The Influence of Sodium Glycocholate and Other Additives on the *in vivo* Transfection of Plasmid DNA in the Lungs

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Purpose. A plasmid containing the luciferase 'marker' cDNA was constructed to test non viral gene delivery formulations *in vivo*.
Methods. A scale up procedure was devised to produce up to gram quantities of plasmid. Sufficient quantities were generated to process and test the DNA with various additives and to generate a spray-dried powder formulation of the plasmid. Male Sprague-Dawley rats (250 g) were intratracheally instilled with 200–250 μl of solution containing 200 μg plasmid ± lipid [DC Chol:DOPE 1:1 molar (2mg/kg)] growth factors [KGF (10 mg/kg), EGF (5 mg/kg)], permeation enhancers [sodium glycocholate (0.01 to 10% w/v)), sodium deoxycholate (1% w/v), beta-cyclodextrin (1% w/v)], surfactant [Tween 80 (1% w/v)], a mucolytic [N-acetylcysteine (10% w/v)] and positively charged synthetic polymers [PVAVAM 6 and 14%]. Animals were sacrificed 24 hr post-dose and the lungs were assayed for luciferase using a chemiluminescent assay.
Results. The relative ability of the materials to promote luciferase production in the lungs was permeation enhancer >> DNA alone ≥ lipid, mucolytic, surfactant, growth factor > polymer. Protein production in the lungs ranged from 10 times below the DNA control (≈16 pg) using the polymers (≈1.5 pg) to ≈125 times greater than the control using the permeation enhancer (≈2050 pg). The transfection capabilities of the majority of additives was low. The enhancing effects of sodium glycocholate were dose-dependent and perhaps associated with the critical micelle concentration. Although the bile salt was the most successful of the tested compounds, it resulted in significant mortality when used at concentrations greater than 1% w/v.
Conclusions. The results suggest that transfection can be significantly enhanced by additives such as NaGC but some toxicity may be unavoidable.

KEY WORDS: DNA; excipients; gene therapy; luciferase; lungs; pulmonary drug delivery; sodium glycocholate.

INTRODUCTION

The *in vivo* transfection of lung tissue after administration of genes to the lungs has been achieved by a variety of means (1–10). Vector systems have included liposome complexes (2,3), adenoviruses (4–6), adeno-associated viruses (7) and retroviruses (8,9). These demonstrations of transfection and production of protein illustrate the promise that gene therapy may have in the future. Presently however, many crucial problems need to be addressed before gene therapy in general, and specifically gene therapy of the lungs, can prove to be a realizable goal. Viral approaches for example, are likely to be severely limited due to immunogenicity especially where repeat dosing is expected. Furthermore, substantial formulation, scale up and manufacturing problems are likely to be encountered. Non viral techniques, although less likely to cause a host reaction (10), have so far demonstrated poor transfection efficiency *in vivo* and large doses of DNA and lipid are necessary to detect even minute levels of protein after instillation in the lungs (3). To compound this issue, aerosol delivery to animals and humans is a highly inefficient process and 80 to 90% of the starting material can be wasted irrespective of the inhalation device employed. To solve the problem requires that large quantities of inexpensive DNA plasmid be readily available and/or a formulation must be found that dramatically improves protein production *in vivo*.

In this text, methodology is described to produce large quantities of purified DNA. Using this material studies were directed toward improving non-viral *in vivo* transfection in the lungs through the use of additives. To serve as a model, the reporter plasmid containing the luciferase gene was constructed and used in all studies. Formulations were subsequently prepared by combining plasmid, in a solution or powdered form, with various additives ranging from lipids to bile salts.

MATERIALS AND METHODS

Plasmid Construct

pGL2CMV, a luciferase expression plasmid vector driven by the human cytomegalovirus promoter, was constructed from pGL2 basic, a firefly luciferase expression vector (Promega, Madison, WI) and CMVFF3, a human cytomegalovirus containing vector (Fox Chase Cancer Center, Philadelphia, PA). Briefly, the plasmid pGL2 basic was linearized with Xho1 (Boehringer Mannheim, Indianapolis, IN) (at base number 33 in the polylinker) and blunted with Klenow (New England Biolabs, Beverly, MA). Similarly, the hCMV promoter was removed from the CMVFF3 plasmid with HindIII and BamH1 (Boehringer Mannheim) and blunted with Klenow. The pGL2CMV was subsequently prepared by ligating the hCMV fragment into the linearized pGL2 Basic plasmid. The orientation of the hCMV promoter in the plasmid was confirmed by a restriction digest of the Sma1 site in the polylinker of the pGL2 basic plasmid and the Nco1 site at the 5′ end of the hCMV fragment. Fragments were run and checked on 1% agarose gels (Seakem LE; FMC, Rockland, ME) @ 100 v, 50 mA for 1 hr (Bio-rad mini sub cell and Bio-rad Power Pac 300; Bio-rad Labs, Hercules, CA).

