



Comparison between intratracheal and intravenous administration of liposome–DNA complexes for cystic fibrosis lung gene therapy

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Intratracheal (i.t.) and intravenous (i.v.) delivery of DNA–vector formulations are two strategies to obtain gene transfer to the lung. It is still uncertain, however, which of these two modes of delivery will be more effective in the treatment of cystic fibrosis and other lung diseases. In this study, we attempted to optimize formulations of the cationic liposome DODAC:DOPE (dioleoyldimethylammonium-chloride:dioleoylphosphatidylethanolamine) complexed to plasmids encoding chloramphenicol acetyltransferase for i.t. and i.v. injection into CD-1 mice and compared the two methods. Our results showed that both methods conferred reporter gene expression in the lung that was significantly higher relative to injection of plasmid DNA alone.

Expression using either mode of administration was maximal 24 h after injection and declined to around 10% of day 1 levels 2 weeks after injection. For i.v. delivery of DODAC:DOPE–DNA complexes multilamellar vesicles were more effective than large unilamellar vesicles in all organs investigated. Recombinant DNA could be detected in the distal lung region following either route of administration. However, i.t. administration predominantly led to DNA deposition in epithelial cells lining the bronchioles, eg in clara cells, whereas i.v. administration resulted in DNA deposition in the alveolar region of the lung including type II alveolar epithelial cells.

Keywords: lung gene therapy; cystic fibrosis; cationic liposomes

Introduction

Somatic gene therapy offers a potential therapeutic strategy for various inherited and acquired human diseases. Cystic fibrosis (CF), the most frequent lethal autosomal recessive disease in the Caucasian population, has been a major focus in this area of research. Mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) lead to dysfunction or absence of a cAMP regulated chloride channel in the apical membrane of epithelial cells.^{1,2} Although various organs of the body are affected, respiratory failure is the cause of death in more than 95% of CF patients. Chronic inflammation, mucus accumulation and persistent bacterial infections cause a progressive decline in lung function.³

Viral and nonviral vector systems, including cationic liposomes, have been widely used to transfer functional genes into the lungs of laboratory animals.^{1,4–11} In most studies the lung has been directly targeted by administering the vector via aerosol or intratracheal and intra-

nasal injections. Direct intrapulmonary administration can be relatively noninvasive; lung epithelial cells that face the airway lumen are readily accessible and potential problems with ectopic expression and toxicity in organs other than the lung are minimized. In CF patients with established lung disease, however, the airway epithelium might be inaccessible for vector–DNA complexes because of thick mucus accumulation, and the chronic inflammation and bacterial infection may impair transgene expression. On the other hand, it has been speculated that inflammation-induced lung injury would enhance gene transfer in CF patients.¹²

Systemic intravenous infusions of liposome–DNA complexes have previously been shown to transfect lung tissue.^{13,14} This mode of administration avoids the mucus barrier in the CF lung, but will also transfect other organs, leading to loss of DNA–liposome material and possibly toxic side-effects. A side by side comparison using the same liposomal vector, will be necessary to determine which route of administration proves to be more effective for gene therapy of CF and other lung diseases.

In this report, we directly compared the expression patterns after intravenous (i.v.) and intratracheal (i.t.) administration of a chloramphenicol acetyltransferase (CAT) reporter gene complexed to the cationic liposome

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DODAC:DOPE (dioleoyldimethylammoniumchloride: dioleoylphosphatidylethanolamine) into adult CD-1 mice. Large unilamellar vesicles (LUV) and multilamellar vesicles (MLV) were used to determine the level and persistence of CAT expression after i.v. and i.t. injection *in vivo*. The distribution of recombinant DNA was subsequently determined in lung and several other organs following either route of administration.

Results

Optimization and persistence of CAT expression in mouse lung

The lipid:DNA ratio could influence transfection efficiencies drastically. To determine the optimal ratio, varying amounts (4.2–30 nmol) of LUV per microgram DNA were injected intravenously into mice and CAT activity was measured in lung tissue 24 h after injection. As shown in Figure 1, CAT activity was maximal at a ratio of 15–18 nmol lipid per microgram of DNA. The same ratio was found to be optimal for intratracheal injections (data not shown). This ratio was used for all subsequent experiments.

Effective gene therapy treatment of CF would presumably require long lasting expression of the administered cDNA. To determine CAT reporter gene expression in the mouse lung after the i.v. or i.t. administration of liposome–DNA complexes, a time-course experiment was conducted for 14 days. As shown in Figure 2a, CAT expression was maximal 1 day following either route of administration. By day 7, CAT expression declined to approximately 16% of day 1 levels in i.v. injected mice and further declined to around 10% by day 14. Following i.t. administration CAT expression also declined with time (Figure 2b), but expression was still detectable by day 7 at a level of 30–40% of that of day 1. Since reporter gene expression is transient following either route of administration repeated treatments of CF patients with DODAC:DOPE–DNA complexes will most likely be necessary.

