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Solvoplex: A New Type of Synthetic Vector for Intrapulmonary Gene Delivery

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ABSTRACT

A novel type of synthetic vector, termed solvoplex, is described that can greatly enhance gene expression in lung after intrapulmonary delivery. Solvoplexes consist of plasmid DNA and organic solvents. Several organic solvents were analyzed, and luciferase reporter gene expression was observed after intrapulmonary delivery of solvoplexes containing DPSO (di-*n*-propylsulfoxide), TMU (tetramethylurea), or BMSO (butylmethylsulfoxide). Expression levels correlated with the amount of solvent used at constant DNA amounts. Highest expression was obtained in the lung after intratracheal injection with 15% DPSO resulting in an increase up to 440-fold compared with DNA alone. DPSO-solvoplexes (15%) gave higher reporter gene expression than polyplexes (ExGen 500) or lipoplexes (DOTAP-cholesterol or DOTAP-DOPE). Solvoplex-mediated gene expression did not depend on the delivery mode, and was observed in both mice and rats. Readministration of DPSO-solvoplexes was possible. A second injection after 4 weeks resulted in expression levels similar to the first administration. Histological analyses using *lacZ* and *GFP* reporter genes demonstrated gene expression in the lung airway epithelium after intratracheal and microspray delivery. When luciferase expression levels in lung homogenates were compared with adenovirus vectors, DPSO-solvoplexes were 4- or 100-fold less efficient, depending on the promoter used in the viral vector. A quantitative histological comparison between solvoplexes and adenovirus vectors in the best expressing regions revealed that solvoplexes yielded about 2% LacZ-positive cells in the lung airway epithelium, and adenovirus vectors about 20%. Using the microsprayer system, we demonstrated that DNA remained intact in solvoplexes on spraying and that reporter gene expression was observed in mice after intrapulmonary delivery of a solvoplex spray. DNA in DPSO-solvoplexes remained stable and functional after prolonged storage at room temperature.

OVERVIEW SUMMARY

We report the development of solvoplex, a new type of synthetic vector, which achieves efficient gene transfer to the lung airway epithelium after intrapulmonary delivery. Solvoplexes may exhibit several advantages compared with other viral and synthetic vectors. They are easy to formulate, can be stored for at least 2 weeks, readministered efficiently, delivered as a spray, and exhibit higher or comparable expression levels in the lung with respect to other synthetic vectors. We conclude that solvoplexes represent an attractive alternative and complementary gene delivery vehicle to the currently available gene transfer vectors.

INTRODUCTION

FOR *in vivo* GENE TRANSFER, synthetic (nonviral) vectors are thought to have several advantages when compared with viral vectors: they can accommodate large-size DNA molecules (therapeutic genes including their endogenous regulatory regions), be used under reduced confinement conditions, be modified with appropriate ligands that allow specific cell targeting, and be administered repeatedly. Many different types of organic compounds have been tested as synthetic gene delivery vectors *in vivo*. The most commonly used vectors are based on cationic lipid-DNA complexes (lipoplexes) or cationic polymer-DNA complexes (polyplexes; reviewed by Abdallah *et al.*, 1995; Fel-

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gner *et al.*, 1995; Lee and Huang, 1997; Mahato *et al.*, 1997; Kabanov *et al.*, 1998; Rolland, 1998; Scherman *et al.*, 1998).

Intrapulmonary gene delivery represents an important route of administration for several applications. One of the most frequent monogenic life-threatening diseases, cystic fibrosis, originates from a defect in the CFTR (cystic fibrosis transmembrane conductance regulator) gene, which is expressed in the epithelia of various organs (reviewed by Tsui, 1992; Frizzell, 1995). However, complications in the lung are the major cause of mortality and morbidity among the patients. Therefore, the airways are the primary target site for delivery of a functional CFTR gene. Preclinical and clinical studies have demonstrated that gene transfer to the lung with viral and synthetic vectors is feasible and that partial restoration of the normal phenotype can be achieved (reviewed by Korst *et al.*, 1995; Rosenfeld and Collins, 1996; Alton *et al.*, 1998). Another important future application could be the treatment of asthma by gene therapy (reviewed by Demoly *et al.*, 1997). Furthermore, gene delivery via the intrapulmonary route would be an attractive noninvasive delivery method for systemic expression of therapeutic proteins by targeting alveolar cells, which then express and secrete the gene products into the blood stream.

Different molecules have been studied for their potential use as gene transfer vectors to achieve expression after intrapulmonary delivery. Application of DNA alone ("naked DNA") has been shown to result in gene expression in trachea and lung (Meyer *et al.*, 1995; Sawa *et al.*, 1996; Zabner *et al.*, 1997). Furthermore, it was demonstrated in animal models that different types of lipoplexes can direct gene expression in lung and trachea after intranasal instillation, tracheal injection or intubation, and aerosolization (Brigham *et al.*, 1989; Stribling *et al.*, 1992; Yoshimura *et al.*, 1992; Alton *et al.*, 1993; Hyde *et al.*, 1993; Canonico *et al.*, 1994; Logan *et al.*, 1995; Aberle *et al.*, 1996; Fortunati *et al.*, 1996; Lee *et al.*, 1996; McLachlan *et al.*, 1996; Wheeler *et al.*, 1996; Eastman *et al.*, 1997a,b, 1998; Fasbender *et al.*, 1997; Oudrhiri *et al.*, 1997; Jiang *et al.*, 1998). Also, delivery of polyplexes directed gene expression in rabbit lungs (Ferrari *et al.*, 1997). In clinical trials, DC-Chol, DOTAP, and GL67 were shown to achieve expression of the CFTR gene and transient correction of the chloride defect (Caplen *et al.*, 1995; Gill *et al.*, 1997; Porteous *et al.*, 1997; Zabner *et al.*, 1997).

Here, we describe a new type of synthetic vector that is based on organic solvents and that can greatly enhance gene delivery to lung epithelium after intrapulmonary delivery.

MATERIALS AND METHODS

Plasmids and recombinant viruses

Plasmid pTG11033 contains the luciferase gene under the control of the cytomegalovirus immediate-early gene 1 (IE1-CMV) promoter, intron 1 of the *HMGCoAR* (hydroxymethylglutaryl-CoA reductase) gene, and the simian virus 40 (SV40) poly(A) signal. Plasmid pTG11034 contains the bacterial *lacZ* gene with a nuclear localization signal (NLS) under the control of the IE1-CMV promoter, the rabbit β_1 -globin *ivs2* intron, and SV40 poly(A). Plasmid pEGFP-C1 contains the green fluorescent protein (*GFP*) gene under the control of the IE1-CMV promoter (Clontech, Palo Alto, CA). Adenovirus vector Ad-RSV-

bGal contains the bacterial *lacZ* gene with an NLS under the control of the Rous sarcoma virus (RSV) promoter, and was obtained from L. Stratford-Perri caudet (Stratford-Perri caudet *et al.*, 1992). Adenovirus vectors AdTG8509 and AdTG6441 contain the luciferase gene under the control of the adenovirus major late promoter and IE1-CMV promoter, respectively.

