene hCFTR Expression in Primate Lungs Using MB1

Sections of lung tissue were analyzed for hCFTR protein expression using immunohistochemical staining with antibody MB-1. Sections of lung 5–7 μm thick were cut with a cryostat, collected on ProbeOn Plus glass slides (Fisher Scientific, Pittsburgh, PA), air-dried for two hours at room temperature and stored at −80°C until used. The samples were then rehydrated in PBS and incubated for 30 seconds in 0.5% periodic acid solution in water to quench endogenous peroxidase. The sections were washed in PBS, and blocked by normal goat serum 10% in PBS. After removing excess goat serum solution, sections were incubated for one hour with varying concentrations of primary antibody diluted in PBS containing normal goat serum (10%). After washing, sections were incubated for an hour in peroxidase conjugated goat-anti-rabbit IgG (Accurate Chemical, Westbury, NY) diluted in PBS/normal goat serum (10%). Slides were then washed once in PBS containing 0.05% w/v Tween-20 and three times in PBS without Tween-20. Color was developed with diaminobenzidine (DAB) substrate (Sigma, St. Louis, MO) and counter-stained with hematoxylin. For negative controls, sections were incubated with the same concentration of irrelevant rabbit IgGs (e.g. rabbit anti-CAT), or with the same concentration of primary antibody absorbed with excess CFTR antigen peptide. Cryosections from primates treated with control plasmids also served as a negative control.

