Enhanced Lung Gene Expression After Aerosol Delivery of Concentrated pDNA/PEI Complexes

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A major limitation of many self-assembling nonviral gene transfer formulations is that they are commonly prepared at relatively low component concentrations. While this typically has little impact on their use in cell culture, it can severely limit the progress of *in vivo* studies. In order to overcome this, we have developed a simple, scalable, pharmaceutically acceptable concentration method that has allowed us to increase the concentration of a commonly used pDNA/PEI formulation from 0.2 to >8 mg/ml plasmid DNA (pDNA). Crucially, the concentration method was found to have only minimal impact on the electrostatic properties or size of the pDNA/PEI particles. When delivered as an aerosol to the mouse lung, the concentrated pDNA/PEI formulations resulted in a 15-fold increase in lung reporter gene expression, with minimal impact in terms of inflammation or toxicity. Importantly, this performance advantage was replicated after aerosol administration to sheep lungs, with reporter gene expression being similarly ~15-fold higher than with the conventional pDNA/PEI formulation, and lung inflammation falling to background levels. These findings demonstrate that concentrated pDNA/PEI formulations offer increased aerosol gene transfer with decreased inflammatory sequelae, and represent a promising advance in the field of nonviral lung gene transfer. It seems likely that similar benefits might be achievable with alternative delivery routes and with other nonviral formulations.

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INTRODUCTION

Lung gene therapy is being investigated as a treatment approach for a range of acute and chronic respiratory diseases[1] as well as for immunization.[2] In the case of chronic disease, successful gene therapy is likely to require repeated application of gene transfer agents (GTAs) to the lung epithelium, and nebulization is likely to be an important delivery route in this regard.[3] Unfortunately, aerosol generation exposes GTAs to considerable mechanical stress and shear-sensitive molecules such as naked plasmid DNA (pDNA)[4] are rapidly degraded during nebulization, leading to a loss in biological activity.[5]

Complexation of pDNA with cationic lipids[6] or cationic polymers[7] can protect pDNA from shear forces during nebulization, and this has led to successful gene expression after aerosol delivery to the lungs of mice,[6] rabbits,[8] sheep,[9] and *Rhesus macaques*.[10] However, not all pDNA/polymer or pDNA/lipid complexes retain biological efficacy after nebulization;[11,12] to date, only one nonviral GTA, a cationic lipid formulation based on Genzyme lipid 67, has been successfully aerosolized to the lungs of patients as part of phase I clinical trials.[13,14]

One GTA that has received considerable attention in respect of aerosol delivery is polyethylenimine (PEI), a highly cationic polymer that efficiently condenses pDNA[15] and has been shown to be effective both in cell culture and *in vivo*.[16] Several forms of PEI are commercially available, with both 22 kd linear[17] and 25 kd branched polymers[18] demonstrating significant levels of gene expression in the lungs of mice when instilled as pDNA/PEI complexes. However, significant expression after aerosol delivery has so far been reported only with the 25 kd branched PEI polymer.[12,19,20] Aerosol delivery of PEI complexes to the lung is associated with minimal detectable toxicity[21] and, unlike many cationic lipid formulations,[22,23] pDNA/PEI complexes are not inhibited by lung surfactants.[24] Unfortunately, the development of clinically viable PEI formulations for aerosol gene therapy has not progressed because of the limited colloidal stability of pDNA/PEI complexes in solution. Unlike other nonviral GTAs that have been developed for aerosol delivery, for which stable aerosols containing over 2 mg/ml pDNA have been reported,[7,25] the preparation of pDNA/PEI complexes at concentrations in excess of 0.5 mg/ml is associated with precipitation and a loss of biological efficacy. Given the relative inefficiency of nonviral gene transfer, clinical applications for lung gene therapy are likely to require the delivery of relatively large doses of pDNA; this requirement emphasizes the need to address the issue of increasing the concentrations of

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formulations without compromising on stability. In this study we have investigated the use of ultrafiltration for generating concentrated PEI (cPEI) formulations. We have also evaluated the efficacy and toxicity of these formulations *in vivo* after aerosol delivery to the lungs of both mice and sheep.