Synthesis of DPSO; preparation of solvoplexes, lipoplexes, and polyplexes

Di-*n*-propylsulfoxide (DPSO) was synthesized by oxidation of di-*n*-propylsulfide with NaO₄: 13.2 g (15.85 ml) of di-*n*-propylsulfide (111 mmol; Aldrich, Milwaukee, WI) was slowly added under stirring to 25 g of NaO₄ (117 mmol) in 245 ml of water at 0°C. Progress of the reaction was followed by thin-layer chromatography (R_f = 0.41; silica plates [Aldrich]: layer 250 μ m, particle size 5–17 μ m, pore size 60 Å; eluent: 90:10 CH₂Cl₂–CH₃OH; detection I₂, KMnO₄). The crude product was recovered by filtration on Celite and washed with 300 ml of water, 200 ml of CH₂Cl₂ followed by two water–CH₂Cl₂ extractions and a wash of the aqueous phase with CH₂Cl₂. The combined organic phases were washed with aqueous Na₂S₂O₃ to reduce the excess of iodine and rinsed with CH₂Cl₂. The solvent was evaporated after drying over anhydrous Na₂SO₄. DPSO was purified by silica gel chromatography (Kieselgel 60, Merck [Rahway, NJ]; column i.d. 4 cm, height 25 cm; eluent: CH₂Cl₂–CH₃OH [98:2]) followed by vacuum distillation at 0.2 mmHg (boiling point: 63°C; yield: 12.3 g, i.e., 91.7 mmol, i.e., 83%). Tetramethylurea (TMU) was obtained from Sigma (l'Isle d'Abeau Chesnes, France). For the preparation of solvoplexes, the corresponding solvent was added to plasmid DNA in 20 mM HEPES, pH 7.5, in the amount necessary to give the desired concentration, and then mixed by pipetting. Solvoplexes were prepared immediately before use. Liposome formulations (DOTAP–Chol or DOTAP–DOPE) were prepared essentially as described elsewhere (e.g., Barron *et al.*, 1998). DOTAP (1,2-dioleoyloxy-3-trimethylammonium propane), DOPE (dioleoylphosphatidylethanolamine), and cholesterol (Chol) were purchased from Avanti Polar Lipids (Atlanta, GA). Briefly, the desired amounts of each lipid in chloroform were mixed in a molar ratio of 1:1 and the organic solvent was evaporated under vacuum for 2 hr at 45°C (Laconco, Rotavap, Uniequip, Munich). The dried lipid films were then hydrated at a DOTAP concentration of 7 g/liter in 5% glucose. The resulting lipid mixtures were extruded through 0.4-, 0.2-, and then 0.1- μ m pore size polycarbonate filters. Lipoplexes at an *N/P* ratio of cationic lipid nitrogen to DNA phosphate (as described by Zanta *et al.*, 1997) of 2.2 were prepared 18 hr before injection by adding 17 μ l of liposomes to 25 μ g of DNA in 33 μ l of 5% glucose solution (final DNA concentration, 0.5 g/liter). PEI–polyplexes were prepared at an *N/P* ratio of 5 and injected 1 hr later. A 3.75- μ l volume of a 0.1 M monomer solution of PEI (Ex-Gen500, linear 22-kDa polyethylenimine; Euromedex, Souffel Weyersheim, France) was diluted in 25 μ l of 5% glucose and then added dropwise to 25 μ g of DNA in 25 μ l of 5% glucose.

Administration of vectors to mice

Mice were kept under specific pathogen-free (SPF) conditions and according to the European regulations for animal experimentation. For intratracheal delivery, mice were anes-

thetized, the skin disinfected with 70% ethanol, and an incision made above the trachea. A volume of 25 or 50 μ l was slowly injected into the trachea, using an injection needle. The breathing rhythm of the animal was closely watched during this period. For the delivery of a spray with the microsyringe system, the blunt-end needle of a microsyringe (IA-IC intrapulmonary aerosolizer; PennCentury, Philadelphia, PA) was inserted through a catheter into the trachea of anesthetized mice to the point of bifurcation (insertion was viewed from the outside after opening the skin overlying the trachea), and the solution was sprayed into the lung.

Luciferase assay

Mice were sacrificed 1 or 2 days postinjection. Lungs and trachea were prepared separately, frozen in liquid nitrogen, and stored at -80°C until further use. For luciferase analysis, tissues were pulverized mechanically by using mortars and pestles chilled on dry ice. The tissue powder was then dissolved in 500 μ l (lung) or 200 μ l (trachea) of lysis buffer (Promega, Charmonnières, France). Alternatively, the tissue was disrupted in the same volumes of lysis buffer with a homogenizer (two 30-sec pulses in a Polytron homogenizer; Kinematica, Littau, Switzerland). After lysis, the homogenate was freeze-thawed three times. The cell debris was removed by centrifugation and 20 μ l of the supernatant were used to determine luciferase activity in a luminometer (Microumat LB 96P; Berthold, Evry, France). Protein content were determined by the bicinchoninic acid (BCA) protein assay (Pierce, Montluçon, France) from an aliquot of the extract supernatant, and luciferase activity was calculated as relative light units (RLU) per minute per milligram protein.

Histological analysis of lacZ and GFP reporter gene expression

For analysis of *lacZ* gene expression, lungs were removed, washed in phosphate-buffered saline (PBS), and fixed in 2% formaldehyde. In some cases, mice were first perfused with PBS, then with 2% formaldehyde, and isolated organs were fixed in this solution for 30 to 60 min. Lungs were then washed in PBS, stained in staining solution [5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 2 mM MgCl_2 , 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal, 1 mg/ml), 0.02% Nonidet P-40 (NP-40), 0.01% sodium deoxycholate in PBS] for 2 days at 37°C , and fixed for 12 to 24 hr in 5% formaldehyde. Lungs were dissected to expose the airways, viewed, and photographed under a dissection microscope. For tissue sections, organs were dehydrated, embedded in paraffin (Histoplast; Life Science International, Eragny, France), cut in 8- μ m sections, in some cases counterstained with hematoxylin and eosin (H&E; Merck), mounted in Mowiol (Calbiochem/Novabiochem, La Jolla, CA) or Eukitt (Labonord, Villeneuve d'Ascq, France), and photographed under a light microscope. For GFP analysis, organs were removed, fixed in 4% formaldehyde (for at least 1 hr), dissected to expose the airways, and examined under a fluorescence microscope (Nikon, Garden City, NY) with the fluorescein isothiocyanate (FITC) filter set. Alternatively, whole animals were perfused with PBS and subsequently with 4% formaldehyde before preparation of lungs. For cryosections, organs were equilibrated in 30% sucrose solution (4°C , overnight), frozen in O.C.T. (Tissue Tek; Bayer Diagnostics,

Munich, Germany), then cut with the cryostat (5- to 8- μ m sections), fixed in methanol (-20°C , 20 min) mounted in Mowiol, and photographed under the fluorescence microscope (Nikon) with the FITC filter set. To determine the number of *lacZ*-positive cells, mice were injected intratracheally with adenovirus or solvoplexes vectors and 1 day after injection, lungs were stained with X-Gal. Subsequently, each lung was cut into 10 pieces (2 per lobe), embedded in paraffin, and sectioned. In this way, the entire lung of a mouse was represented in about 1000 tissue sections, with 100 sections per piece. From each piece, 10 sections were taken (evenly distributed over the entire number of sections from each piece), and one microscopic field was selected from each section and recorded. On each section, the microscopic field that contained the highest number of positive cells in the respiratory epithelium was selected.

RESULTS

Solvoplexes greatly enhance intrapulmonary gene delivery

To identify new synthetic vectors for intrapulmonary gene delivery, we tested various molecules that may increase transfection efficiency by either interacting with DNA and/or influencing cellular DNA uptake. We hypothesized that organic solvents may favorably change the properties of airway epithelial cells, and/or DNA conformation. When testing different solvents, we found that the addition of polar aprotic solvents to plasmid DNA considerably enhanced gene expression in lungs after intrapulmonary delivery. This mixture of DNA and solvent was termed *solvoplexes*.

Mice were injected intratracheally with solvoplexes or DNA alone and luciferase expression in lungs was analyzed 2 days later. Intratracheal injection of DPSO- or TMU-solvoplexes yielded considerably higher reporter gene expression than DNA alone. Figure 1A represents the accumulated data from several experiments. In this combined data set, 15% DPSO-solvoplexes resulted, on average, in up to 150-fold increased expression levels when compared with DNA alone, and 15% TMU-solvoplexes increased expression about 40-fold. Injection of the same amount of DNA in 25 μ l instead of 50 μ l resulted in only slightly higher expression levels, but the 25- μ l volume was better tolerated by the mice. In some experiments (see below) an increase in expression levels of up to 440-fold, compared with naked DNA, could be observed on day 1 postinjection.