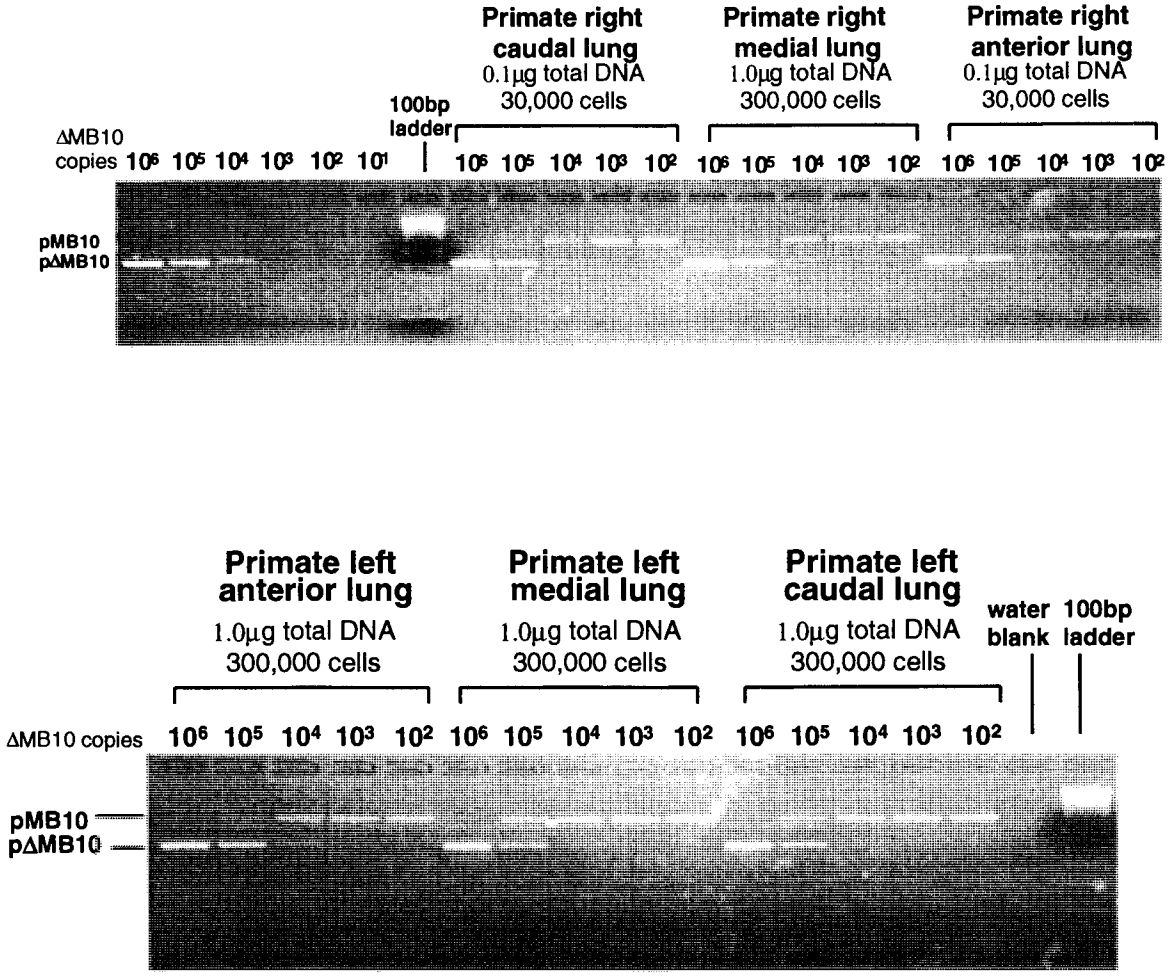


Fig. 3. Quantitative PCR analysis from Primate. To determine the lowest amount of total DNA necessary to detect the target plasmid β-galactosidase (pMB10) by PCR, end-point dilution analysis was performed on total DNA from lung tissue from animal 2. The top panel contains samples from the right lung sections and the bottom panel contains samples from the left lung. After determination of the amount of total DNA necessary for pMB10 detection, a series of samples were co-amplified with a dilution series of the plasmid containing a deletion i.e. pΔMB10. Following the PCR reaction, the PCR products were size separated by agarose gel electrophoresis. pMB10 generates a band of 800 bp and pΔMB10 generates a band of 428 bp. The first six lanes on the top panel contain the serial dilution standard curve for pMB10 from 10^6 to 10^1. The 100 bp ladder serves as a size marker. To detect the transfected DNA in each samples a serial dilution was done previously to determine the correct dilution needed for this quantitation step; the amount of total DNA needed to assay each sample is indicated.