DNA Preparation and Methodology

The spray-drying procedure used to prepare powders typically consumed a minimum of 50 mg of DNA for every batch produced, hence methodology was required to produce large quantities (≥0.5g) of DNA to satisfy the formulation requirements. Cell paste was grown in a 10L fermentor to an optical density >100. Bacterial hosts were grown in Terrific broth supplemented with 100 ug/ml ampicillin. Eight hundred grams of the resulting cell paste was added to 25 L of resuspension buffer (100 μl/ml RNase A, 50 mM Tris base, and 10 mM EDTA at pH 8.0). Cells were mixed for 45 minutes at 4°C

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whereupon 25 L of lysing buffer (200 mM NaOH and 1% w/v SDS) was added and stirred for a further 20 min.. Lysate was then precipitated with 25 L of 3 M potassium acetate, pH 5.5 held at 4°C. The mixture was stirred for 60 min. 750 mg of filter aid 521 was added to the precipitated lysate to aid filtering through a pre-wetted CUNO 10SP filter (Cuno, France) at a flow rate of 2 L/min. Back-pressure was monitored and held at <35 psig. The DNA was precipitated from the filtered lysate with 0.8 volumes of isopropanol (4°C) and was then 'dead-end-screen' filtered onto a Sartopure GF 1.2 μm filter (Sartorius, Edgewood, NY) at a flow rate of 500 ml/min.. After loading, DNA was back-flushed off the filter with 10 L of loading buffer (10 mM Tris-HCL, 1 mM EDTA, 50 mM NaCl, pH 8.5) at a flow rate of 250 ml/min. The time required to back-flush was determined by monitoring the UV absorbance at 260 and 280 nm. Once absorbance dropped to background, flow was halted and the DNA was removed for purification.

DNA Purification and Characterization

DNA separation was carried out on a Biopilot FPLC (Pharmacia Biotech Inc., Piscataway, NJ) in a BPG 200/500 column (Pharmacia Biotech) using Q Sepharose high performance anion exchange gel (Pharmacia Biotech). Briefly, the DNA was loaded onto the column at 13 cm/hr. The excess debris was subsequently washed through the column with 10 column volumes (CV) of the loading buffer. The loading buffer was then exchanged with a 'low-salt' buffer (10 mM Tris-HCL, 1 mM EDTA, 0.6 M NaCl, pH 8.0) using a 5 CV linear gradient. DNA was eluted over a 20 CV linear gradient with 'high-salt' buffer (10 mM Tris-HCL, 1 mM EDTA, 0.9 M NaCl pH 8.0) at 13 cm/hr. The DNA eluted at approximately 0.73 M NaCl. A commercial endotoxin (LPS) removal kit (Qiagen, Chatsworth, CA) was used to remove bacterial endotoxins from the DNA. The resultant 'clean' material was then screened for LPS using the limulus amebocyte lysate (LAL) test (11).

Representative samples from each lot of DNA were run on 1% agarose gels to insure the DNA was intact. This was established by comparing with DNA control samples obtained from mini-prep techniques that had been tested *in vitro* in Cos-7 cells and were known to result in transfection.

DNA Formulations

lipid-DNA DNA-Lipid mixtures were prepared with lipid concentrations of dioleoyl phosphatidylethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) to [N-(N',N'-dimethyl aminoethane)-carbamoyl] cholesterol (DC Chol) (1:1 mole ratio). The DC Chol was synthesized and the liposomes were formed as described by Gao and Huang (12). The DC Chol was characterized by thin layer chromatography (TLC) and infra red spectroscopy (IR). For TLC, a mobile phase of 65:35 $CHCl_3$:MeOH by volume was used and samples were identified with iodine vapor and ninhydrin. A single spot was resolved indicating that the lipid was intact. For IR (Perkin Elmer 1600 series; Norwalk, CT), a nugol mull was prepared and scanned from 450–4500 cm^{-1}. To form liposomes, the DOPE and the DC Chol were combined in chloroform and dried over nitrogen gas. Further drying was performed for 3 hr in an evacuated desiccator. The lipid film was subsequently hydrated with distilled water over a period of 2 hr. During this hydration period, the dispersion was sonicated at intervals of 5 min. (15 min total) using an ultrasonic water bath. The dispersion was allowed to cool on ice between each sonication period. DNA, at the appropriate amount, was mixed in solution with the formed liposome dispersion within 30 min. of dosing cells or animals.

DNA powder. Spray-dried powders were prepared using a modified Buchi 190 spray-drier as described elsewhere (13). Solutions containing 0.2% w/v plasmid and 1.8% w/v trehalose in water were atomized at a rate of 1.5 ml/min into the drier using an atomization air pressure of 70 psig (10 Lair/min) and a drying air-volume of ≈600 L/min. Inlet and outlet air temperatures were 135 and 91°C respectively. The dew point of the drying air was ≈−75°F. The resulting powder was assessed for size and morphology using centrifugal photosedimentation (Horiba CAPA 300; Horiba Inc., Irvine, CA), and scanning electron microscopy (JSM 5200; Jeol Inc. Peabody, MA). The stability of the plasmid after reconstitution of the powder in water was checked using agarose gels as described above.

DNA and Additives

Besides lipid, 200 μg DNA was combined with a number of materials before dosing animals as described in Table 1. The bile salt sodium glycocholate (NaGC) was tested using a range of concentrations in water (200 μl total dose volume). Sodium deoxycholate at a 1% w/v concentration resulted in significant mortality in animals and therefore testing was discontinued.