Highest levels of luciferase reporter gene expression in the lung were obtained with solvoplexes containing 15% DPSO and 50 μ g of DNA. The efficiency of solvoplexes was concentration dependent with respect to the DPSO and TMU solvents (Fig. 1B). A 10% DPSO concentration was about 30-fold more efficient than 5% DPSO. A 15% DPSO concentration was about 4-fold better than 10%, and about 130-fold higher than 5% DPSO. Administration of 10% TMU-solvoplexes yielded only slightly better results than DNA alone (data not shown). Several other solvents were tested, and luciferase expression levels, slightly higher than for DNA alone, were observed for DESO (diethylsulfoxide), MESO (methylethylsulfoxide), PPG (propyleneglycol), TEMSO (tetramethylenesulfoxide), and sul-

A RLU/mg **B RLU/mg**

FIG. 1. (A) DPSO- and TMU-solvoplexes yield high levels of luciferase expression in lung, compared with DNA alone. Female B6 or B6SF1 mice were injected intratracheally with solvoplexes or DNA alone (50 μ g of pTG11033 plasmid DNA in 50 or 25 μ l) and luciferase expression in lung homogenates was determined 2 days after injection. As control, naive mice (not injected) were analyzed. Mean values were calculated from several independent experiments and statistical analyses were performed using the Mann-Whitney rank sum test. * $p < 0.05$ in a pairwise comparison with DNA alone. (B) Solvoplex-mediated gene expression is dependent on the concentration of solvent. Female B6SF1 mice were injected intratracheally with DNA alone (0% DPSO) or with solvoplexes containing increasing concentrations of DPSO (50 μ g/25 μ l of plasmid pTG11033 in each case), and luciferase expression in lung homogenates was determined 1 day after injection. * $p < 0.04$ in all pairwise comparisons of solvoplex-injected mice. (C) Solvoplex-mediated gene expression works also in rats. OF1 rats were injected intratracheally with solvoplexes, PEI-polyplexes, or DNA alone (pTG11033 plasmid DNA) and luciferase expression in lung homogenates was determined 1 day after injection. As control, naive rats (not injected) were analyzed. Mean values were calculated from two independent experiments. n , Number of mice analyzed in each group; SEM, standard error of the mean.

FIG. 1 (continued)

folane (data not shown). BMSO (butylmethylsulfoxide)-containing solvoplexes gave expression levels similar to those of TMU-solvoplexes (data not shown).
Gene expression, after solvoplex delivery, was highest on

day 1 and declined rapidly until day 7, when expression levels were only slightly above background (data not shown). Thus, for most subsequent analyses, we determined expression levels on day 1 postinjection.

FIG. 2. DPSO-solvoplexes are more efficient than DOTAP-lipoplexes or PEI-polyplexes. Female B6SF1 mice were injected intratracheally with solvoplexes or DNA alone (25 μ g/50 μ l of plasmid pTG11033 in each case) and luciferase expression in lung homogenates was determined 1 day after injection. As control, naive mice (not injected) were analyzed. Mean values were calculated and statistical analyses were performed using the Mann-Whitney rank sum test. * p < 0.04 in a pairwise comparison of the lipoplexes and polyplexes with DPSO-solvoplexes. n , Number of mice analyzed in each group; SEM, standard error of the mean.

FIG. 3. *lacZ* reporter gene expression is observed in the lung airway epithelium after intratracheal injection of solvoplexes. One day after injection of female B6SF1 mice, lungs were prepared. After staining with X-Gal, lungs were cut into large pieces, and the cut open surface was photographed under a dissection microscope. (A) Noninjected control mice; (B) mice injected with 5×10^8 PFU of Ad-RSV-bGal adenovirus; (C) mice injected with 15% DPSO-solvoplexes; (D) mice injected with 15% TMU-solvoplexes. For solvoplexes, $50 \mu\text{g}/50 \mu\text{l}$ of pTG11034 plasmid DNA was used. Lungs from 15% DPSO-solvoplex-injected mice were embedded in paraffin, sectioned, and viewed under a light microscope using Nomarski (E) or bright-field illumination after H&E staining (F and G). (G) Lung sections of mice treated with a solvoplex microspray.

FIG. 4. *GFP* reporter gene expression is observed in the lung airway epithelium after intratracheal injection of solvoplexes. One day after injection of female B6 or B6SF1 mice, lungs were prepared, cut into large pieces, and directly viewed under a fluorescence microscope. Cut open surfaces displaying positive *GFP* expression were photographed. (A) Noninjected control mice; (B) mice injected with 15% DPSO-solvoplexes; (C) mice injected with 15% TMU-solvoplexes. For solvoplexes, 50 $\mu\text{g}/25\ \mu\text{l}$ of plasmid pEGFP-C1 DNA was used. (D and E) Cryosections of lungs injected with 15% DPSO-solvoplexes and photographed with a fluorescence microscope. Examples of *GFP*-positive cells are indicated by arrows in (C)–(E).

We could also detect luciferase activity in trachea after intrapulmonary application of DNA alone and solvoplexes. In general, for DNA alone, expression levels were higher in trachea than in lung, whereas for solvoplexes, expression levels in trachea were lower or comparable to those in lung (data not shown). Since we were mainly interested in gene delivery to the lung airway epithelium, we focused our analysis on the lung.

To investigate whether intrapulmonary delivery of solvoplexes could also result in gene expression in other species, we administered DPSO-solvoplexes to rats. After intratracheal delivery of 15% DPSO-solvoplexes, expression of the luciferase reporter gene was observed in rat lungs (Fig. 1C). In this case, although larger amounts of DNA were used to adjust for the

larger size of rats, the relative expression levels (RLU/mg tissue extract) were lower than in mice.

Lungs from mice, injected with 5, 10, and 15% DPSO-solvoplexes or with DNA alone, were analyzed for pathological alterations on day 1 and day 7 postinjection. Mild to moderate inflammation (peribronchial and perivascular lymphocyte infiltration) was observed on day 1 in mice injected with 5 and 10% DPSO-solvoplexes. For 15% DPSO-solvoplexes, moderate inflammation with some focal lesions and edema were apparent. In mice injected with DNA alone, no inflammation was observed in one experiment, whereas in another experiment, moderate to intense inflammation and edema, similar to solvoplex-injected mice, were detectable. Seven days after injection, no or weak residual inflammation was seen for solvoplexes and

DNA alone. Changes in relative weight loss were not significantly different between injected and noninjected mice, and no increase in serum transaminase levels (asparagine transaminase and alanine transaminase) was observed in the serum on day 1 or day 7 after intratracheal injection (data not shown).

Comparison of solvoplexes with other synthetic vectors

The efficiency of DPSO-solvoplexes was compared with lipoplexes and polyplexes. Formulation of lipo- and polyplexes at DNA concentrations higher than 25 $\mu\text{g}/50\ \mu\text{l}$ was not possible, because of precipitation. Therefore, we used 25 μg instead of 50 μg of DNA in a volume of 50 μl for all complexes. Under these conditions, DPSO-solvoplexes gave the highest expression levels in the lung on day 1 after injection (Fig. 2). Luciferase gene expression levels obtained after intratracheal injection of 15% DPSO-solvoplexes were 260-, 25-, or 4-fold higher than those achieved with DOTAP/DOPE-, DOTAP/cholesterol-lipoplexes, or PEI-polyplexes, respectively. It should also be noted that in this experiment, DPSO-solvoplex-mediated expression levels were 440-fold higher than those of naked DNA. Thus, solvoplexes represent a more efficiently performing vector for intrapulmonary gene delivery than many of the currently used synthetic vectors.

Histological analysis of lungs after intrapulmonary delivery of solvoplexes

To localize the cells in the lung that exhibit expression after solvoplex delivery, *lacZ* and the GFP reporter genes were in-

jected into mouse lungs as DPSO- or TMU-solvoplexes. A first macroscopic examination was performed by cutting the lungs with a scalpel and observing the surface of the respiratory epithelium directly under a dissection microscope. *lacZ*-expressing, blue cells were detectable after X-Gal staining, in areas of the proximal bronchi and bronchiole of mice injected with the Ad-LacZ control vectors (Fig. 3B), but not in noninjected control mice (Fig. 3A). In mice injected with DPSO- or TMU-solvoplexes, *lacZ*-positive cells were found in proximal regions of the lung airways (Fig. 3C and D, respectively). Paraffin sections demonstrated *lacZ* expression within the lung airway epithelium of mice injected with either DPSO- or TMU-solvoplexes (Fig. 3E and F). When DPSO-solvoplexes were delivered as a microspray, a similar expression pattern in the lung airway epithelium was observed (Fig. 3G).