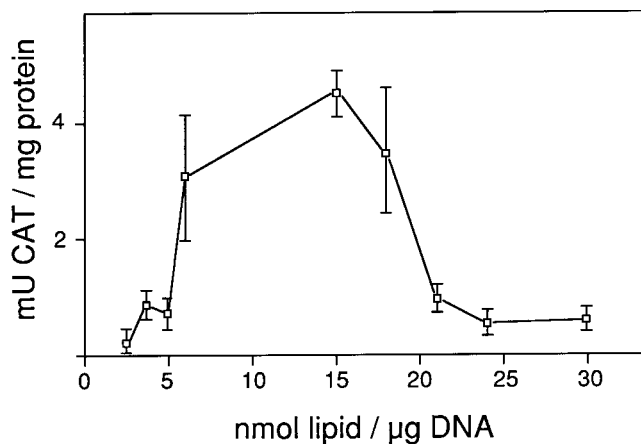


Figure 1 Optimization of lipid:DNA ratios. CD-1 mice were injected i.v. with large unilamellar DODAC:DOPE vesicles complexed to DNA and killed 24 h later. Approximately 2.4–30 nmol lipid per µg DNA were used for each mouse. Lung homogenates were assayed for CAT activity. The mean value derived from five animals is shown for each time-point with ± s.e.m.

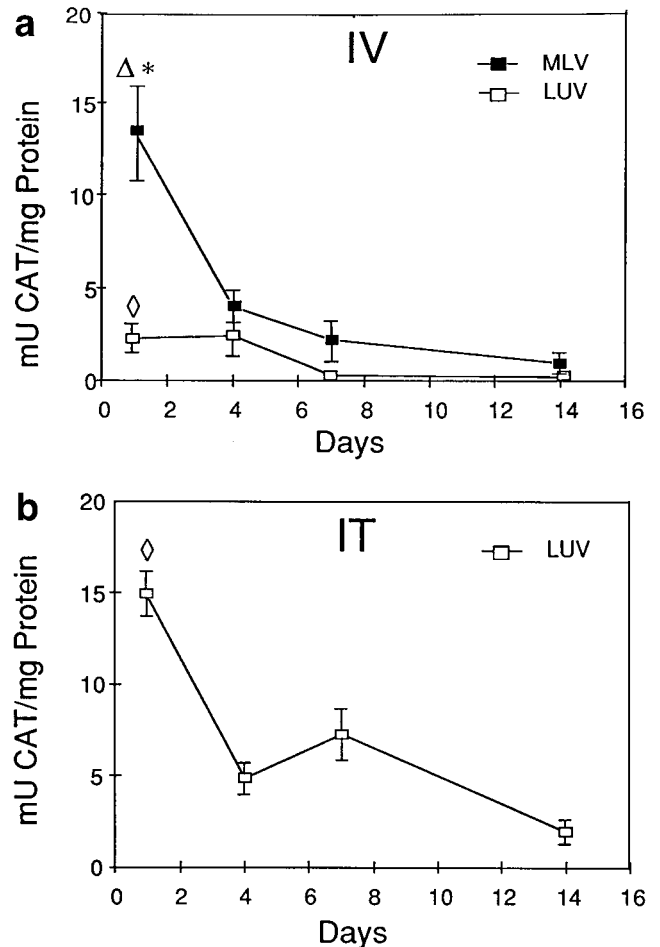


Figure 2 CAT expression in mouse lungs. (a) CD-1 mice were injected i.v. with multilamellar or large unilamellar DODAC:DOPE vesicles complexed to DNA and killed 1–14 days later. Lungs were assayed for CAT activity. (b) Following i.t. administration with LUV–DNA complexes CAT activity was measured in lungs. Each data point represents the mean ± s.e.m. ($n = 8$). Δ indicates significant difference between expression obtained following LUV and MLV injection. * Indicates significant difference between expression at day 1 and day 7 after MLV injection. \diamond Indicates significant difference between day 1 and day 7 after LUV injection.

Comparison of LUV and MLV delivery

Size and structure of cationic liposomes has been shown to influence expression levels *in vitro*. To determine if these parameters also influence *in vivo* expression DODAC:DOPE MLV or LUV were used to form the complexes. As shown in Figure 2a i.v. injection of MLV resulted in higher reporter gene expression than injection of LUV with up to seven-fold increase in CAT activity in the lung 24 h after administration. Significant activity was also detected in spleen and liver tissues following i.v. injection of either type of vesicle (data not shown). The i.t. administration of MLV–DNA complexes resulted in mortality rates of more than 60% of the injected mice, but the surviving mice ($n = 4$) did not show enhanced expression with MLV (data not shown). Size and structure of liposomes did influence expression levels *in vivo*, although the optimal lipid formulation was dependent on the mode of administration. In subsequent experiments MLV were used for i.v. injections and LUV were used for i.t. injections unless otherwise indicated.