RESULTS

Aerosol delivery of standard PEI formulations to the mouse lung

In order to evaluate gene expression from pDNA/PEI formulations, the luciferase expression plasmid pCIKLux was complexed with 25 kd branched PEI over a range of pDNA concentrations from 0.1 to 0.5 mg/ml and aerosolized to the lungs of mice. Although previous studies have demonstrated that aerosol delivery of pDNA/PEI formulations to mice is relatively inefficient, with only 0.2% of aerosolized material depositing in the lungs of individual mice,[26] robust luciferase activity was detected in the lungs of all the mice 24 hours after treatment. Expression levels increased with increasing concentration of the complex (**Figure 1a**), with maximal luciferase activity being achieved when using complexes formulated at 0.4 mg/ml. The formulation of complexes at a concentration of 0.5 mg/ml resulted in some visible precipitation of material and no further increase in luciferase activity. Attempts to prepare complexes at even higher concentrations resulted in the complete precipitation of pDNA/PEI complexes, and aerosol delivery of these formulations was not feasible. When mice were exposed to increasing volumes of pCIKLux/PEI aerosol, a dose-related increase in lung gene expression was observed (**Figure 1b**). These data indicated that aerosol delivery of concentrated pDNA/PEI complexes should lead to increased levels of gene transfer in the lung.

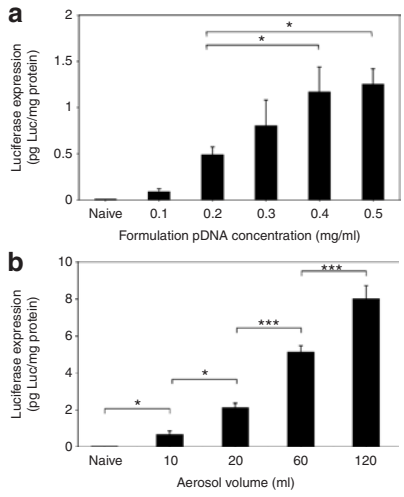

Figure 1 Aerosol delivery of pDNA/PEI formulations to the mouse lung. (**a**) Plasmid pCIKLux was complexed with polyethylenimine (PEI) (N:P ratio 10:1) at a range of plasmid DNA (pDNA) concentrations from 0.1 to 0.5 mg/ml and 10 ml of each formulation was aerosolized to the lungs of BALB/c mice ($n = 6$) with a predicted aerosol deposition of 0.2% (20 µl) per mouse. (**b**) pCIKLux/PEI complexes were prepared at 0.2 mg/ml and increasing volumes were aerosolized to the lungs of mice ($n = 6$). Luciferase activity in lung samples was determined 24 hours later. Data represent mean values ± SEM. * and *** represent statistical significance with $P < 0.05$ and $P < 0.0001$, respectively.

Concentration of pDNA/PEI complexes

In order to examine whether stable pDNA/PEI formulations can be prepared at higher concentrations, pDNA/PEI complexes were concentrated by ultrafiltration. Quantification of pDNA in the filter retentate demonstrated that pDNA/PEI complexes at concentrations >15 mg/ml could be generated in the absence of any visible signs of precipitation of the complex (**Figure 2**). However, these formulations were associated with measurable losses, with only 82 ± 2% of the starting material being recoverable after increasing the concentration to 8 mg/ml, as against 98 ± 5% recovery at 1 mg/ml. The rate of concentration of the complex was dependent upon the applied relative centrifugal force (**Figure 2a**), remained relatively unaffected by the concentrations of the initial complexes (**Figure 2b**), and varied significantly between complexes formed at different N:P ratios (**Figure 2c**). Minimal concentrations of complexes prepared at a ratio of 5:1 were observed, possibly associated with the increase in pDNA/PEI particle size reported for complexes at N:P ratios of 3 to 5.[27] Similar size-related effects have been reported after attempts to purify pDNA/PEI complexes at lower N:P ratios using size exclusion chromatography.[28] No pDNA was ever detected in the filtrate of pDNA/PEI complexes after ultrafiltration. However, significant amounts of "free" or uncomplexed PEI, amounting to 25–35% of the initial PEI dose, were removed from pDNA/PEI formulations during ultrafiltration and could be detected in the filtrate (**Figure 2d**). No precipitation of cPEI formulations was observed during ultrafiltration.