After intratracheal injection of solvoplexes that contained a GFP reporter gene plasmid, GFP-expressing cells were readily detectable under a fluorescence microscope in bronchi of mice injected with DPSO- or TMU-solvoplexes in whole lung sections (Fig. 4B and C, respectively). The distribution of positive cells was the same as previously seen for the *lacZ* reporter gene. In cryosections from injected lungs, we confirmed that GFP-positive cells were localized within the respiratory epithelium (Fig. 4D and E).

Readministration of solvoplexes

One of the presumed advantages of synthetic vectors over viral vector systems is the possibility of administering them re-

FIG. 5. Readministration of solvoplexes results in gene expression levels similar to single administration; comparison of efficacy between solvoplexes and adenovirus vectors. Female B6SF1 mice were injected intratracheally with solvoplexes (50 $\mu\text{g}/25\ \mu\text{l}$ of plasmid pTG11033) or with 1×10^8 PFU of AdTG8509 adenovirus and analyzed for luciferase expression 1 or 29 days later. Two groups were reinjected with solvoplexes or with adenovirus on day 28 and 1 day later, luciferase expression in lung homogenates was determined. Ad MLP-Luc (AdTG8509) or Ad CMV-Luc (AdTG6441): luciferase reporter driven by MLP or CMV promoter, respectively. Mean values were calculated and statistical analyses were performed using the Mann-Whitney rank sum test. * $p < 0.01$ for the pairwise comparisons of the following groups of adenovirus-injected mice: day 1 and day 29, day 1 and day 29 reinjected on day 28; n, Number of mice analyzed in each group; SEM, standard error of the mean.

peatedly. To investigate whether solvoplex vectors could be readministered, we injected mice with 15% DPSO-solvoplexes and again, 28 days later (Fig. 5). Luciferase expression was then analyzed on day 29. Mice injected with solvoplexes showed luciferase expression on day 1, but not 29 days after injection. In contrast, mice that were injected on day 0, reinjected on day 28, and analyzed on day 29, exhibited the same expression levels on day 29 as mice injected on day 0 and analyzed at day 1.

In parallel, mice injected with adenovirus luciferase vectors were subjected to the same protocol. Initial expression on day 1 after injection was higher than for solvoplexes, and on day 29 some residual expression was still detectable. However, mice that were injected at day 0, and then reinjected with the adenovirus vector on day 28, did not show an increase in gene expression compared with mice injected only once and then analyzed on day 29 (Fig. 5).

FIG. 6. Quantitation of *lacZ*-positive cells in the lung. Female B6SF1 mice were injected intratracheally with **(A)** 5×10^8 PFU of Ad-RSV-bGal adenovirus (in 25 μ l) or **(B)** solvoplexes (50 μ g/25 μ l of plasmid pTG11034) and stained for *lacZ* expression in the lung 1 day after injection. Paraffin sections were prepared, about 100 optical fields were selected (as described in text), *lacZ*-positive cells were counted, and the distribution of the number of *lacZ*-positive cells over the optical fields was plotted. On the X axis, optical fields were ranked according to the number of positive cells observed in this field. This number is thus artificial and does not correspond to the sequence of sections along the tissue.

TABLE 1. QUANTITATIVE HISTOLOGICAL ANALYSIS OF *lacZ*-POSITIVE CELLS AFTER DELIVERY OF SOLVOPLEXES COMPARED WITH ADENOVIRUS VECTOR

Vector (mouse no.)	Mean number of positive cells/field	Relative efficiency compared with best Ad	No. of positive/ total cells ^a	Percentage of positive/ total cells ^a
Ad (1)	76.40	1	1,591/8,521	18.7
Ad (2)	71.07	0.93	1,641/10,716	15.3
DPSO (13)	1.72	0.023	ND	ND
DPSO (14)	1.04	0.014	ND	ND
DPSO (15)	2.70	0.035	101/5,615	1.8

^aIn the 10 best fields; ND, not determined.

Efficacy of solvoplexes compared with adenovirus vectors

After having shown that solvoplexes represent efficient vectors compared with other synthetic vectors, we also compared their efficacy with that of viral vectors, such as adenovirus. Since these are two different types of vectors, the expression levels in individual cells and the total number of cells expressing the reporter gene may be different. Therefore, we decided to compare expression levels in lung homogenates and, more importantly, the number of *lacZ*-expressing cells after delivery of solvoplex versus adenovirus vectors.

For the comparison of expression levels in lung homogenates, two viral vectors were used. AdTG8509 and AdTG6441 contain the luciferase reporter gene under the control of the MLP promoter and the CMV promoter, respectively.

One day after intratracheal delivery, 15% DPSO–solvoplexes gave 4-fold and about 100-fold lower expression levels compared with 1×10^8 PFU of AdTG8509 and AdTG6441, respectively (Fig. 5). The plasmid used for solvoplexes contained the CMV promoter driving the luciferase gene.

To compare the number of cells expressing a foreign gene after solvoplex versus adenovirus delivery, we injected two mice intratracheally with 5×10^8 PFU of recombinant AdTG-RSV-bGal vector and three mice with 15% DPSO–solvoplexes containing the *lacZ* reporter gene (plasmid pTG11034). One day after injection, lungs were stained for *lacZ*-expressing cells and sectioned. The number of *lacZ*-positive cells from 99 to 110 (depending on the individual mouse) representative microscopic fields was counted for each mouse and the mean number of *lacZ*-expressing cells per field was calculated for each mouse (see Materials and Methods for details). We counted

FIG. 7. (A) DNA in solvoplexes is stable on spraying. Solvoplexes (15% DPSO) were prepared with pTG11033 (0.5 μ g of DNA/ μ l) and sprayed with a microsprayer. Subsequently, samples were collected, diluted in TE, and 20 μ l of untreated DNA (1 μ g; lane C), or DNA from solvoplexes sprayed once (lane 1) or twice (lane 2) were separated on a 1% agarose gel next to a DNA size marker (lane M). (B) Microsprayed solvoplexes yield gene expression in mouse lungs. A microsprayer device was inserted into the trachea of female B6 or B6SF1 mice, and solvoplexes (50 μ g/25 μ l of pTG11033 plasmid DNA) were sprayed directly into the lung. Luciferase expression in lung homogenates was determined 1 day after administration. As control, naive mice (not injected) were analyzed. Mean values were calculated from two independent experiments. *n*, Number of mice analyzed in each group; SEM, standard error of the mean.

only *lacZ*-positive cells in the lung airway epithelium, because in alveolar regions, positive cells were rarely observed. This protocol allowed us to compare the relative number of *lacZ*-positive cells for the whole lung between mice injected with solvoplexes to those injected with adenovirus, without determining for each mouse the total number of positive cells. The distribution of the number of *lacZ*-expressing cells over all sections analyzed is shown in Fig. 6. On average, in the three mice injected with 15% DPSO–solvoplexes, the number of *lacZ*-positive cells represented 1.4 to 3.5% of positive cells (Table 1) compared with the number of positive cells found in the adenovirus-injected mouse that exhibited the highest expression level (set to 100%).

To determine the percentage of *lacZ*-positive cells relative to the total number of airway epithelial cells in transfected regions of the lung, we counted the number of epithelial cells in the 10 microscopic fields that showed the highest number of *lacZ*-positive cells (see Fig. 6). For the adenovirus vector, reporter gene expression in 15 to 19% of the airway epithelial cells was observed, and in the DPSO–solvoplex-injected mouse, about 2.0% of the cells in the airway epithelium expressed the *lacZ* reporter gene (Table 1). It should also be noted that for all three mice, the percentage of *lacZ*-positive cells compared with the total number of epithelial cells was similar in all 10 fields analyzed.