As controls, free plasmid DNA or liposome complexed DNA were also injected either i.v. or i.t. The amount of free DNA and the volume injected were similar to the amounts injected into the liposome–DNA receiving mice (i.v.: 50 μ g DNA in 400 μ l 5% sucrose; i.t.: 13 μ g DNA in 100 μ l 5% sucrose). In addition, a control group of mice was injected i.v. with 900 nmol DODAC:DOPE in sucrose (5%) only. The mice were killed 24 h after injection. Figure 3 shows that DNA complexed to DODAC:DOPE liposomes yielded a marked increase in CAT expression compared with DNA alone. A 40-fold and a 10-fold increase in CAT expression was measured following i.t. and i.v. injection, respectively. Injection of liposomes without DNA did not lead to measurable CAT activity.

Regional distribution of CAT activity in the lung

To prevent the progressive lung damage in CF patients the ion transport defect will ideally have to be corrected in the entire lung. The regional accessibility of DNA–liposome complexes in the mouse lung was examined for both administrations. The intrapulmonary distribution of CAT activity was determined at the gross anatomical level by removing individual lobes and trachea 24 h after i.v. or i.t. administration of the complexes. The lobes of the lung and the trachea were then assayed individually for CAT expression. As shown in Figure 4, both i.v. and i.t. administration led to a uniform distribution of CAT activity per milligram of tissue protein in the individual lobes of the lung. CAT activity in the trachea, however, was consistently lower than lobar values.

In situ localization of recombinant ³⁵S-labelled DNA following i.t. and i.v. administration

Autoradiographic and immunohistochemical analyses were next carried out to determine the distribution of uptake of the recombinant DNA 24 h after mice had received an i.t. or i.v. injection of DODAC:DOPE–DNA complexes. Figure 5 shows labelled DNA in the distal

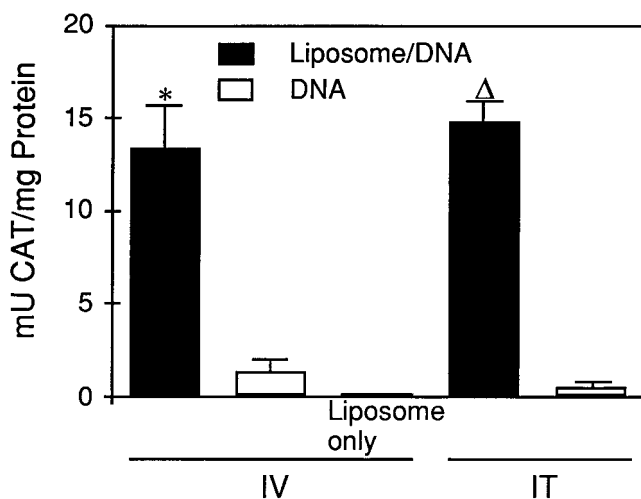


Figure 3 Comparison of transfection with free DNA or DNA complexed to DODAC:DOPE. Mice were injected i.v. with MLV or i.t. with LUV complexed to DNA, or with equivalent amounts of free DNA or i.v. with equivalent amounts of MLV liposomes only. Twenty-four hours after injection mice were killed, lungs were harvested and CAT activity was assayed. Each data point represents the mean \pm s.e.m. (n = 6–8). *Indicates significant difference following i.v. injection. Δ Indicates significant difference following i.t. injection.