Table 1. Treatment Data[a]

Compound	n	Dose[b] mg/ml	Dose[b] mg/kg[c]	Mass ratio
DNA	17	1.0	0.8	—
trehalose powder[d]	10	—	7.2	1:9
NaGC	6	100	80.0	1:100
	5	10.0	8.0	1:10
	5	5.0	4.0	1:5
	5	1.0	0.8	1:1
	5	0.1	0.08	10:1
KGF[e]	5	10.0	10.0	—
EGF	5	5.0	5.0	—
Tween 80	5	10.0	8.0	1:10
DC Chol:DOPE[f]	6	8.0	6.4	1:8
N-acetyl cysteine	6	100	80.0	1:100
β-cyclodextrin	9	10.0	8.0	1:10
PVAVAM 14%[g]	4	35.2	8.8	1:44
PVAVAM 6%	4	35.2	8.8	1:44

[a] A total of 200 μg DNA was admixed with additive in water within 30 min. of dosing the animals.

[b] A total volume of 200 μl was dosed to each animal unless stated otherwise. With the exception of the first row regarding DNA alone, the data refers to the additive only.

[c] Mean rat weight was ≈ 250 g.

[d] Amount of sugar (1.8 mg) combined in the spray dried particles containing the DNA.

[e] Rats dosed 24 hr before dosing DNA with 250 μl of 10mg/ml KGF or 5 mg/ml EGF.

[f] Liposomes formed in a 1:1 mole ratio.

[g] Poly (vinylalcohol-co-vinylamine) (29) was dosed in a total volume of 250μl. The PVAVAM molecular weight was <5000 for these studies.

Keratinocyte growth factor (KGF), (Amgen manufacturing) and epidermal growth factor (EGF) (Sigma) were administered 24 hr before dosing the plasmid alone. Experiments were also conducted *in vivo* to compare transfection of DNA before and after treatment to remove LPS.

In Vitro Gene Transfer

Cos-7, an SV40 virus transformed monkey kidney cell line, was obtained from the American Type Culture Collection (ATCC, CRL 1651) and maintained in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% v/v FBS and 100 units/ml penicillin and 100 ug/ml streptomycin (Gibco BRL). For transfection studies, the Cos-7 cells were allowed to grow to ≈80% confluency in a 6 well plate (Falcon Ware, Becton Dickinson, Franklin Lakes, NJ). Cells were then transfected with various lipid types in combination with DNA. The magnitude of transfection, in these studies, was quite similar with each lipid (data not shown). Consequently, the majority of experiments were conducted with DC Chol:DOPE (1:1) as it was readily synthesized in quantity and was one of the least toxic of the tested lipids. A total of 1 μg of the test plasmid, pGL2CMV, or control plasmid pCMV-β-Gal, was added to cells after mixing the DNA with lipid. The complex was added to an appropriate volume of medium (Opti-mem serum free medium, Gibco BRL) and 1 ml of the mixture was added to each well. After 24 hr the media was removed and 300 μl of lysis buffer was added to each well. Cells were incubated for 5 min and a 50 μl sample was then assayed using a commercial luciferase assay kit (Enhanced luciferase assay kit (1800K; Analytical Luminescence Labs., San Diego, CA). Luciferase was detected using a luminometer (Monolight 2010; Analytical Luminescence Labs) with samples being read for 10 seconds.

In Vivo Gene Transfer

Adult male rats (Spague-Dawley; approximate weight 250 g) were used for all *in vivo* experiments. Animals were quarantined for 1 week before being released for experimental use. Chow and water was provided *ad libitum*. The animals were dosed via intratracheal instillation (IT) or intratracheal insufflation (IF). The rats were initially intubated with the teflon sheath of a Quik-Cath 18G 2″ catheter (Baxter Health Care Corp., McGaw Park, IL). To instill, the dosing solution was dispensed via a 20 G 4″ pipetting needle inserted into the catheter. To insufflate, a 'tube' containing 1 to 2 mg of powder (see above) was lodged inside the barrel of the catheter. A 5 ml syringe was then attached to the Luer end of the catheter and a 4 ml air-bolus was used to force the powder into the lungs. The majority of experiments were performed using the instillation technique. Time course (24, 48 and 72 hr) and dose response (50, 200 and 500 μg DNA) studies were conducted using the DNA plasmid alone. In subsequent studies, using the different formulations, a nominal dose of 200 μg DNA was administered to rats and they were sacrificed 24 hr post-dose.

Following euthanasia, the chest wall of the rats was opened and the lungs were resected *en bloc*. The pulmonary circulation was perfused via the pulmonary artery with 15–20 ml buffer (136 mM NaCl, 5.3 mM KCl, 5.6 mM Glucose, 10mM Hepes buffer, and 3 mM $NaPO_4$) gravity fed with 40 cm water pressure until clear of blood. The lungs were then dissected into 2 parts. The 'left' side included the left and mediastinal lobes and the 'right' side included the right upper, middle and lower lobes. The lung lobes were homogenized (PT-MR 3000 homogenizer; Polytron, Kinematica, Littau, Switzerland) in 2 ml lysis buffer (0.1 ml potassium phosphate pH7.8, 1% w/w Triton X100, 1 mM DTT and 2 mM EDTA; Analytical Luminescence Labs) for 30 seconds at 25000 RPM. The homogenate was centrifuged at 15,000 RPM (Tomy MTX-150 centrifuge; Tomy Tech, Palo Alto, CA) at 4°C for 5 min.. A 50 μl aliquot was taken from each sample supernatant and assayed for luciferase as described above. An estimate of luciferase recovery from lung homogenate was determined by spiking known amounts of the protein into the trachea of dissected lungs and then following the same homogenization procedure. Assay values were compared with spiked values. Only some 10% of protein is recovered from the tissue samples. The experimental results to be shown are *not* adjusted for this estimated loss.