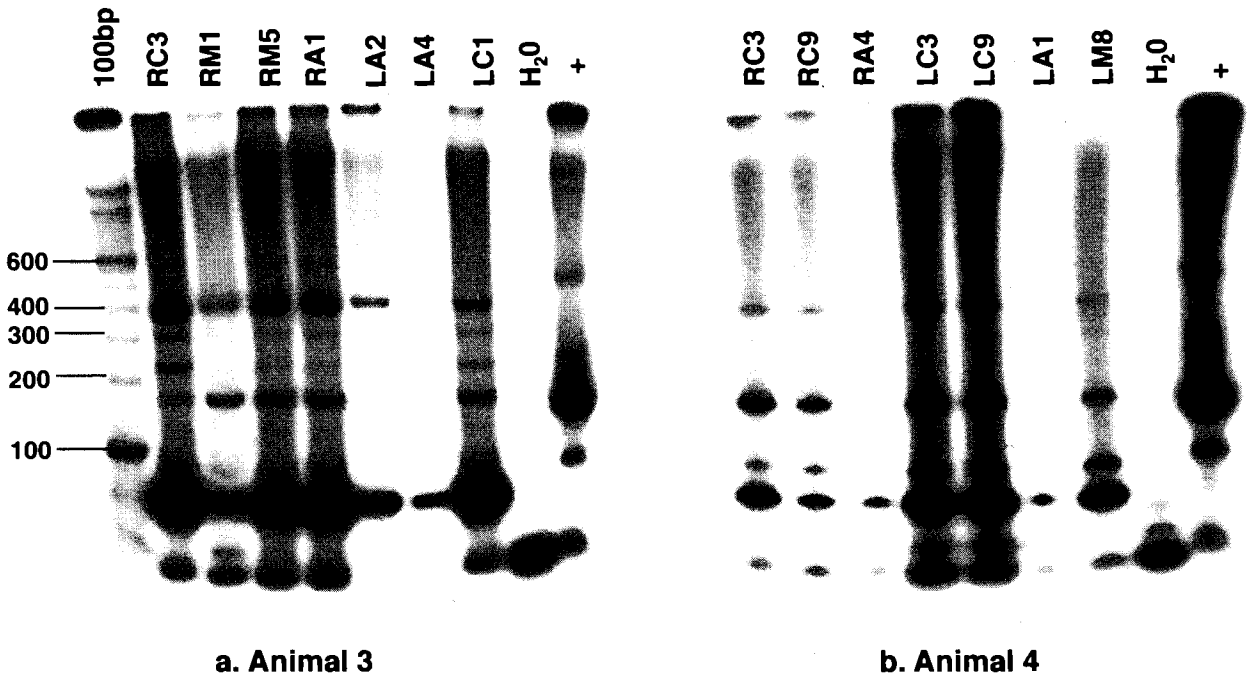


Fig. 4. Analysis of transgene specific mRNA sequences for the hCFTR treated animals. RT-PCR was performed on RNA samples isolated from primate lung samples from animal 3 and animal 4 using the 5′ vector primer set. These primers detect plasmid DNA yielding a 501 bp band and a 159 bp band for correctly spliced CFTR RNA from pMB113. The ^{32}P labeled RT-PCR products were size separated on a TBE 6% acrylamide gel. The gels were dried and exposed to X-ray film for 15 min (a) Samples from animal 3: M, 100 bp marker; RC3, right caudal section 3; RM1, right medial section 1; RM5, right medial section 5; RA1, right anterior section 1; LA2, left anterior section 2; LA4 left anterior section 4; LC1, left caudal section 1; H_20, water blank, negative control; positive control, RT-PCR from 293 cells transfected with pMB113; (b) samples from animal 4: RC3, right caudal section 3; RC9, right caudal section 9; RA4, right anterior section 4; LC3, left caudal section 3; LC9, left caudal section 9; LA1, left anterior section 1; LM13, left medial section 13; H_20, water blank, negative control; C positive control, PCR from 293 cells transfected with pMB113.

RESULTS

Analysis of Lipid:DNA Complexes

Naked DNA or EDMPC:DNA complex were aerosolized at 40 psig for either 25 or 60 minutes depending on the initial volume of DNA in the nebulizer. Agarose gels showed that the naked DNA sample had significant smearing throughout the gel as compared to much less smearing when lipid:DNA complexes were examined. The size of the lipid:DNA complexes was apparently unaffected, as measured by photon correlation spectroscopy, and the complexes produced a positive chloride efflux after transfection of 293 cells in tissue culture (data not shown). Measurement of biological activity of collected samples was performed by intralobal instillation (15).

Evaluation of Animals

No treatment related effects were evident during the aerosolization procedure and all animals were clinically normal following aerosol treatment and prior to necropsy. Arterial blood gas values were normal with the exception of mild hypercarbia noted in one animal. Blood chemistries and complete blood counts on blood sampled immediately prior to necropsy were within normal limits. The nasal mucosa, pharynx, trachea and lungs from each animal were normal and were free of histological lesions. Sections of lung taken from each quadrant of the collection grid (Fig. 1) (minimally 23 sites per animal) were histologically normal in all animals with no evidence of inflammation. Sections from the anterior, middle and caudal lobes of the lungs were sampled for DNA vector specific RNA or for protein using the MB-1 anti-CFTR antibody.