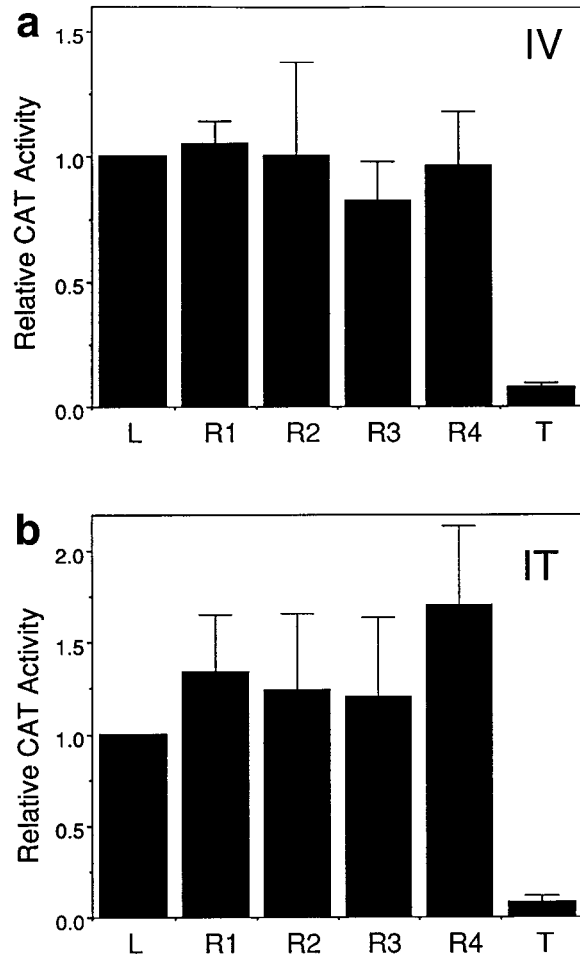


Figure 4 Regional distribution of CAT activity in the lung. Mice were killed 1 day after injection and the individual lobes of the lung and the trachea were harvested and assayed for CAT activity. CAT activities were normalized for expression in the left lobe of the mouse lung (L = 1 relative CAT activity). Expression in all lobes of the right lung (R1–R4) was similar to the left lung following either i.v. (a) or i.t. (b) injection. CAT expression in the trachea (T) was much lower even though results were recorded as mU/mg protein. Each data point represents the mean \pm s.e.m. (n = 4).

region of the lung following i.t. injection of DODAC:DOPE–DNA complexes. The majority of the injected DNA that reached the distal airways, was localised in bronchioles and terminal bronchioles. Only a comparatively small amount was detectable in respiratory bronchioles and alveoli. The distribution of DNA was similar in all five lobes of the mouse lung.

Immunohistochemical analysis using antibodies against surfactant protein A (anti-SP-A), was used as a marker for bronchiolar clara cells and type II alveolar cells in lung tissue sections.^{15,16} Figure 5c shows that terminal bronchioles are lined by clara cells and in an i.t. injected animal these cells also contained labelled DNA.

Figure 6a and b show that i.v. injection of the DODAC:DOPE–DNA complexes resulted in DNA uptake within the alveolar region of the distal lung. Type II cells were identified through immunohistochemical staining using anti-SP-A antibodies. Figure 6c shows that DNA was taken up into type II cells after i.v. injection of DODAC:DOPE–DNA complexes. Type II cells are usu-