mRNA Isolation

To confirm transfection and gauge how long expression levels were maintained in the lungs, mRNA was isolated after dosing with a 1% w/v NaGC and 200μg DNA mixture as described earlier and sacrificing animals at 12, 24 and 48 hr. Lungs were immediately removed and frozen in liquid nitrogen. After thawing (2 min.) the tissue was homogenized as described above. Total RNA was then isolated from lung samples (RNeasy total RNA purification kit, Qiagen) and the luciferase mRNA was amplified by reverse transcription polymerase chain reaction (RTpcr) using a GeneAmp EZ rTth RNA PCR kit (Perkin Elmer, Norwalk, CT). Oligonucleotide primers (Amgen, Boulder, CO) used for RTpcr were 5′ GGAACCTTACTTCTGTGG TGTG-3′ and 5′CTTGGGGTCTTCTACCTTTCTC-3′. The RTpcr product was run on a 4% agarose gel (Nusieve 3:1 agarose gel, FMC, Rockland ME).

Toxicity

To evaluate the effects of the intratracheal instilled DNA in conjunction with 1% w/v NaGC, lung lavage and lung histology were performed 24 hr after dosing. After cannulating the trachea with an 18 G needle, 5 × 3 ml washes were lung lavage was performed with Dubellco's phosphate buffered saline (Gibco BRL) through a 5 cc syringe (5 × 3 ml washes) and cytospin slides were prepared as described elsewhere (14). Samples for histological analysis were also prepared by first cannulating the trachea as above, then perfusing the lungs with ≈2 ml of zinc-formalin buffer (Baxter Health Care) using a pressure head of ≈20 cm water. The lungs were then fixed in 50 ml zinc-formalin for 6 hours. After embedding and sectioning, slides were mounted and stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

Charateristics of the Purified DNA Plasmid

Agarose gels and anion exchange chromatography demonstrate that the plasmid is resolved as several bands and peaks, with one dominating, in the gels and chromatograms respec-

tively (Fig 1). A 'denatured' form of the DNA, induced by enzyme nicking is shown in lane 2 of Fig 1a illustrating open-circular DNA and perhaps the presence of some genomic DNA. Open-circular DNA is also resolved as a fronting-peak in the chromatogram (15, 16). Genomic DNA may also be represented on the chromatogram as the shoulder on the back-side of the main peak. The fact that the 'purified' DNA causes transfection *in vitro* and *in vivo* as seen below suggests that the majority of plasmid is 'biologically active'.

In Vitro Gene Transfer

The optimal lipid:DNA mass ratio for the DC Chol:DOPE lies between mass ratios of 20:1 to 50:1 with no significant transfection apparent at ratios less than 15:1 (Figure 2). Higher ratios than 50:1 cause a reduction in transfection due to toxicity. The greatest quantities of luciferase are apparent after 24–48 hours. The *in vitro* analysis also indicates that reconstituted spray-dried powder is capable of transfection after mixing with the lipid at a ratio of 40:1 (Figure 2). However there is a reduction in transfection suggesting that some biological activity is lost during spray-drying or during reconstitution of the plasmid. The DNA does not cause luciferase production alone. DNA that was not treated for LPS removal did not cause a substantial increase in transfection compared with treated plasmid.

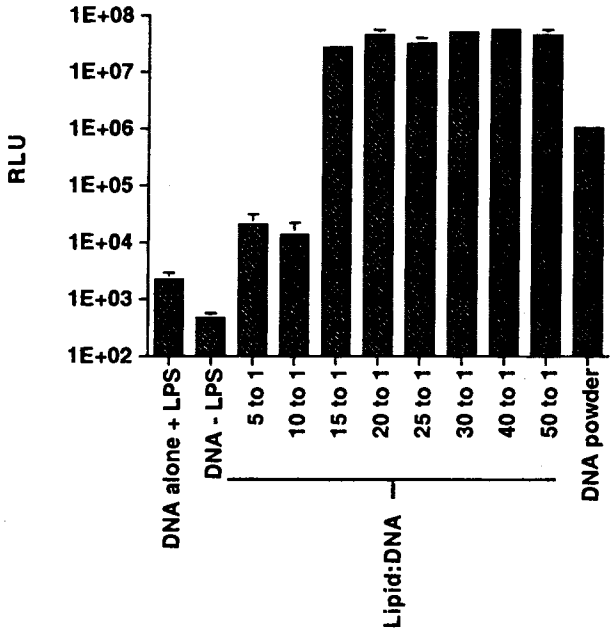

Fig. 2. The *in vitro* transfection of DC Chol:DOPE 1:1 with DNA at different mass ratios in Cos-7 cells. The last column also shows that reconstituted and spray-dried DNA mixed with lipid in a ratio of 40:1 also results in transfection of the cells. Data shown is the mean ± sem ($n \geq 5$).