In order to investigate the effect of the concentration process on the physical properties of the final product, pCIKLux/PEI complexes (0.2 mg/ml; N:P of 10:1) were concentrated by ultrafiltration and samples were removed from the ultrafiltration cell at intervals. Electron microscopy showed that both standard PEI formulations at 0.2 mg/ml (**Figure 2e**) and cPEI formulations prepared at concentrations of up to 8 mg/ml (**Figure 2f**) appeared to contain spherical particles of ~100 nm in diameter with no evidence of particle aggregation at higher concentrations. Particle size and surface charge (ζ potential) were also determined using dynamic light scattering (**Figure 2g**). The increase in concentration of pDNA/PEI complexes from 0.2 to 8 mg/ml was associated with a small but significant increase in particle size (from 81.9 ± 2.1 nm to 124.9 ± 4.9 nm; $P = 0.001$) and surface charge (+28.8 ± 0.1 mV to +36.7 ± 0.1 mV; $P = 0.003$).

Gene transfer after delivery of cPEI formulations to the mouse lung

In order to assess the efficacy of cPEI formulations *in vivo*, complexes of pCIKLux/PEI at different concentrations were delivered to the lungs of mice by instillation or aerosol. Formulations were prepared by direct mixing (0.2 and 0.4 mg/ml) or by concentration (≥1 mg/ml). While instillation of pCIKLux/PEI formulations between 0.2 and 1 mg/ml resulted in similar levels of luciferase activity in the lung (**Figure 3a**), significant toxicity was observed at the highest dose, with three animals dying within 24 hours of delivery. Because of this finding, higher concentrations were not assessed by instillation. Overt indications of toxicity were observed in mice in the 24-hour period after instillation of pDNA/PEI complexes. These included hunching, pronounced piloerection,