Delivery of solvoplexes as microspray

The ultimate way of delivering vectors to the respiratory epithelium will be in the form of an aerosol. This noninvasive route of administration was successfully employed for adenovirus-mediated CFTR gene delivery to the airways of cystic fibrosis patients (Bellon *et al.*, 1997). However, DNA in solvoplexes was degraded when aerosolized by a jet stream nebulizer (data not shown). We thus explored the use of a microsprayer system that consists of a high-pressure syringe connected to a system of nozzles and that allows generation of a fine mist. To test whether DNA in solvoplexes remained intact, DPSO–solvoplexes were sprayed with the microsprayer, the mist was collected, and the DNA analyzed on an agarose gel. Under these conditions, only minor DNA degradation was seen in the form

of a low molecular weight smear (Fig. 7A). When the microsprayer device was used to deliver a DPSO–solvoplex spray directly into mouse lungs, luciferase expression was observed on day 1 after administration (Fig. 7B).

Stability of DNA in solvoplexes

An important parameter for the clinical use of synthetic vectors is the stability of DNA upon storage. When DPSO–solvoplexes were kept at -20°C upon at room temperature, no degradation of DNA was observed within a time period of 15 days. Only after storage for more than 5 days at 37°C was slight DNA degradation detected (Fig. 8). The stored DNA could still be digested with restriction enzymes, and the correct restriction pattern was obtained (data not shown). Furthermore, plasmid DNA (pTG11033) that was stored as 15% DPSO–solvoplex at room temperature for up to 15 days resulted in the same levels of reporter gene expression as nonmanipulated DNA when transfected as lipoplexes into cultured A549 cells (data not shown).

DISCUSSION

We identified a novel synthetic vector for intrapulmonary gene delivery, termed *solvoplex*. Solvoplexes consist of DNA and an organic solvent, they are easy to formulate at high DNA concentrations, are stable for an extended time period, can be administered as a spray, achieve gene expression in cells of the lung airway epithelium, yield expression levels that are comparable or higher than other synthetic vectors, and can be administered repeatedly.

Many different types of vectors have been tested for intrapulmonary delivery of either the CFTR gene or various reporter genes. For lipoplexes, the cationic lipid DOTAP (Alton *et al.*, 1993; McLachlan *et al.*, 1995, 1996; Aberle *et al.*, 1996; Porteous *et al.*, 1997), other cationic lipids, or PEI–polyplexes have been used (reviewed by Abdallah *et al.*, 1995; Felgner *et al.*, 1995; Lee and Huang, 1997; Mahato *et al.*, 1997; Kabanov *et al.*, 1998; Rolland, 1998; Scherman *et al.*, 1998). We preferred to directly compare DPSO–solvoplexes with DOTAP–

FIG. 8. DNA in solvoplexes is stable upon storage. Solvoplexes (15% DPSO) were prepared with pTG11033 (1 μg of DNA/ μl), and stored at -20°C , at room temperature, or at 37°C . From each of the three samples, aliquots were taken on days 3, 5, 10, and 15. Subsequently, 20 μl (1 μg of DNA) from each sample were separated on a 1% agarose gel next to a plasmid control (lane C) and a DNA size marker (lane M). Days of storage are indicated.

lipoplexes and PEI–polyplexes under the same conditions within the same experiment. We demonstrated that 15% DPSO–solvoplexes resulted in much higher levels of gene expression than did DOTAP–lipoplexes formulated with either cholesterol or DOPE as colipid, and higher levels than PEI–polyplexes.

DNA in solvoplexes remained intact and functional at room temperature for at least 2 weeks, representing a distinct advantage of solvoplexes compared with concentrated lipoplexes. Many of the currently used lipid–DNA complexes need to be prepared shortly before use, a cumbersome and difficult process during clinical studies.

Intrapulmonary delivery of lipoplexes can result in adverse inflammatory reactions that are associated with the lipid compound or the DNA (Scheule *et al.*, 1997; Yew *et al.*, 1999). Here, we observed for DPSO–solvoplexes and DNA alone, an inflammatory response in the lung that increased with increasing DPSO concentrations. However, after 7 days, lungs exhibited no or only weak signs of residual inflammation. Since similar effects were seen for both DNA alone and solvoplexes, the pathological changes are most likely caused by a combination of the manipulation (intratracheal injection) itself, the DNA, and the solvent (at higher concentrations). Furthermore, it is conceivable that the pathological changes observed are due to the administration of solvoplexes as a bolus, which could lead to transient obstruction of the airways, and that they will be less pronounced if the vector is delivered by an aerosol. No liver toxicity or weight loss was observed 1 or 7 days after injection. Thus, there are no indications of adverse effects in other organs after intratracheal administration of solvoplexes. It is important to note that the amount of DPSO that is applied in our protocol is about 5000-fold less than the median lethal dose (LD₅₀) determined for mice after intraperitoneal injection (Tatken and Lewis, 1983).

Maximal expression levels were reproducibly observed with 15% DPSO. However, DNA remains in solution at even higher solvent concentrations and for some solvoplexes, we have tested concentrations of up to 20%. Such formulations resulted in higher expression levels in some mice but variations between individual mice were much more pronounced. This effect is most likely due to the increased viscosity of solvoplex solutions at high DPSO concentrations, with consequent difficulties in achieving reproducible delivery to the lung.

In histological sections, we demonstrated that gene expression after intrapulmonary delivery is localized in cells of the intact airway epithelium of the lung, and not in detached or isolated cells or in regions where the epithelium is damaged. Expression of both *lacZ* and *GFP* reporter genes was observed. In future, it will be necessary to determine the exact cell type that expresses the foreign gene, and to associate gene transfer and expression with biological functions (e.g., after CFTR gene delivery).

After whole lung staining, we detected expression of the *lacZ* reporter gene mainly in proximal bronchi and bronchioli of the lung airways, indicating that the injected material did not penetrate well into deeper regions of the lung. In addition, we observed some variation in gene expression levels in individual mice. We attribute these differences and restricted expression to the fact that it is difficult to administer such formulations in a reproducible way into the airways of small rodents, using the

currently available technology. For adenovirus vectors, intrapulmonary delivery was increased by adding surfactant (Jobe *et al.*, 1996; Katkin *et al.*, 1997) whereas for the delivery of lipoplexes, an inhibitory effect was noted (Tsan *et al.*, 1997; Raczka *et al.*, 1998). Furthermore, addition of fluorocarbon liquid resulted in an improved distribution of injected adenovirus vectors (Lisby *et al.*, 1997). The use of such additives may further improve the efficacy of solvoplex vectors.

We also compared the efficacy of solvoplexes with that of adenovirus vectors by determining the overall levels of gene expression in lung homogenates as well as the number of cells that express a histologically detectable reporter gene after intrapulmonary delivery. If we compare the levels of luciferase gene expression in mice that received 1×10^8 PFU of recombinant adenovirus, expression levels in lung homogenates were about 4- to 100-fold lower for solvoplexes when compared with adenovirus containing either an MLP- or CMV-driven luciferase reporter gene. For solvoplexes, a CMV-luciferase plasmid was used.

The comparison of efficiency between the two vector systems can be misleading if only the overall expression levels in lung homogenates are considered. For some therapeutic genes, such as CFTR, the absolute level of expression in the lung may be less important than the number of cells expressing the exogenous gene. Endogenous levels of the CFTR transcript are low in the lung epithelium (Trapnell *et al.*, 1991) and it has been demonstrated that, *in vitro*, expression in 5% of cells could restore the transmembrane potential difference (Johnson *et al.*, 1992). Furthermore, a comparison between adenovirus and lipoplex vectors suggests that the two types of vectors may target different cell types and thus have different therapeutic effects (Zhang *et al.*, 1998). Therefore, we also compared the two vector systems on the basis of the number of *lacZ*-expressing cells after gene delivery.