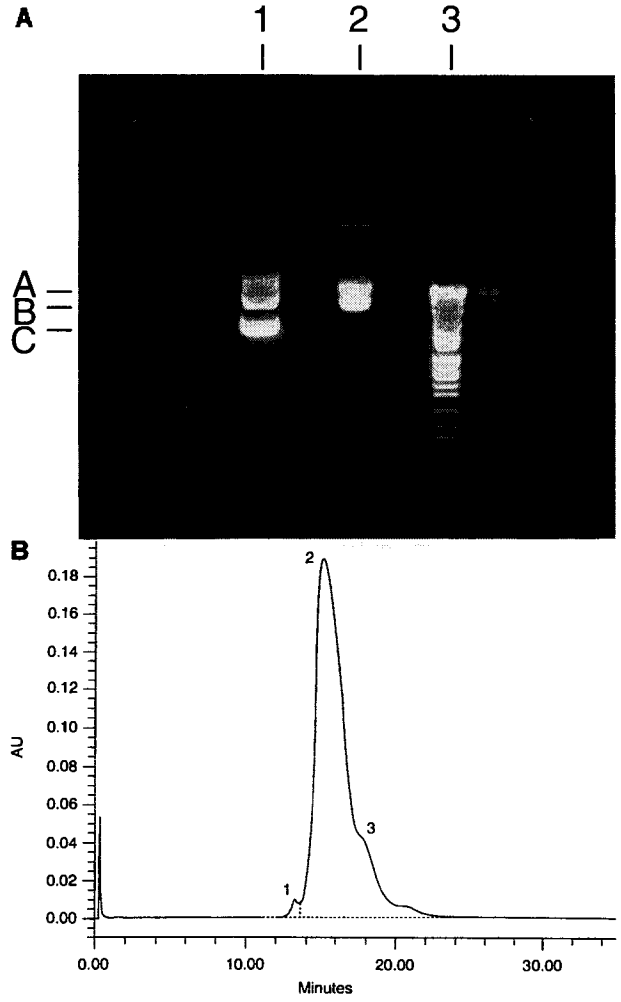

Fig. 1. A. An agarose gel illustrating, from left to right, 1—the supercoiled DNA plasmid [C]; 2—a denatured sample showing the open-circular form of DNA [B] together with a band possibly showing genomic DNA [A]; 3 molecular weight standards. The standards range in size from several hundred bp to ≈12 Kbp. B. An anion exchange chromatogram showing the 'purified' form of the plasmid. The chromatogram also illustrates the open-circular form of DNA [1] and a main peak [2] of supercoiled DNA. The shoulder on the back-side of the peak [3] possibly represents genomic DNA.

In Vivo Gene Transfer

In contrast with the results obtained *in vitro,* DNA that is not treated to remove LPS causes a significant increase in luciferase production compared to treated DNA ('t'-test, $p < 0.05$; Figure 3). Lungs dosed with untreated DNA exhibit macroscopic hemmorhaging and edema. These observations are not apparent in treated preparations. The addition of LPS [200 μg and 1mg/kg (250 to 2500 EU/kg)] to DNA just before intratracheal instillation does not restore transfection levels to those observed with untreated DNA (Figure 3). One plausible explanation for these results is that contaminants, in addition to LPS, in the untreated DNA are influencing the transfection levels in the lungs. If true, the removal kits are capable of removing these other contaminants together with the LPS.

The dose-response data using 'purified' DNA alone obtained 24 hr after dosing indicated that a dose of 200 μg DNA is capable of producing detectable levels of luciferase ($50\mu g = 8.9 \times 10^3 \pm 7.3 \times 10^2$ [n = 5]; $200\mu g = 3.3 \times 10^5 \pm 5.8 \times 10^4$ [n = 17]; $500\mu g = 1.4 \times 10^4 \pm 8.2 \times 10^2$ [n = 4]. mean ± sem). A subsequent time course study also suggested that 24 hr is optimal for detection of the luciferase although the protein is detectable for at least 72 hr. A 'baseline'