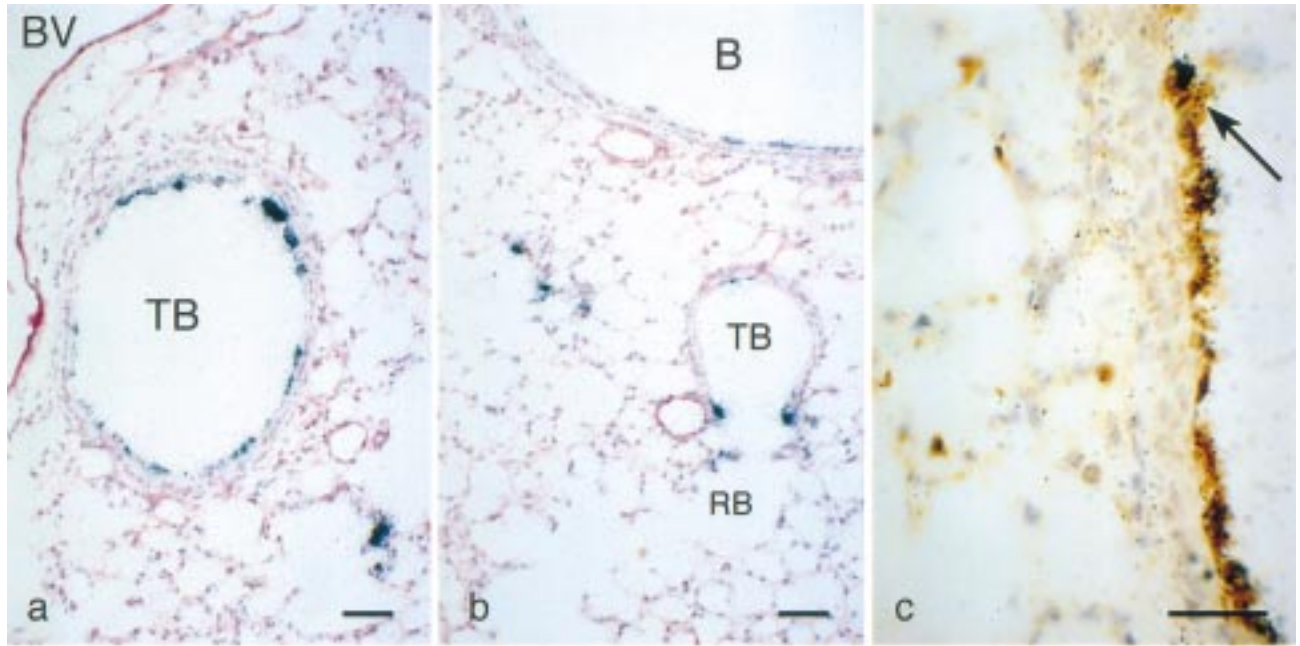


Figure 5 Detection of ^{35}S -labelled DNA through autoradiography in lung on day 1 following i.t. administration of liposome–DNA complexes. (a) Terminal bronchiole and part of a respiratory bronchiole showing the presence of injected DNA (black dots). (b) Bronchiole, terminal bronchiole and respiratory bronchiole contain injected DNA. (c) Immunostaining using anti-SP-A antibodies (surfactant protein A) and the horseradish peroxidase ABC kit were performed (brown colour) in addition to autoradiography. The cytoplasm of clara cells in bronchioles contains SP-A and injected DNA. Original magnification $\times 1000$. B, bronchiole; TB, terminal bronchiole; RB, respiratory bronchiole; BV, blood vessel (scale bar = $20\ \mu\text{m}$).

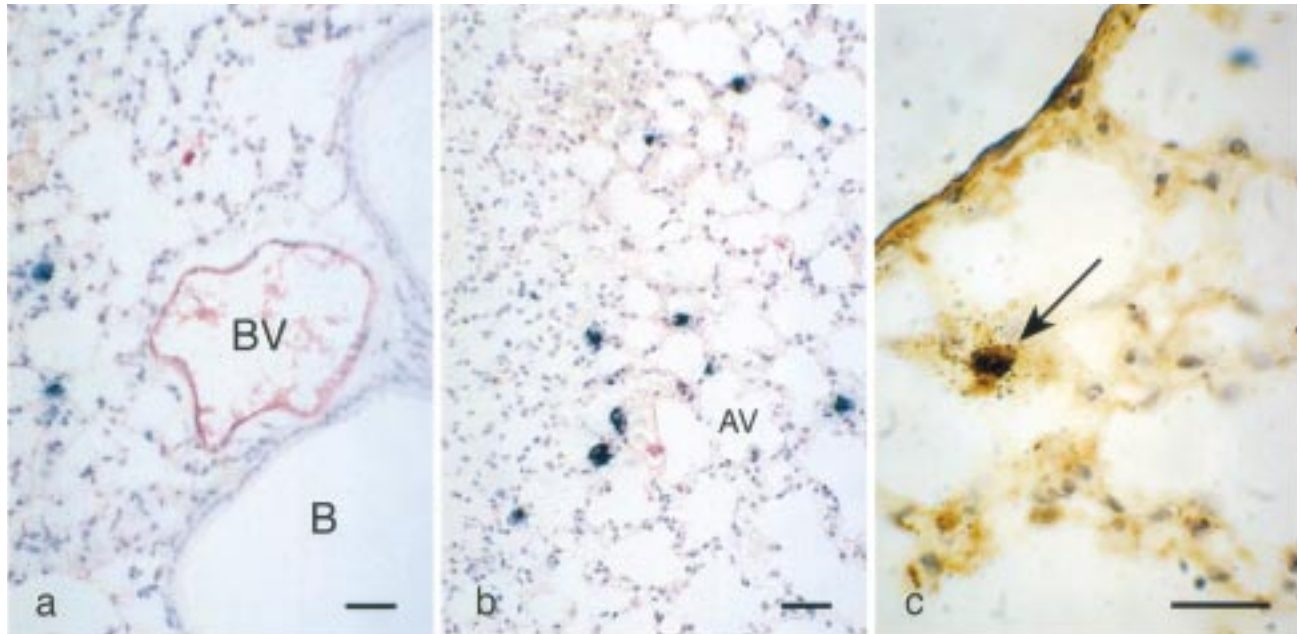


Figure 6 Detection of ^{35}S -labelled DNA through autoradiography in lung following i.v. injection of liposome–DNA complexes. Immunostaining (brown colour) with anti-factor VIII antibodies and the alkaline phosphatase ABC kit was performed in addition to autoradiography in (a) and (b) to identify endothelial cells that line the blood vessels. Anti-SPA antibodies (surfactant protein A) and the horseradish peroxidase ABC kit were used for (c). (a) Injected DNA (black dots) is visible in alveolar region of the distal lung, but not in terminal bronchioles. (b) Alveoli in distal lung containing injected DNA. (c) Type II alveolar epithelial cells which produce SP-A contain injected DNA. BV, blood vessel; AV, alveoli. The arrow marks a type II alveolar epithelial cell (scale bar = $20\ \mu\text{m}$).

ally found at points where the alveolar walls unite and form angles, which are the part of the alveoli where most of the injected DNA appears to be located. Anti-human factor VIII antibodies were used to identify endothelial cells that line the blood vessels. Figure 6a shows that

there was no ^{35}S -labelled DNA detectable in larger blood vessels. However, in this study, it was not possible to differentiate between epithelial and endothelial uptake of injected DNA in the alveolar region of the lung.