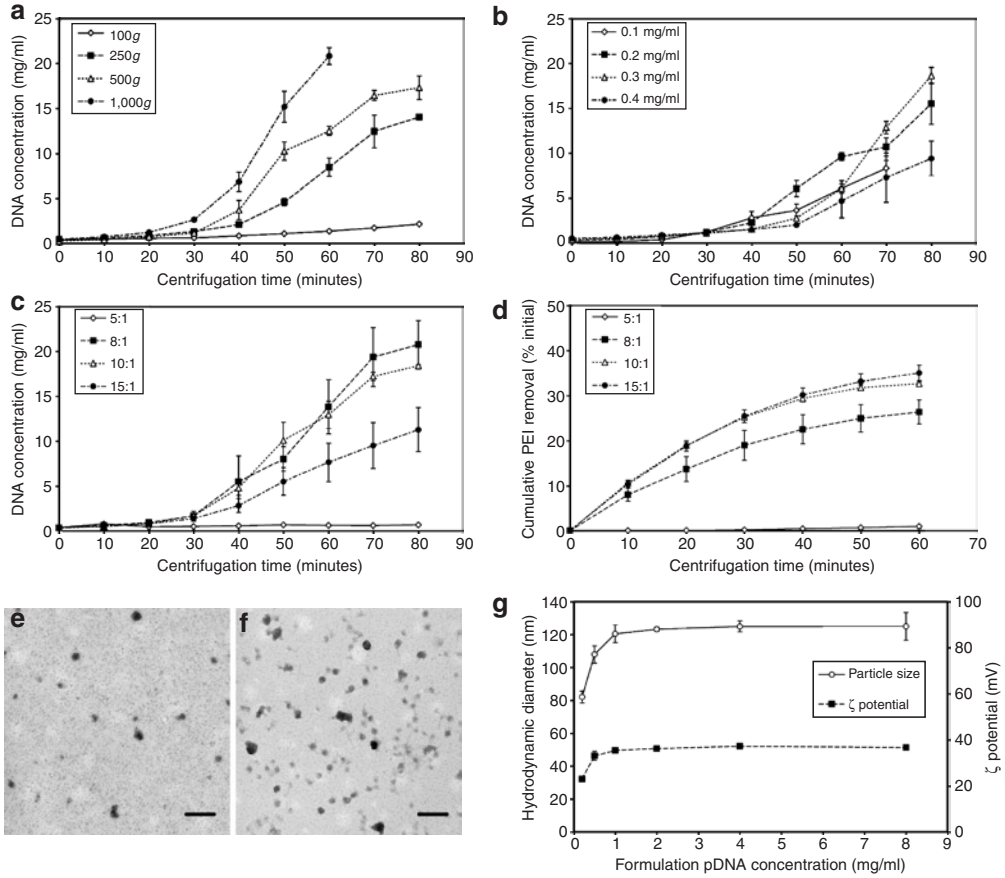

Figure 2 Concentration of pDNA/PEI complexes by ultrafiltration. Standard pDNA/PEI complexes (10 ml) were concentrated by ultrafiltration, and the concentration of plasmid DNA (pDNA) in the filter retentate was monitored. The effects of (**a**) relative centrifugal force, (**b**) starting concentration, and (**c**) N:P ratio upon the rate of complex concentration were examined. The amount of uncomplexed polyethylenimine (PEI) removed from pDNA/PEI formulations during ultrafiltration was also determined and (**d**) expressed as a percentage of the initial amount of PEI used for pDNA complexation. Electron micrographs of standard pCIKLux/PEI complexes prepared at (**e**) 0.2 mg/ml, and (**f**) after concentration to 8 mg/ml. Bar = 200 nm. (**g**) Particle sizes and surface charges of concentrated PEI formulations were determined using dynamic light scattering. The data represent mean values ± SEM for three separate samples in each group.

extended recovery times, and weight loss of 5–10% of initial body weight; these were not seen in naive or control mice instilled with sterile water (**Figure 3b**). In contrast, aerosol delivery of pDNA/PEI complexes at concentrations up to 8 mg/ml was well tolerated. Moreover, at the highest concentration, lung luciferase activity was ~15-fold higher than that achieved with standard 0.2 mg/ml formulations (**Figure 3a**).

An assessment of bronchoalveolar lavage fluid (BALF) for inflammatory markers revealed elevated levels of total protein in all the mice that had been instilled with pCIKLux/PEI complexes (as compared to naive mice) but this was not observed in mice exposed to aerosols (data not shown). Similarly, BALF from complex-instilled mice contained significantly more inflammatory cells than that from naive mice. The majority of the infiltrating cells were identified as neutrophils (**Figure 3c**), although small increases in both lymphocytes and macrophages were also observed (data not shown). In contrast, aerosol delivery of complexes resulted in no significant increase in BALF neutrophils, even when the highest concentration of 8 mg/ml was administered. No increase in cell infiltrate was observed after instillation or aerosolization of sterile water to control animals. Histological analysis of lungs from mice instilled with complexes revealed significant dose-dependent inflammatory changes (**Figure 3d**); mice that received either 0.2 or 0.4 mg/ml pCIKLux/PEI demonstrated prominent foci of interstitial inflammation and necrosis associated with the infiltration of large numbers of neutrophils and, to a lesser extent, lymphocytes (**Figure 4c–f**). Mice instilled with 1 mg/ml formulations developed a much more severe and extensive inflammatory response involving a large proportion of the lung. Widespread congestion, hemorrhage, and necrosis were observed in all the treated mice (**Figure 4g** and **h**). In contrast, aerosol delivery of pCIKLux/PEI formulations at all concentrations up to 8 mg/ml resulted in only minimal inflammatory responses involving mild hemorrhagic oedema in alveolar spaces and congestion of some alveolar capillaries (**Figure 4i** and **j**). Similar minor inflammatory changes were observed in control animals when sterile water was delivered by lung instillation or aerosol (data not shown).

GFP expression after aerosol delivery of PEI formulations to mouse lung

In order to investigate the distribution of gene expression and to identify cell types transfected after aerosol delivery, 60 ml of cPEI formulations containing 2 mg/ml pEGFP-N1 were aerosolized to