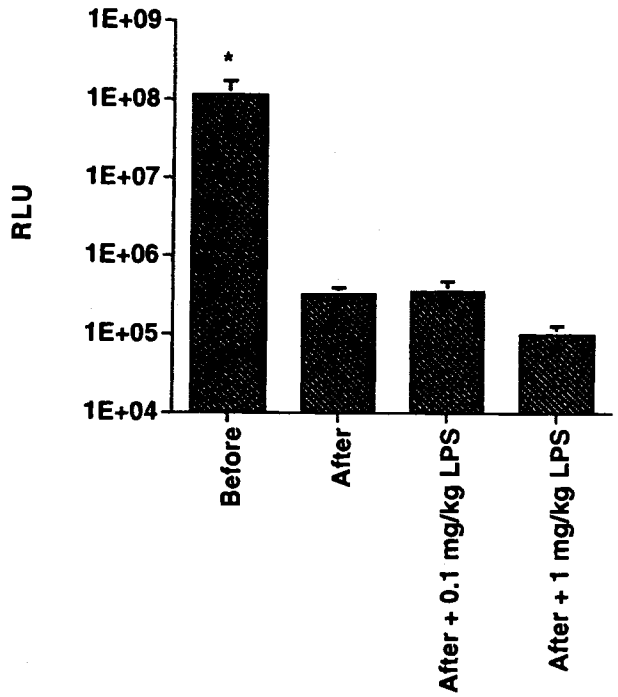

Fig. 3. The effect of DNA 'purity' on *in vivo* transfection. Before reducing the quantity of endotoxin and other protein contaminants from the plasmid using the Qiagen endotoxin-removal-kit, an 'artificially' high and variable luciferase production is noted in the lungs. This value is reduced an order of magnitude after using the kit. Endotoxin does not appear to be singularly responsible for the increased transfection. Adding endotoxin to purified DNA in quantities as high as 1mg/kg does not reproduce the values noted before the purification step. Data shown is the mean ± sem. The 'before' and 'after' treated DNA is significantly different by Students t-test (*indicates $p < 0.05$).

level of protein is always found after dosing with the plasmid alone. Of note is the fact that dosing the plasmid-complexed with lipid (DCchol:DOPE 1:1) does not increase transfection levels at the 8:1 lipid:DNA mass ratio employed (lipid-DNA = $3.3 \times 10^5 \pm 1.2 \times 10^5$; DNA = $3.3 \times 10^5 \pm 5.8 \times 10^4$ RLU, p = NS). However, the amount of lipid used was not optimal with respect to the results obtained *in vitro*. Ratios of 20:1 were found to be impractical to dose as there are limitations in the volume of solution that can be dosed to the animals without causing asphyxiation (≈400 μl), and limitations in the concentration of lipid and DNA that can be used without causing precipitation of the complex. This is one area where formulation efforts may in the future prove rewarding but there is no guarantee that a correlation between the *in vivo* and *in vitro* data will exist. Subsequent studies were directed to finding alternative formulations, without lipid, that would enhance transfection.

mRNA Detection

The presence of luciferase mRNA is detectable in lung tissue after 12 hr and up to at least 48 hr after dosing the animals (Figure 4). The amplified mature mRNA differs from luciferase DNA by approximately 60bp due to an intron that is excised during post transcriptional processing. This provided a check that the mRNA and not luciferase DNA contamination was being detected on the gels. The presence of the mRNA confirms that the luciferase protein has been produced within lung cells and also indicates that the protein is being continuously produced and that its presence is not simply due to a prolonged half-life in lung tissue.

In Vivo Effects of Additives

To determine if the cell-cycle stimulants KGF and EGF would influence transfection, rats were preteated 24 hr before dosing the plasmid. KGF was dosed at 10 mg/kg at which levels the protein is known to induce hyperplasia of alveolar type II cells in rats (17). EGF was dosed at 5 mg/kg and is known to stimulate growth in lung epithelial cells (18). Neither protein however causes a significant increase in the appearance of luciferase vs. animals pretreated with buffer followed by DNA plasmid (Figure 5).

Bile salts are known penetration enhancers that can increase the absorption of macromolecules across membranes (19) and also act as protease inhibitors (20). We hypothesized that these natural cholesterol-based chemicals might influence membrane bilayer stability and thus could influence cellular uptake of DNA. NaGC at a dose of 1% w/v causes a marked increase in luciferase production (125×) vs. controls. The effects of the bile salt are also dose-dependent as shown in Table 2. The amount of protein being assayed after using 1% w/v NaGC is around 2 ng, assuming 18000 RLU ≈ 1pg of protein (approximate conversion given in luminescence assay kit). As stated in the methods, the assayed amount represents only ≈10% of the luciferase in the tissue, hence levels could be as much as 20 ng. This suggests that therapeutic amounts of protein are feasible by inserting genes of potent cytokines into the plasmid. Unfortunately, higher NaGC concentrations results in mortality. This could be due to the increase in viscosity of the concentrated NaGC preparations and perhaps some animals were asphyxiated as most deaths occurred shortly after dosing. In those animals examined for acute toxicity, the response was variable. Cytospins demonstrated the presence of granulocytes in 2 of 3 animals indicating that some inflammatory response occurs but certainly nothing resembling the extreme response that is observed after endotoxin administration which in contrast does not elicit substantially increased levels of luciferase. Hematoxylin and eosin slides from 3 other animals are characterized by compartmentalized but mild to heavy inflammation. Some tissue regions are almost normal; in others, heavy protein exudation and hemmorhaging is evident. Type II cell hyperplasia and some macrophage granuloma formation is also identifiable. Control animals receiving NaGC alone exhibit similar pathology whereas animals receiving only the plasmid DNA do not show any abnormal histological features. This result indicates that the NaGC is responsible for the observed tissue damage. The variable response may be due to the inhomogeneous deposition of fluid after intratracheal instillation. It is not yet known whether DNA transfection is dependent on the toxic response to the bile salt.