Large quantities of intravenously injected DODAC:

DOPE–DNA complexes were also deposited in liver and spleen tissue. In the liver, the injected DNA was localized in sinusoids between liver plates (data not shown). Liver parenchymal cells did not appear to take up the liposome–DNA complexes. In the spleen, injected DNA was found in the marginal zone between white and red pulp (data not shown). Macrophages (Kupffer cells) have previously been shown to be responsible for DNA uptake in the liver and in both the liver and spleen DNA was localized in areas known to be rich in macrophages.^{17,18}

Discussion

In this study, we compared an i.v. and an i.t. route of administration of the cationic liposome DODAC:DOPE complexed to DNA as a means for *in vivo* lung transfection. DODAC:DOPE was a suitable vector for the i.v. and i.t. delivery of plasmid expression cassettes as it was associated with a significant increase in CAT expression over the injection of naked DNA following either route of administration. Expression was comparable in all lobes of the lung. For i.v. injections MLV were more effective than LUV in all organs that were investigated. CAT activity in the lung was similar for i.v. and i.t. injections. Either route of administration has been shown to be able to transfect cells in the distal region of the lung, but marked differences in the relative distribution of injected DNA were noted. Clara cells were shown to be major recipients for i.t. injected DNA. In addition, we provide evidence that type II alveolar epithelial cells are able to take up i.v. injected DNA.

Persistence of expression in our study was transient and comparable to other reports^{8,10,17,19,20} which utilize a variety of cationic lipid formulations. Possible explanations that may account for the rapid decline in expression observed here and in other studies include: (1) loss of plasmid DNA and/or RNA from the transfected cells; (2) down-regulation of the CMV promoter; or (3) loss of transfected cells due to necrosis.

Previous studies^{21,22} reported that MLV will improve gene delivery over LUV using various cationic liposome formulations *in vitro* and *in vivo*. Our study also showed a better performance of MLV *in vivo* following i.v. injection of DODAC:DOPE. Several explanations may account for the increase in transfection efficiency through MLV–DNA complexes following i.v. administration: (1) DNA might be better protected against degradation in MLV complexes; (2) uptake of MLV–DNA complexes through endocytosis might be increased; and (3) MLV–DNA complexes might become trapped in capillaries of lung, spleen and liver when injected intravenously due to their larger size. As speculated by Rahman *et al.*¹⁸ LUV may circulate faster than MLV and consequently the contact time between liposome and cell surface is reduced, resulting in decreased transfection efficiency. It is interesting to note that the liposome structure does not appear to influence expression following i.t. injections, because we did not detect a difference in LUV- and MLV-mediated transfection after i.t. administration.

Reports comparing the transfection efficiency of liposome–DNA complexes and free DNA are not consistent and might depend on the particular lipid composition used. Some studies^{9,23–27} report either no expression when free DNA is injected or expression levels similar to those achieved with liposome–DNA complexes. Balasubraman-

iam *et al.*²⁸ show that free DNA transfects respiratory tissue more efficiently than DC-cholesterol–DNA or DOTAP–DNA, which are cationic lipids in clinical use, indicating the poor transfection efficiency of those lipids. Other studies^{7,13,17,19,29} do not include free DNA controls. Recently, two reports^{10,11} describe a drastic improvement in transfection efficiency after intranasal administration of optimized liposome–DNA formulations. In this study, we describe a cationic lipid that leads to a significant increase (at least 10-fold over free DNA) in reporter gene expression in the lung after i.v. administration. In addition, the same liposome composition is shown to enhance reporter gene expression at least 40-fold over free DNA following direct i.t. administration.

Our study showed that either route of administration could achieve a uniform distribution of reporter gene expression in individual lobes of the mouse lung. Expression in the trachea, however, was consistently much lower. The trachea is supplied mainly by bronchial vessels and not through the pulmonary artery. It has, therefore, little exposure to i.v. injected liposome–DNA complexes. Effective mucociliary transport might reduce the contact time between liposome–DNA complexes and cells lining the trachea and therefore, reduce cellular uptake following i.t. injection. In addition to poor transfection efficiency, poor retrieval of protein lysates from cells lining the trachea might account for the reduced CAT activity. The only other study²³ describing distribution of expression in individual parts of the lung reported highest expression in the trachea and the left lobe of the lung after i.t. administration. However, Meyer *et al.*²³ used DNA that was not complexed to liposomes and delivered the DNA through an i.t. catheter that may have damaged the epithelium.