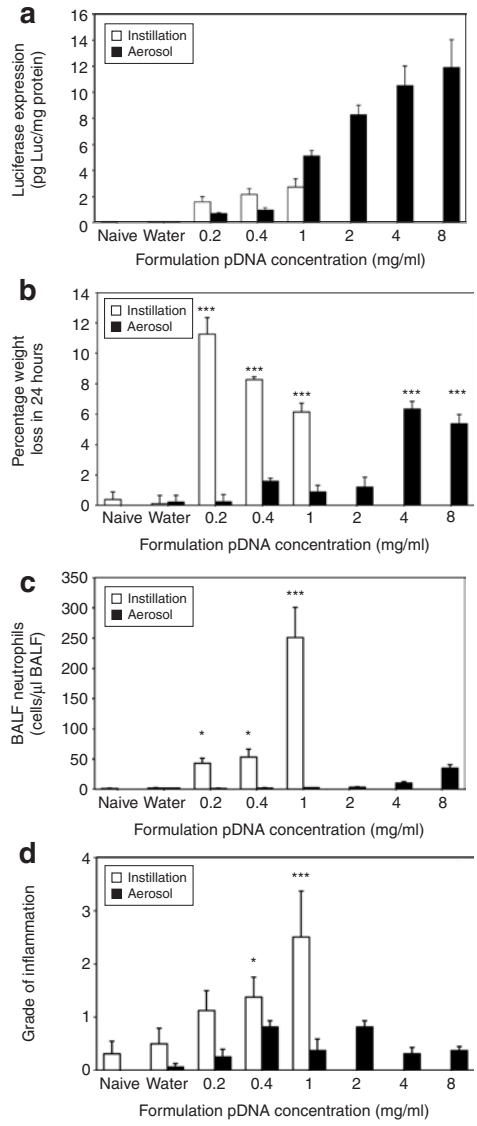

Figure 3 Delivery of concentrated polyethylenimine (cPEI) formulations to the mouse lung. Formulations of pCIKLux/PEI were prepared at concentrations between 0.2 and 8 mg/ml, and instilled into (100 µl), or aerosolized to (10 ml aerosol) the lungs of mice, the predicted aerosol deposition being 0.2% (20 µl) per mouse. (**a**) Luciferase activity in lung homogenates, (**b**) weight loss, (**c**) bronchoalveolar lavage fluid (BALF) neutrophils, and (**d**) lung histology were assayed 24 hours after delivery. The data represent mean values ± SEM for $n = 6$ animals in each group, except for weight ($n = 8$) and histology grading ($n = 4$). * and *** represent statistical significance with $P < 0.05$ and $P < 0.0001$, respectively. pDNA; plasmid DNA.

mice ($n = 6$). Green fluorescent protein (GFP)-positive cells were observed throughout the lungs of all the treated mice 24 hours after delivery, although the number of positive cells was relatively low at 6.4 cells/cm^2 of lung tissue. A proportion of GFP-positive cells detected in the mouse lung (11.2%) were located in the epithelium of the conducting airway, where many displayed characteristic apical cilia indicative of airway epithelial cells (**Figure 4m**). The remaining GFP-positive cells (88.8%) were detected in the lung parenchyma and were identified as primarily type I pneumocytes based on their size and morphology (**Figure 4n**). No GFP-positive alveolar macrophages were observed.