Some further experiments, that are not definitive but do provide some further insight into the use of NaGC, were completed. Doses of NaGC and DNA were administered separately to the lungs two hours apart. One group received the NaGC first and the other the DNA first. In both cases, transfection levels are reduced to almost background levels. These results

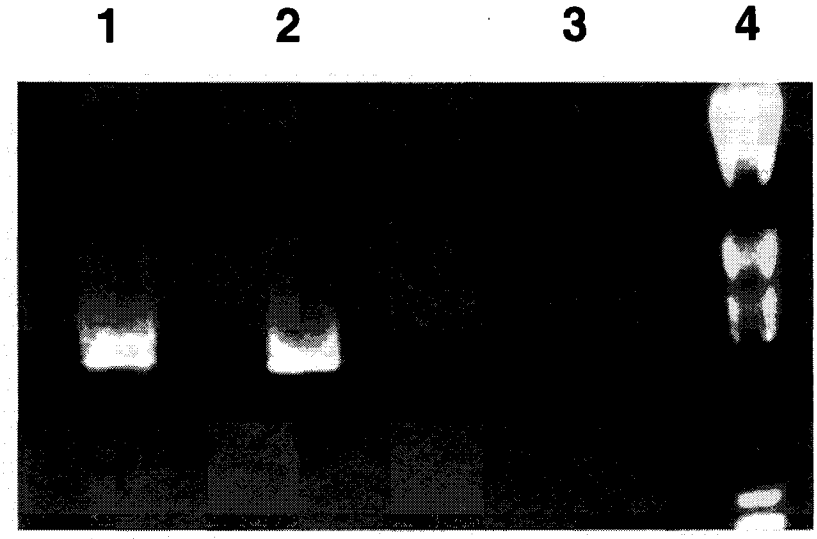

Fig. 4. A 4% agarose gel illustrating luciferase mRNA (RTpcr product) isolated from lung tissue. The lanes from left to right depict (1) mRNA 24 hr post-dose and (2) mRNA 48 hr post-dose (3) a control lung tissue sample 24 hr after treating with buffer (4) the molecular weight ladder.

suggest that in order to be effective the DNA and NaGC must be instilled together or concomittantly.

The reason for the dramatic increase in transfection within the concentration range of 0.1 to 1.0% w/v may relate to the formation of micelles. The critical micelle concentration (CMC) of NaGC in aqueous solution is around 9 mM or 0.45% w/v (21). However, Tween 80 was employed at concentrations well above the CMC of the surfactant, yet no change in transfection occurred when Tween 80 was mixed with the DNA and then dosed to animals. It would appear therefore that the nature of the NaGC-DNA complex may be an important aspect of *in vivo* performance.

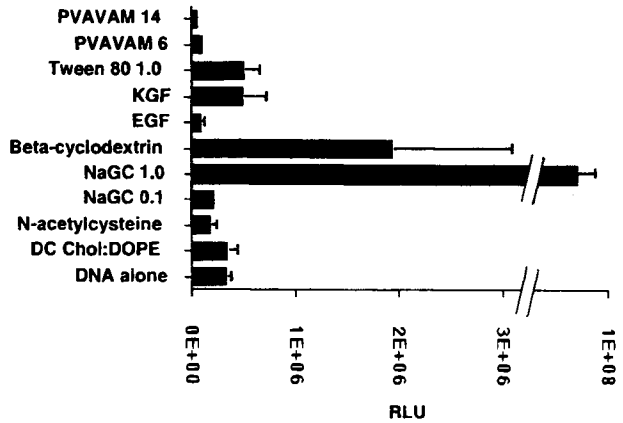

Fig. 5. The effect of additives on the transfection of pCMVlux in the lungs. With the exception of the cytokines EGF and KGF where rats were pretreated 24 hrs before dosing the DNA, all others were formulated with DNA before instillation or insufflation as described in the text. Data shown is the mean ± sem. The 'n' for each additive is given in Table 1. The amount of luciferase protein is represented by relative light units (RLU's; 1 pg luciferase = 18000 RLU and each rat lung [left and right] contain ≈60 mg total protein).

Although NaGC is a known protease inhibitor, it is unlikely that the reason for the increase in luciferase presence is due to inhibition of protease activity in the extracellular domain, since luciferase is apparently not secreted but is restricted to intracellular peroxisomes (22).

As an alternative to the bile salts, β-cyclodextrin was also chosen as a potential enhancer. This category of molecule has been used primarily as a 'solubilizing' agent for drugs of low aqueous solubility (23). They also have been found to enhance uptake of albuterol from the lungs and thus possess penetration enhancement capabilities (24). A dose of 5 mg/kg results in ≈6× increase in luciferase production. This is not as significant a change as observed with NaGC. On the other hand there was no apparent toxicity associated with this dose-level.

N-acetyl cysteine is a known mucolytic agent. Given the viscous nature of airway mucus and DNA, it was thought that the mucolytic agent might improve access of the plasmid to cell surfaces. However, a dose of 10% w/v, adjusted to pH 7.0 (normal for clinical use), with the DNA does not increase luciferase levels above baseline.

Table 2. Influence of NaGC Concentration on DNA Transfection in the Lungs

Concentration % w/v	RLU average	RLU sem
0	3.27×10^5	5.80×10^3
0.01	8.15×10^4	2.87×10^3
0.1	2.16×10^5	2.64×10^3
0.5	7.10×10^6	2.44×10^6
1.0	3.65×10^7	2.07×10^6
10[a]		

[a] A 10 %w/v NaGC concentration resulted in mortality in most rats. Experiments were therefore discontinued.