In the comparison of the adenovirus and solvoplex vector systems for the entire lung, we estimate that the adenovirus vector yielded 25-fold more *lacZ*-positive cells than did DPSO–solvoplexes. If, however, the percentage of *lacZ*-positive cells in the best expressing regions was analyzed, the adenovirus vector resulted in expression of about 20% of lung airway epithelial cells, whereas the DPSO–solvoplexes achieved expression in about 2% of the epithelial cells. If one assumes that for regions where maximal gene expression was observed, the conditions for gene transfer were optimal, then solvoplexes are only 10-fold less efficient than adenovirus vectors. It should be noted that adenovirus vectors contain auxiliary genes, and it has been demonstrated that the E4 region represents an important *trans*-activating component for high-level gene expression from various promoters (Armentano *et al.*, 1997; Dedieu *et al.*, 1997; Lusky *et al.*, 1998). A comparison with solvoplexes containing the reporter gene and, in addition a second plasmid encoding the viral E4 gene, could further elucidate the relative performance of solvoplexes versus adenovirus vectors.

It should be noted that for adenovirus vectors, the RSV promoter and for plasmid vectors, the CMV promoter were used in this comparison, knowing that the CMV promoter exhibits a higher transcriptional activity. A CMV-*LacZ* adenovirus vector is not available in our laboratory. Nevertheless, in a different context, we could show that the use of a murine leukemia virus (MuLV) promoter versus RSV promoter in adenovirus

lacZ vectors resulted in an increase of expression levels in individual cells after intrapulmonary delivery, but did not influence the number of *lacZ*-positive cells (data not shown). Thus, for the comparison described here, the use of two different promoters should not have a significant impact on the number of expressing cells, and we, therefore, conclude that the difference in the number of positive cells mainly reflects the transfection efficacy of the two vector systems.

Guillaume-Gable *et al.* (1998) reported about 21% of reporter gene-expressing cells in the lung after intrapulmonary delivery of phosphonolipid-containing synthetic vectors. In this case, cell suspensions were prepared from lungs and then analyzed by fluorescence-activated cell sorting for the expression of the *lacZ* reporter gene, using a fluorescence substrate. Under these conditions, high background levels of about 13% in noninjected mice and 10% positive cells for mice injected with DNA alone were observed. Under the conditions used in our studies, we observed no background expression of the *lacZ* or GFP reporter gene in nontreated animals, and the number of *lacZ*-positive cells was determined by counting histologically stained airway epithelial cells.

At present, we can only speculate about the mechanism of transfection of airway epithelial cells by solvoplexes. Organic solvents could change the composition of the cell membrane by altering its fluidity, or they may change the properties and distribution of receptors. Also, it is conceivable that the basolateral cell surfaces of epithelial cells in the lung become exposed in the presence of solvent, thus allowing DNA uptake via the basolateral membrane. It was shown that pretreatment of mice with the nonionic detergent polidocanol (PODC) can greatly enhance gene expression after administration of adenovirus vectors (Parsons *et al.*, 1998). PODC was shown to permeabilize tight junctions between airway epithelial cells. A similar enhancement effect was demonstrated for EGTA or Ca^{2+} -free medium *in vitro* with polarized epithelial cells (Chu *et al.*, 1999). It will thus be interesting to test PODC and related compounds together with solvoplexes in future. As an alternative to effects at the cellular level, the conformation of DNA may be altered in the presence of solvents in such a way that uptake is facilitated.

Gene expression after solvoplex-mediated delivery lasted for only a few days. This is a general phenomenon that is observed for all types of synthetic vectors after intrapulmonary or intravenous delivery (reviewed by Abdallah *et al.*, 1995; Felgner *et al.*, 1995; Lee and Huang, 1997; Mahato *et al.*, 1997; Kabanov *et al.*, 1998; Rolland, 1998; Scherman *et al.*, 1998). Thus, treatment with solvoplexes would require multiple dosing in patients. If, however, toxicity is low, multiple dosing could be acceptable, as it is currently necessary in standard treatment regimens for patients with severe lung diseases. We showed that, in contrast to delivery by adenovirus vectors, successful gene expression after multiple administration of solvoplexes is possible. This feature represents one of the most important advantages of solvoplexes. It will, however, be necessary to study in detail the potential toxic effects of multiple dosing and long-term toxicity before proceeding to clinical studies.

We demonstrated gene expression in the lung airway epithelium after intrapulmonary delivery, but the activity of solvoplexes may not be restricted to this particular mode of administration. It will thus be interesting to analyze the transfection

efficiency of solvoplexes for intravenous, intratumoral, intradermal, intramuscular, subcutaneous, or other applications.

In conclusion, solvoplexes represent an interesting alternative or complementary method for intrapulmonary gene delivery compared with currently existing synthetic and viral vectors and this potential should be explored further.

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REFERENCES

- ABDALLAH, B., SACHS, L., and DEMENEIX, B.A. (1995). Non-viral gene transfer: Applications in developmental biology and gene therapy. *Biol. Cell* **85**, 1–7.
- ABERLE, A.M., BENNETT, M.J., MALONE, R.W., and NANTZ, M.H. (1996). The counterion influence on cationic lipid-mediated transfection of plasmid DNA. *Biochim. Biophys. Acta* **1299**, 281–283.
- ALTON, E.W., MIDDLETON, P.G., CAPLEN, N.J., SMITH, S.N., STEEL, D.M., MUNKONGE, F.M., JEFFERY, P.K., GEDDES, D.M., HART, S.L., WILLIAMSON, R., FASOLD, K.I., MILLER, A.D., DICKINSON, P., STEVENSON, B.J., McLACHLAN, G., DORIN, J.R., and PORTEUS, D.J. (1993). Non-invasive liposome-mediated gene delivery can correct the ion transport defect in cystic fibrosis mutant mice. *Nature Genet.* **5**, 135–142.
- ALTON, E.W., GEDDES, D.M., GILL, D.R., HIGGINS, C.F., HYDE, S.C., INNES, J.A., and PORTEOUS, D.J. (1998). Towards gene therapy for cystic fibrosis: A clinical progress report. *Gene Ther.* **5**, 291–292.
- ARMENTANO, D., ZABNER, J., SACKS, C., SOOKDEO, C.C., SMITH, M.P., ST. GEORGE, J.A., WADSWORTH, S.C., SMITH, A.E., and GREGORY, R.J. (1997). Effect of the E4 region on the persistence of transgene expression from adenovirus vectors. *J. Virol.* **71**, 2408–2416.
- BARRON, L.G., MEYER, K.B., and SZOKA, F.C., JR. (1998). Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid–DNA complexes. *Hum. Gene Ther.* **9**, 315–323.
- BELLON, G., MICHEL-CALEMARD, L., THOUVENOT, D., JAGNEAUX, V., POITEVIN, F., MALCUS, C., ACCART, N., LAYANI, M.P., AYMARD, M., BERNON, H., BIENVENU, J., COURTNEY, M., DÖRING, G., GILLY, B., GILLY, R., LAMY, D., LEVREY, H., MOREL, Y., PAULIN, C., PERRAUD, F., RODILLON, L., SENÉ, C., SO, S., TOURAINE-MOULIN, F., SCHATZ, C., and PAVIRANI, A. (1997). Aerosol administration of a recombinant adenovirus expressing CFTR to cystic fibrosis patients: A phase I clinical trial. *Hum. Gene Ther.* **8**, 15–25.
- BRIGHAM, K.L., MEYRICK, B., CHRISTMAN, B., MAGNUSON, M., KING, G., and BERRY, L.C., JR. (1989). In vivo transfection of murine lungs with a functioning prokaryotic gene using a liposome vehicle. *Am. J. Med. Sci.* **298**, 278–281.
- CANONICO, A.E., PLITMAN, J.D., CONARY, J.T., MEYRICK, B.O., and BRIGHAM, K.L. (1994). No lung toxicity after repeated