Our study confirmed that injection of ³⁵S-labelled DNA could be used effectively as a means to monitor DNA distribution after i.t. and i.v. delivery. It should be noted that the signal intensity reflects only 10% of the total injected DNA (the proportion of DNA that was radioactively labelled). Clara cells in the distal bronchioles were shown to internalize i.t. injected DNA. However, the technique does not discriminate between transcribed and degraded DNA. These cells express CFTR and contain CFTR-like chloride channels.^{30,31} A comparatively small amount of i.t. injected DNA was visible in the alveolar region and because of low signal intensity it was not possible to characterize further the cell types that contained injected DNA in this region.

In previous studies,^{7,14,32} recombinant DNA or protein were localized in the alveolar region of rabbits and mice following i.v. injection of liposome–DNA complexes. In these studies, however, cell types containing the recombinant DNA were not further characterized. We have shown here that type II alveolar epithelial cells internalize recombinant DNA, indicating that the alveolar wall is permeable for i.v. injected DODAC:DOPE–DNA complexes. However, the mechanism of liposome–DNA uptake into type II cells remains unclear and requires further investigation. Alveolar type II cells are progenitors for type I alveolar epithelial cells and potential target cells for the gene therapeutic treatment of various lung pathologies such as oxygen toxicity, in which increased antioxidant enzyme activities in transgenic animals or protein therapy have been shown to be protective or α 1-antitrypsin deficiency.^{33–36} Recently it has been suggested

that CFTR is expressed in alveolar type II cell precursors and in differentiated alveolar type II cells.³⁷ CFTR activity has also been demonstrated in cultured rat distal lung epithelium cells.³⁸ Alveolar type II cells might be suitable target cells for CF gene therapy. Functional studies will determine if expression of recombinant CFTR in these cells will improve the disease phenotype in CF patients.

In contrast to other studies⁷ we did not detect i.v. injected DNA in endothelial cells lining larger pulmonary blood vessels. DODAC:DOPE-DNA liposome complexes might not be suitable for the transfection of pulmonary endothelium. It is also possible that endothelial cells take up DNA which we were not able to detect because in our autoradiographic analysis only 10% of the injected DNA were ³⁵S-labelled. We can also not exclude, however, that endothelial cells in the alveolar septum are transfected, because these cells are thin and hard to distinguish from epithelial cells.

In order to determine if recombinant DNA is expressed, it will be necessary to colocalize recombinant DNA and protein. We have recently used immuno-gold labelled antibodies to localize reporter protein and a general concordance could be observed (data not shown). Because of generally low transfection efficiency, further quantitative analysis will be required. Barrier of gene transfer is thought to be primarily due to inefficient migration of DNA from the cytoplasm into the nucleus.³⁹

CFTR is expressed in a subpopulation of epithelial cells at every level of the distal lung, including in clara cells and alveolar type II cells. These cells in the distal lung may play a critical role in the pathogenesis of CF and are, in addition to cells in proximal airway structures, potential target cells for CF gene therapy as well as for lung diseases like hyperoxic lung damage or α 1-antitrypsin deficiency. We have shown here that i.t. and i.v. administration of DODAC:DOPE-DNA complexes leads to DNA uptake into clara cells and type II cells, respectively. Although mice were thought not to be a good model for CF lung disease, recent studies showed that CF knockout mice in a defined genetic background develop a pulmonary phenotype resembling the lung pathology in CF patients in certain aspects.⁴⁰ These mice will be helpful to achieve a better understanding of the pathophysiology of CF and provide the basis for functional assays that will ultimately determine which mode of administration will be more effective in correcting the CF phenotype in the lung.

Materials and methods

Recombinant plasmid and DNA preparation

A modified pCMV β -galactosidase plasmid (Clontech, Palo Alto, CA, USA) containing the 0.7 kb CAT cDNA instead of the 3.4 kb β -galactosidase cDNA, a 45 bp translational enhancer from the alfalfa virus and a 100 bp SV40 hybrid intron in the 5' untranslated region was used (Inex Pharmaceuticals Corporation, Vancouver, BC, Canada). Plasmid DNA was prepared using alkaline lysis and potassium acetate precipitation followed by CsCl-gradient ultracentrifugation and extensive dialysis against water.⁴¹ The endotoxin contamination in this preparation was 2.4 endotoxin units (EU) per μ g DNA (E-Toxate Kit; Sigma, St Louis, MO, USA).

Preparation of liposomes and liposome-DNA complexes
DODAC and DOPE were mixed in a 1:1 molar ratio in chloroform. DODAC was obtained from Inex Pharmaceuticals Corporation and DOPE from Avanti Polar Lipids, Alabaster, AL, USA. The solvent was removed under vacuum and the lipid mixture rehydrated in sterile distilled water. The resulting MLV had a size range from 400 to 700 nm diameter as determined by quasi elastic light scattering methods.⁴² LUV with a size range of 70–130 nm diameter were obtained by extruding MLV through a 100 nm polycarbonate filter.⁴³ Liposome-DNA complexes were prepared 15–30 min before injection. For i.v. injections 50 μ g of DNA in 200 μ l of 5% sucrose were mixed rapidly with 900 nmol of DODAC:DOPE liposomes in 200 μ l of 5% sucrose per mouse. For i.t. instillations, a quarter of the above mixture which contained 13 μ g DNA and 225 nmol liposomes was used. Optimal ratios of DNA and liposomes for *in vivo* transfections were determined in previous experiments (data not shown).