Poly(vinylalcohol-co-vinylamine) polymers (PVAVAM) (25) of differing positive charge (6% and 14% amine residues per mole) were combined with the DNA in the hope that they might further condense the plasmid and perhaps improve transfection beyond levels that have been obtained with cationic lipids. The results however indicate quite the opposite and a significant reduction in transfection occurs with the PVAVAM 14% vs. DNA alone ($p < 0.05$). This again suggests that the nature of the DNA-additive complex is an important determinant for *in vivo* performance.

Finally, DNA powder was formulated by spray-drying in conjunction with trehalose. It was hoped that this change of state might provide a means to concentrate DNA molecules within localized regions of the airway and alveolar epithelium during dissolution of deposited particles. This in turn, might increase the cellular uptake of DNA simply on the basis of a large number of DNA molecules being present in the vicinity of the cell surface.

The volume median size of the spray dried powder is 5.14 μm as measured by centrifugal sedimentation. SEM results confirm the particle dimensions and show that the presence of the DNA is affecting the morphology of the powder. This has been seen with proteins combined with trehalose (13) and the particles exhibit a rough and indented surface. A substantial quantity of the plasmid remains supercoiled as found on agarose gels after reconstitution of the DNA in water or PBS (data not shown). The reconstituted powder, when instilled into animals at a dose of ≈200 μg plasmid alone, results in significant luciferase production albeit a levels lower than those achieved with the pre-spray dried material (Figure 6). This result correlates directly with the *in vitro* data. Unfortunately, no response to the insufflated DNA powder could be obtained (n = 10). This is probably due to the difficulty in administering sufficient powder as a bolus to the regions of the lungs where transfection is occurring. The distribution of insufflated powder in the lungs can differ considerably from that of an instilled dose and much of the powder will deposit in the trachea (13). When powder is insufflated and then immediately followed by an instilled dose of 200 μl of 1% w/v NaGC, luciferase levels are increased in some, but not all, tested animals (3 out of 6; Figure 6). This result nevertheless confirms that DNA powder can cause transfection in the lungs and also illustrates that much work remains to be done in improving the initial formulation as well as the insufflation technique. Insufflation with any drug-powder formulation is a significant challenge. This stems from the fact that a 'bolus' dose of powder must be dispersed, deagreggated and distributed into a small volume, of high surface area and almost 100% relative humidity at 37°C. Such conditions are not conducive to effective dosing and the answer to this problem probably lies in finding a means to aerosolize and deaggregate the powder in a small (<10 ml) volume just before inhalation: something that we have not been able to accomplish to date.

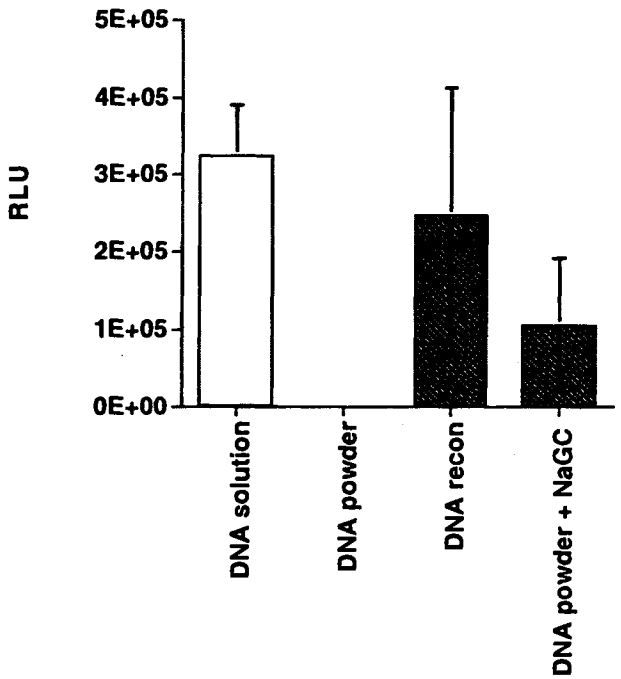

Fig. 6. The effect of an insufflated spray-dried pCMVlux:trehalose (1:9) powder on the transfection of lung tissue. The powder was administered directly, as a powder reconstituted in distilled water, and as a powder immediately followed with an instilled dose of 1.25 mg NaGC in phosphate buffered saline. For comparison, the results for DNA that was not spray-dried are shown (□). Data shown is the mean ± sem.

Another reason for the poor performance may relate to the viscosity of the DNA during hydration in airway fluids. If deposition of powder is predominant in the upper airways, much of the dose may not have sufficient time to solubilize and penetrate the mucus blanket, especially given the high viscosity of concentrated DNA, and may simply be expectorated via the mucociliary escalator which in the upper airways is clearing insoluble particulates at rates of nearly 1 mm/min. (26).

In summary, the results highlight some of the issues surrounding the preparation and dosing of DNA to the lungs but demonstrate that transfection can be achieved using DNA in conjunction with the appropriate additive. Along with efforts at the molecular and cellular level to enhance intracellular translocation of plasmid and increase protein copies, there are means whereby relatively simple formulations, that are readily scaleable to manufacturing levels, can also improve transfection. The particular use of bile-salts, opens a new area of investigation and although their use in the lungs may be limited due to toxicity, their use with injectable preparations into tumors for example may be acceptable. More importantly, the results imply that other less toxic derivatives might be developed once a better understanding of the mechanisms of action are obtained.

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