- aerosol or intravenous delivery of plasmid-cationic liposome complexes. *J. Appl. Physiol.* **77**, 415–419.
- CAPLEN, N.J., ALTON, E.W., MIDDLETON, P.G., DORIN, J.R., STEVENSON, B.J., GAO, X., DURHAM, S.R., JEFFERY, P.K., HODSON, M.E., COUTELLE, C., HUANG, L., PORTEOUS, D.J., WILLIAMSON, R., and GEDDES, D.M. (1995). Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nature Med.* **1**, 39–46.
- CHU, Q., TOUSIGNANT, J.D., FANG, S., JIANG, C., CHEN, L.H., CHENG, S.H., SCHEULE, R.K., and EASTMAN, S.J. (1999). Binding and DNA uptake of cationic lipid:pDNA complexes by polarized airway epithelial cells. *Hum. Gene Ther.* **10**, 25–36.
- DEDIEU, J.F., VIGNE, E., TORRENT, R.C., JULLIEN, C., MAHFOUZ, I., CAILLAUD, J.M., AUBAILLY, N., ORSINI, C., GUILLAUME, J.M., OPOLO, P., DELAERE, P., PERRICAUDET, M., and YEY, P. (1997). Long-term gene delivery into the livers of immunocompetent mice with E1/E4-defective adenoviruses. *J. Virol.* **71**, 4626–4637.
- DEMOLY, P., MATHIEU, M., CURIEL, D.T., GODARD, P., BOUSQUET, J., and MICHEL, F.B. (1997). Gene therapy strategies for asthma. *Gene Ther.* **4**, 507–516.
- EASTMAN, S.J., LUKASON, M.J., TOUSIGNANT, J.D., MURRAY, H., LANE, M.D., ST. GEORGE, J.A., AKITA, G.Y., CHERRY, M., CHENG, S.H., and SCHEULE, R.K. (1997a). A concentrated and stable aerosol formulation of cationic lipid:DNA complexes giving high-level gene expression in mouse lung. *Hum. Gene Ther.* **8**, 765–773.
- EASTMAN, S.J., TOUSIGNANT, J.D., LUKASON, M.J., MURRAY, H., SIEGEL, C.S., CONSTANTINO, P., HARRIS, D.J., CHENG, S.H., and SCHEULE, R.K. (1997b). Optimization of formulations and conditions for the aerosol delivery of functional cationic lipid:DNA complexes. *Hum. Gene Ther.* **8**, 313–322.
- EASTMAN, S.J., TOUSIGNANT, J.D., LUKASON, M.J., CHU, Q., CHENG, S.H., and SCHEULE, R.K. (1998). Aerosolization of cationic lipid:pDNA complexes—in vitro optimization of nebulizer parameters for human clinical studies. *Hum. Gene Ther.* **9**, 43–52.
- FASBENDER, A., MARSHALL, J., MONINGER, T.O., GRUNST, T., CHENG, S., and WELSH, M.J. (1997). Effect of co-lipids in enhancing cationic lipid-mediated gene transfer in vitro and in vivo. *Gene Ther.* **4**, 716–725.
- FELGNER, P.L., TSAI, Y.L., SUKHU, L., WHEELER, C.J., MANTHROPE, M., MARSHALL, J., and CHENG, S.H. (1995). Improved cationic lipid formulations for in vivo gene therapy. *Ann. N.Y. Acad. Sci.* **772**, 126–139.
- FERRARI, S., MORO, E., PETTENAZZO, A., BEHR, J.P., ZACHELLO, F., and SCARPA, M. (1997). ExGen500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Ther.* **4**, 1100–1106.
- FORTUNATI, E., BOUT, A., ZANTA, M.A., VALERIO, D., and SCARPA, M. (1996). In vitro and in vivo gene transfer to pulmonary cells mediated by cationic liposomes. *Biochim. Biophys. Acta* **1306**, 55–62.
- FRIZZELL, R.A. (1995). Functions of the cystic fibrosis transmembrane regulator protein. *Am. J. Respir. Crit. Care Med.* **151**, 554–558.
- GILL, D.R., SOUTHERN, K.W., MOFFORD, K.A., SEDDON, T., HUANG, L., SORGI, F., THOMSON, A., MACVINISH, L.J., RATCLIFF, R., BILTON, P., LANE, D.J., LITTLEWOOD, J.M., WEBB, A.K., MIDDLETON, P.G., COLLEDGE, W.H., CUTHBERT, A.W., EVANS, M.J., HIGGINS, C.F., and HYDE, S.C. (1997). A placebo-controlled study of liposome-mediated gene transfer to the nasal epithelium of patients with cystic fibrosis. *Gene Ther.* **4**, 199–209.
- GUILLAUME-GABLE, C., FLOCH, V., MERCIER, B., AUDREZET, M., GOBIN, E., LEBLOCH, G., YAOUANC, J., CLEMENT, J., DES ABAYES, H., LEROY, J., MORIN, V., and FEREC, C. (1998). Cationic phosphonolipids as nonviral gene transfer agents in the lungs of mice. *Hum. Gene Ther.* **9**, 2309–2319.
- HYDE, S.C., GILL, D.R., HIGGINS, C.F., TREZISE, A.E., MACVINISH, L.J., CUTHBERT, A.W., RATCLIFF, R., EVANS, M.J., and COLLEDGE, W.H. (1993). Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. *Nature (London)* **362**, 250–255.
- JIANG, C., O'CONNOR, S.P., FANG, S.L., WANG, K.X., MARSHALL, J., WILLIAMS, J.L., WILBURN, B., ECHELARD, Y., and CHENG, S.H. (1998). Efficiency of cationic lipid-mediated transfection of polarised and differentiated airway epithelial cells in vitro and in vivo. *Hum. Gene Ther.* **9**, 1531–1542.
- JOBE, A.H., IKEGAMI, M., YEI, S., WHITSETT, J.A., and TRAPNELL, B. (1996). Surfactant effects on aerosolized and instilled adenoviral-mediated gene transfer. *Hum. Gene Ther.* **7**, 697–704.
- JOHNSON, L.G., OLSEN, J.C., SARKADI, B., MOORE, K.L., SWANSTROM, R., and BOUCHER, R.C. (1992). Efficiency of gene transfer for restoration of normal airway epithelial function in cystic fibrosis. *Nature Genet.* **2**, 21–25.
- KABANOV, A.V., FELGNER, P.L., and SEYMOUR, L.W. (1998). *Self-Assembling Complexes for Gene Delivery*. John Wiley & Sons, Chichester, UK.
- KATKIN, J.P., HUSSER, R.C., LANGSTON, C., and WELTY, S.E. (1997). Exogenous surfactant enhances the delivery of recombinant adenoviral vectors to the lung. *Hum. Gene Ther.* **8**, 171–176.
- KORST, R.J., McELVANEY, N.G., CHU, C.S., ROSENFELD, M.A., MASTRANGELI, J.H., BRODY, S.L., EISSA, N.T., DANIEL, C., JAFFE, H.A., and CRYSTAL, R.G. (1995). Gene therapy for the respiratory manifestations of cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **151**, S75–S87.
- LEE, E.R., MARSHALL, J., SIEGEL, C.S., JIANG, C., YEW, N.S., NICHOLS, M.R., NIETUPSKI, J.B., ZIEGLER, R.J., LANE, M.B., WANG, K.X., WAN, N.C., SCHEULE, R.K., HARRIS, D.J., SMITH, A.E., and CHENG, S.H. (1996). Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum. Gene Ther.* **7**, 1701–1717.
- LEE, R.J., and HUANG, L. (1997). Lipidic vector systems for gene transfer. *Crit. Rev. Ther. Drug Carrier Syst.* **14**, 173–206.
- LISBY, D.A., BALLARD, P.L., FOX, W.W., WOLFSON, M.R., SHAFFER, T.H., and GONZALES, L.W. (1997). Enhanced distribution of adenovirus-mediated gene transfer to lung parenchyma by perfluorochemical liquid. *Hum. Gene Ther.* **8**, 919–928.
- LOGAN, J.J., BEBOK, Z., WALKER, L.C., PENG, S., FELGNER, P.L., SIEGAL, G.P., FRIZZELL, R.A., DONG, J., HOWARD, M., MATALON, S., LINDSEY, J.R., DUVAL, M., and SORSCHER, E.J. (1995). Cationic lipids for reporter gene and CFTR transfer to rat pulmonary epithelium. *Gene Ther.* **2**, 38–49.
- LUSKY, M., CHRIST, M., RITTNER, K., DIETERLE, A., DREYER, D., MOURROT, B., SCHULTZ, H., STOECKEL, F., PAVIRANI, A., and MEHTALI, M. (1998). In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. *J. Virol.* **72**, 2022–2032.
- MAHATO, R.I., ROLLAND, A., and TOMLINSON, E. (1997). Cationic lipid-based gene delivery systems: Pharmaceutical perspectives. *Pharm. Res.* **14**, 853–859.
- McLACHLAN, G., DAVIDSON, D.J., STEVENSON, B.J., DICKINSON, P., DAVIDSON-SMITH, H., DORIN, J.R., and PORTEOUS, D.J. (1995). Evaluation in vitro and in vivo of cationic liposome-expression construct complexes for cystic fibrosis gene therapy. *Gene Ther.* **2**, 614–622.
- McLACHLAN, G., HO, L.P., DAVIDSON-SMITH, H., SAMWAYS, J., DAVIDSON, H., STEVENSON, B.J., CAROTHERS, A.D., ALTON, E.W., MIDDLETON, P.G., SMITH, S.N., KALLMEYER, G., MICHAELIS, U., SEEBER, S., NAUJOKS, K., GREENING, A.P., INNES, J.A., DORIN, J.R., and PORTEOUS, D.J. (1996). Laboratory and clinical studies in support of cystic fibrosis gene therapy using pCMV-CFTR-DOTAP. *Gene Ther.* **3**, 1113–1123.