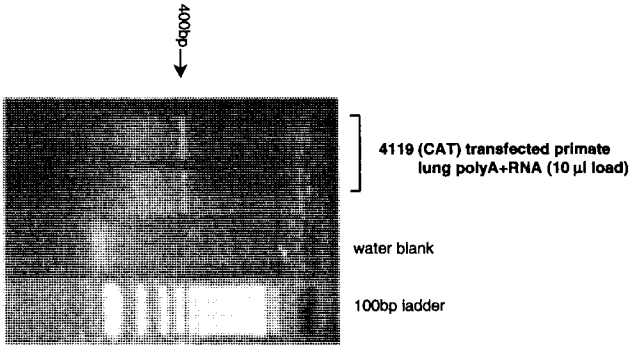
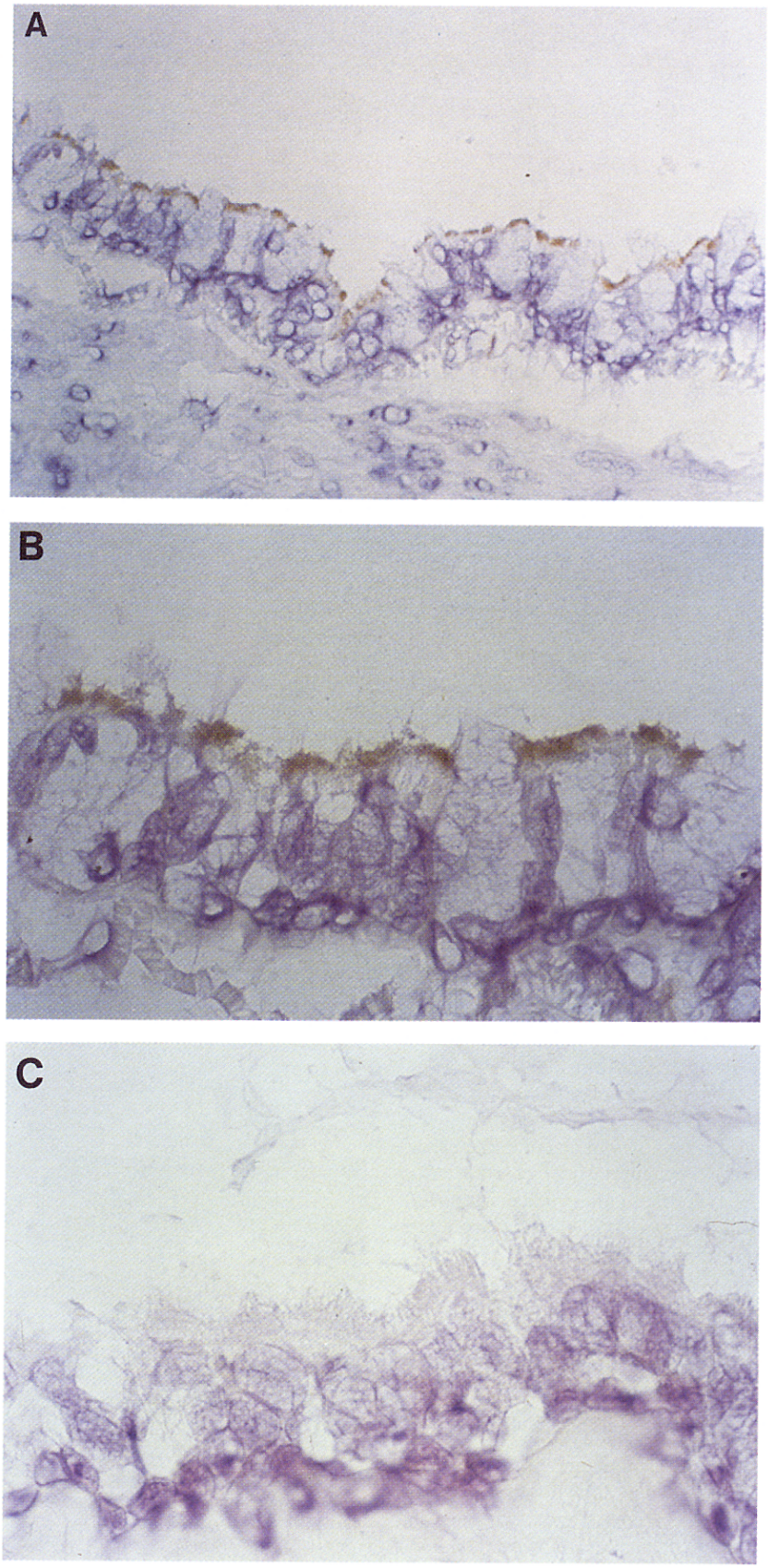


Fig. 5. Analysis of transgene specific mRNA sequences for the CAT treated animal. RT-PCR was performed on RNA samples isolated from primate lung samples from animal 1, transfected with the CAT reporter gene vector (P4119), using a nonradioactive RT-PCR method. The RT step was performed as described, using random hexamer to make cDNA. The RT-PCR product was visualized by electrophoresis on a 2% agarose TBE gel stained with ethidium bromide. An RNA specific band of 400 bp is visible in samples analyzed for the present of CAT RNA following DNA:lipid transfection.