Administration of liposome-DNA complexes into mice
CD-1 mice, aged 6–8 weeks, received liposome-DNA complexes either by i.v. or i.t. routes. For i.v. injections the tail was warmed up slightly in 60°C water in order to expose the vein and complexes were injected using a 30-gauge syringe. For i.t. injections mice were anaesthetized (Avertin, 25 μ l per gram body weight) and the trachea was surgically exposed. A 30-gauge needle was then inserted directly into the trachea for i.t. instillation of the complexes. Following injection the mice were placed in an oxygen tent for about 30 min. For i.v. and i.t. administration 50 μ g DNA in 400 μ l and 13 μ l DNA in 100 μ l total volume were administered, respectively. Six to eight mice were injected for each experiment and all animal experiments were performed in accordance with the Guidelines of the Canadian Council on Animal Care.

Determination of CAT activity

The mice were killed at various times after administration of the complexes and several organs (lung, spleen, heart and 100–200 mg liver) were harvested and processed as previously described.⁹ The protein concentration of the organ extracts was determined (Bradford Protein Assay; Biorad, Hercules, CA, USA) and extract samples containing 50–200 μ g total protein were used for each CAT assay. Ten microliters acetyl-CoA (20 mM stock in homogenization buffer; Sigma), 0.15 μ Ci ¹⁴C-labelled chloramphenicol (ICN, Costa Mesa, CA, USA) and homogenization buffer to a final volume of 100 μ l were added to the protein lysates and the reactions were incubated for 8–12 h at 37°C. Acetylated and unacetylated chloramphenicol were then extracted and CAT activity was measured.²³ The amount of CAT activity in the samples was determined through comparison with CAT standards (Promega, Madison, WI, USA) that were processed in parallel with each experiment using 0.05–30 mUnits (mU) purified CAT per reaction. CAT units were then calculated as mU CAT per mg of total protein.

Immunohistochemical staining and autoradiography

For *in situ* detection the DNA was labelled with ³⁵S-dATP using the Nick Translation System (Promega). Ethanol precipitation was carried out twice to remove unincorporated nucleotides and labelled DNA was mixed 1:10

with unlabelled DNA before addition of DODAC:DOPE LUV liposomes. Twenty-four hours after i.v. or i.t. administration the mice were killed with Euthanol (MTC Pharmaceuticals, Toronto, Canada), and 2 ml of heparin (1 U/ml in PBS) was injected into the right ventricle of the heart. The mice were then perfused with freshly prepared cold 4% paraformaldehyde in PBS, pH 7.6 through the right ventricle, while the lungs were inflated to 10 cm H₂O transpulmonary pressure through an intratracheal catheter. The organs were removed and processed as described previously.⁴⁴ Sections, 5 µm thick, were cut and mounted on silane-coated slides.

Immunohistochemistry was carried out using the avidin-biotin complex (ABC) detection procedures.⁴⁵ Incubation with either rabbit polyclonal antibody against human surfactant protein A (SP-A) (1:100 dilution in blocking solution) or against human factor VIII (1:400 dilution in blocking solution) was carried out overnight at 4°C (anti-factor VIII; Dakopatts, Glostrup, Denmark and anti-SP-A; Dr M Post, Toronto, ON, Canada). After washing in PBS, sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (Calbiochem, La Jolla, CA, USA) for 1–2 h at room temperature. Following a thorough rinsing in PBS horse-radish peroxidase Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was used for SPA localization. The alkaline phosphatase Vectastain ABC kit (Vector Laboratories) was used for factor VIII detection. Horseradish peroxidase reactive sites were stained with diaminobenzidine (DAB). Immuno-labelled alkaline phosphatase reactive sites reacted with Vector Red (Vector Laboratories). Sections were then washed in PBS, dehydrated through an ethanol gradient and air dried before dipping into photographic emulsion (NBT2; Kodak, New Haven, CT, USA). The slides were exposed for 1 or 5 days at 4°C and developed (Kodak). Slides were counterstained with Carazzi haematoxylin or haematoxylin/eosin, dehydrated, and mounted with Permount (Fisher, Nepean, ON, Canada).

Statistical analysis

Results are reported as means ± standard error of the mean (s.e.m.). The statistical significance of the data has been determined with a one-way ANOVA test followed by Student–Neuman–Keul test when multiple comparisons were made and Student's unpaired *t* test, as appropriate. Probability (*P*) < 0.05 was considered significant.

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