- MEYER, K.B., THOMPSON, M.M., LEVY, M.Y., BARRON, L.G., and SZOKA, F.C. (1995). Intratracheal gene delivery to the mouse airway: Characterisation of plasmid DNA expression and pharmacokinetics. *Gene Ther.* **2**, 450–460.
- OUDRHRI, N., VIGNERON, J.P., PEUCHMAUR, M., LECLERC, T., LEHN, J.M., and LEHN, P. (1997). Gene transfer by guanidinium-cholesterol cationic lipids into airway epithelial cells in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 1651–1656.
- PARSONS, D.W., GRUBB, B.R., JOHNSON, L.G., and BOUCHER, R.C. (1998). Enhanced in vivo airway gene transfer via transient modification of host barrier properties with a surface-active agent. *Hum. Gene Ther.* **9**, 2661–2672.
- PORTEOUS, D.J., DORIN, J.R., MCLACHLAN, G., DAVIDSON-SMITH, H., DAVIDSON, H., STEVENSON, B.J., CAROTHERS, A.D., WALLACE, W.A., MORALEE, S., HOENES, C., KALLMEYER, G., MICHAELIS, U., NAUJOKS, K., HO, L.P., SAMWAYS, J.M., IMRIE, M., GREENING, A.P., and INNES, J.A. (1997). Evidence for safety and efficacy of DOTAP cationic liposome mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Gene Ther.* **4**, 210–218.
- RACZKA, E., KUKOWSKA-LATALLO, J.F., RYMASZEWSKI, M., CHEN, C., and BAKER, J.R., JR. (1998). The effect of synthetic surfactant Exosurf on gene transfer in mouse lung in vivo. *Gene Ther.* **5**, 1333–1339.
- ROLLAND, A.P. (1998). From genes to gene medicines: Recent advances in nonviral gene delivery. *Crit. Rev. Ther. Drug Carrier Syst.* **15**, 143–198.
- ROSENFELD, M.A., and COLLINS, F.S. (1996). Gene therapy for cystic fibrosis. *Chest* **109**, 241–252.
- SAWA, T., MIYAZAKI, H., PITTET, J.F., WIDDICOMBE, J.H., GROPPER, M.A., HASHIMOTO, S., CONRAD, D.J., FOLKESSON, H.G., DEBS, R., FORSAYETH, J.R., FOX, B., and WIENER-KRONISH, J.P. (1996). Intraluminal water increases expression of plasmid DNA in rat lung. *Hum. Gene Ther.* **7**, 933–941.
- SCHERMAN, D., BESSODES, M., CAMERON, B., HERSCOVICI, J., HOFLAND, H., PITARD, B., SOUBRIER, F., WILS, P., and CROUZET, J. (1998). Application of lipids and plasmid design for gene delivery to mammalian cells. *Curr. Opin. Biotechnol.* **9**, 480–485.
- SCHEULE, R.K., GEORGE, J.A., BAGLEY, R.G., MARSHALL, J., KAPLAN, J.M., AKITA, G.Y., WANG, K.X., LEE, E.R., HARRIS, D.J., JIANG, C., YEW, N.S., SMITH, A.E., and CHENG, S.H. (1997). Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. *Hum. Gene Ther.* **8**, 689–707.
- STRATFORD-PERRICAUDET, L.D., MAKEH, I., PERRICAUDET, M., and BRIAND, P. (1992). Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. Clin. Invest.* **90**, 626–630.
- STRIBLING, R., BRUNETTE, E., LIGGITT, D., GAENSLER, K., and DEBS, R. (1992). Aerosol gene delivery in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11277–11281.
- TATKEN, R.L., and LEWIS, S.R., JR. (1983). *Registry of Toxic Effects of Chemical Substances*. Tractor Jitco, Rockville, MD.
- TRAPNELL, B.C., CHU, C.S., PAAKKO, P.K., BANKS, T.C., YOSHIMURA, K., FERRANS, V.J., CHERNICK, M.S., and CRYSTAL, R.G. (1991). Expression of the cystic fibrosis transmembrane conductance regulator protein in the respiratory tract of normal individuals and individuals with cystic fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6565–6569.
- TSAN, M.F., TSAN, G.L., and WHITE, J.E. (1997). Surfactant inhibits cationic liposome-mediated gene transfer. *Hum. Gene Ther.* **8**, 817–825.
- TSUI, L. (1992). The spectrum of cystic fibrosis mutations. *Trends Genet.* **8**, 392–398.
- WHEELER, C.J., FELGNER, P.L., TSAI, Y.J., MARSHALL, J., SUKHU, L., DOH, S.G., HARTIKKA, J., NIETUPSKI, J., MANTHORPE, M., NICHOLS, M., PLEWE, M., LIANG, X., NORMAN, J., SMITH, A., and CHENG, S.H. (1996). A novel cationic lipid greatly enhances plasmid DNA delivery and expression in the mouse lung. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11454–11459.
- YEW, N.S., WANG, K.X., PRZYBYLSKA, M., BAGLEY, R.G., STEDMAN, M., MARSHALL, J., SCHEULE, R., and CHENG, S.H. (1999). Contribution of plasmid DNA to inflammation in the lung after administration of cationic lipid:DNA complexes. *Hum. Gene Ther.* **10**, 233–234.
- YOSHIMURA, K., ROSENFELD, M., NAKAMURA, H., SCHERER, E., PAVIRANI, A., LECOCQ, J., and CRYSTAL, R. (1992). Expression of the human cystic fibrosis transmembrane conductance regulator gene in the mouse lung after in vivo intratracheal plasmid-mediated gene transfer. *Nucleic Acids Res.* **20**, 3233–3240.
- ZABNER, J., CHENG, S.H., MEEKER, D., LANSPACH, J., BALFOUR, R., PERRICONE, M.A., MORRIS, J.E., MARSCHALL, J., FASBENDER, A., SMITH, A.E., and WELSH, M.J. (1997). Comparison of DNA-lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia in vivo. *J. Clin. Invest.* **100**, 1529–1537.
- ZANTA, M.A., BOUSSIF, O., ADIB, A., and BEHR, J.P. (1997). In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. *Bioconj. Chem.* **8**, 839–844.
- ZHANG, Y., JIANG, Q., DUDUS, L., YANKASKAS, J.R., and ENGELHARDT, J.F. (1998). Vector-specific complementation profiles of two independent primary defects in cystic fibrosis airways. *Hum. Gene Ther.* **9**, 635–648.

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