A
B
C

Delivery of Plasmid DNA

Aliquots were taken from the series of lung tissue samples collected at necropsy from the animals treated with the pMB113 formulations (animals Three and Four) as shown in Fig. 1. Each of eight samples from animal Three and eight samples from animal Four tested positive for hCFTR DNA (Fig. 2). The PCR products from the eight samples from animal Three were fairly consistent in intensity but the signals from each sample from animal Four were quite variable—suggestive of some degree of non-uniformity in delivery. Since the primers are transgene specific, they do not cross react with endogenous CFTR.

DNA delivery to the lungs of animal Two, treated with the pMB10 formulation, was studied by quantitative PCR. Samples from the right and left-anterior, medial, and caudal lung regions were analyzed (Fig. 3). At that dilution point in which the internal standard competes equally with the transfected gene, copy numbers should be equal. Given the size of the primate genome (3.11×10^9 bp), the number of equivalent genome copies present for the amount of total DNA used in the assay was calculated. By using this method of analysis, it was determined that the β-gal plasmid was present in various regions of the lung tissue at a level of 10^4 copies in 30,000 lung cells.

Analysis Expression of the Transgenes Following Aerosol Delivery

RNA Analysis

Transgene specific RT-PCR was used to detect the expression of hCFTR and CAT mRNA, using β- actin as an internal standard.

To assay for the presence of CFTR transgene RNA, primers which span the intron were used. The vector-specific primers were designed to distinguish between a vector generated DNA PCR product (501 bp) and a mRNA generated product (159 bp) using primers which span the 5′ intron present in the pMB113 vector (Fig. 4). In animal Three, samples LA2 and LA4 showed no specific transgene RNA. Samples RA4 and LA1, from animal Four, were weakly positive for the presence of the 159 bp transgene mRNA band. Radiolabeled primers migrate at the gel front and the intense band that migrated at below 100bp is most likely dimerized primers. In all, 24 samples were positive for transgene specific mRNA: 13 from animal Four and 11 from animal Three. In addition to these samples, three samples which had intact actin RNA were negative for vector specific RNA and six samples showed degraded RNA.

Analysis of primate RNA from animal One transfected with the CAT reporter gene vector (P4119), was performed. The forward primer spans from 782 bp to 802 bp was utilized with an unlabeled reverse primer found 215–236 bp 3′ to the translation start site of the CAT gene. A single band of the expected 400 bp RT-PCR product was obtained (Fig. 5). Since these primers also span the intron present in these vectors, this band is specific for the presence of transgene RNA. Non-transfected primate tissues were negative when screened with these primers, indicating specificity for the transgene mRNA.

Protein Analysis

Characterization of MB1 antibody by immunoblot analysis. The MB-1 antibody detected a single protein band of 160–190 kD in immunoblots of HT29 and T84 cell extracts. A similar protein band was recognized in wild type cell preparations, and a band of 140–150 kD was found in ΔF508 cell extracts. There was no detectable reaction in mock cell extracts.

Detection of hCFTR Transgene Expression in Primates Following Aerosol Delivery of Lipid:DNA Complexes by Immunhistochemistry

CFTR protein antigen was detected in the same tissue samples evaluated by PCR analysis using standard immunhistochemistry techniques with a CFTR specific antibody (MB-1). Since MB-1 reacts with both human and non-human primate CFTR protein, samples from the control and hCFTR treated animals were stained and compared side by side to differentiate hCFTR from endogenous primate CFTR. The controls ensured that the CFTR signal observed was not due to the lipid:DNA complex causing an up regulation of endogenous CFTR protein. The primary antibody was employed at dilutions (of up to 1:1000) where no endogenous CFTR was observed in the control animals.

From each primate, a minimum of 10 sections were prepared from each of four tissue blocks taken from the anterior, middle and caudal lobes of the left and right lungs. Tissue sections were incubated with MB-1 as the primary antibody as described above. At a dilution of 1:1000 for the primary antibody, CFTR protein was detected on the apical surface of airway epithelia only in the two pMB113 treated animals (Fig 6). The lack of CFTR staining seen in the control animals is consistent with expression of hCFTR arising from the delivered DNA, rather than upregulation of endogenous CFTR expression occurring as a treatment artifact.

In animals Three and Four, the presence of CFTR on epithelial cells varied from diffuse to patchy in all regions of the trachea and lung above the level of terminal bronchioles. In more diffusely involved segments it was estimated that up to 50% of the airway surface area was stained. It was also common to find unstained and stained airways within the same section. Staining was judged more intense in animal Three, which received the higher amount of DNA delivered to the lungs (estimated to be 4.0 mg) as compared to animal Four, which was estimated to have received 1.5 mg (Table II).

In addition to negative-control primate tissues, other immunohistochemical controls included substitution of an irrel-

Fig. 6. (*opposite*) Immunohistochemical analysis of rhesus monkey lung sections. Immunohistochemical staining patterns of epithelium lining the bronchi of primates treated with either CFTR-DNA (A, 250X; B,2500X) or CAT-DNA (C, 2500X) complexes and probed with a primary anti-CFTR antibody concentration of 1:1000 and counterstained with hematoxylin. The brown-stained, CFTR positive reaction product is confined to the apical surface of the luminal epithelium (A,B) with the exception of a few mucus-laden goblet cells which do not stain. Sections of airway from primates treated with CAT-DNA control gene (C) lack the positive reaction product. Similar negative results were seen when the controls consisted of an irrelevant primary antibody, a CFTR protein absorbed antibody or no primary antibody applied to airways of CFTR-DNA treated primates. Figure 6A reduced to 81% for reproduction; 6B and C reduced to 95%.

evant primary antibody (anti-CAT) in place of MB-1, exposure to the secondary antibody alone and exposure to MB-1 absorbed with a blocking peptide (the CFTR peptide to which MB-1 was raised in rabbits). In all of these cases, no detection of CFTR protein antigen was observed in the treated or control samples.

A weak CFTR signal could be detected in the CAT or β-gal treated animals when higher concentrations (1:200 dilution) of the primary antibody were employed, possibly indicating presence of endogenous (cross-reactive) CFTR protein and/or non-specific background staining.

DISCUSSION

The present study shows that a transgene, hCFTR cDNA, can be successfully delivered to primate airways and results in expression of the transgene protein on the apical surface of airway epithelium, three days post dosing. All samples taken from various regions of the primate lung were positive for the presence of the transgene DNA indicating widespread exposure to the DNA delivered by aerosol. Quantitative PCR data indicate that the transgene was present at a level of 10^4 copies per 30,000 total lung cells. Since this analysis is averaged over the cell population, we have no way of determining the frequency of uptake into cells. Expression of the hCFTR protein in the lung, as assayed by the MB1 antibody, was uneven and involved areas of locally diffuse and patchy staining. Given the variables of aerosol delivery, mucociliary transport and airway epithelial cell type and dynamics it is unlikely that expression of the transgene would be uniformly distributed. The location of the protein in the lung was limited to the apical surface of pulmonary epithelial cells, which is the desired site of localization of hCFTR. These findings, nevertheless, do suggest that the formulations of EDMPC cholesterol used in this study may be able to transfect at a minimum, the 5–10% airway cell transfection thought necessary for the treatment of cystic fibrosis (1,9,26). *In vitro* studies of the gene product demonstrated activity in a chloride efflux assay.

As well as having the capability to produce the required gene-product *in vivo,* any effective gene therapy must be non-toxic. This has been a limitation for adenoviral therapies used to date. Similarly, a number of cationic lipid based gene delivery systems, while showing protection of the DNA upon nebulization (14) and adequate expression, resulted in inflammation (10). The present study indicated that no visible signs of inflammation or toxicity were observed using the EDMPC:DNA complexes in primates, three days post dosing. Nevertheless, further longer term studies in animals and subsequently in humans will be required to demonstrate the ability to successfully apply multiple dosing regimes using lipid vectors.

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