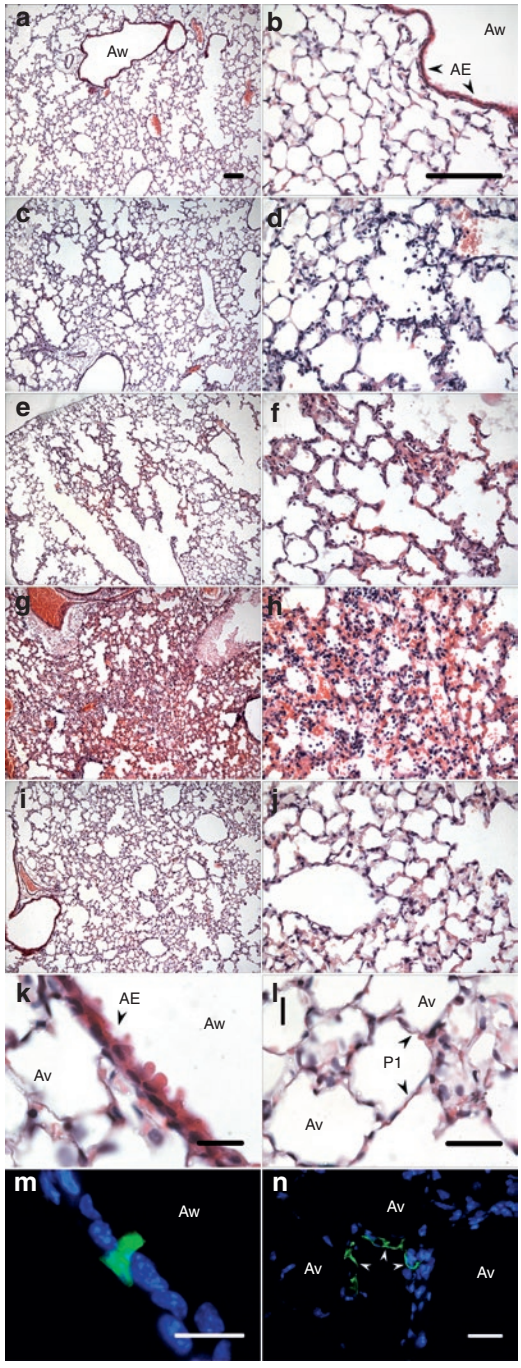

Figure 4 Histological changes after administration of polyethylenimine (PEI) formulations. Low- and high-magnification images of representative hematoxylin and eosin sections from the lungs of (**a**,**b**) naive mice, (**c**,**d**) mice instilled with 0.2 mg/ml, (**e**,**f**) mice instilled with 0.4 mg/ml, (**g**,**h**) mice instilled with 1 mg/ml pCIKLux/PEI, and (**i**,**j**) mice treated with pCIKLux/PEI aerosol at 8 mg/ml. Examples of conducting airways (Aw), airway epithelium (AE), and individual alveoli (Av) are indicated. Bar = 100 µm (**a**–**j**). Representative high-magnification images of (**k**) airway epithelia and (**l**) alveolar regions are shown. (**m**) An individual green fluorescent protein (GFP)-positive ciliated cell in the airway epithelium and (**n**) a single GFP-positive type I pneumocyte (P1) in the alveoli of mice treated with pEGFP-N1/PEI. The filamentous cytoplasm of a single P1 cell is indicated (white arrows). 4′,6-Diamidino-2′-phenylindole dihydrochloride–stained nuclei are shown in blue. Bar = 20 µm (**k**–**n**).

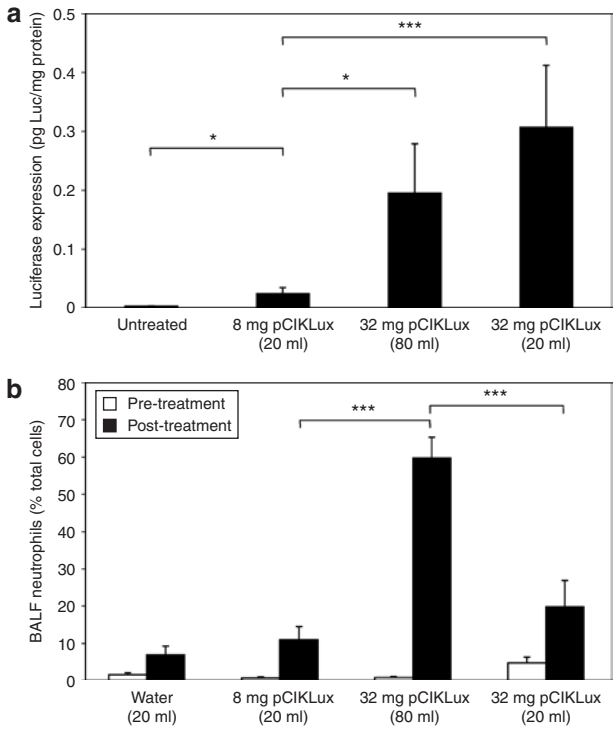

Figure 5 Delivery of polyethylenimine (PEI) formulations to sheep lung. Standard pCIKLux/PEI complexes prepared at 0.4 mg/ml (20 or 80 ml volumes) and concentrated pCIKLux/PEI complexes prepared at 1.6 mg/ml (20 ml) were aerosolized to the lungs of anesthetized sheep, and (**a**) lung luciferase activity and (**b**) bronchoalveolar lavage fluid (BALF) neutrophil numbers were quantified 24 hours later. The data represent mean values ± SEM for all groups. * and *** represent statistical significance with $P < 0.05$ and $P < 0.0001$, respectively.

Aerosol delivery of cPEI formulations to the sheep lung

In order to investigate the viability of cPEI formulations in a more clinically relevant large animal model, 20 ml of standard pCIKLux/PEI formulation prepared at 0.4 mg/ml (8 mg total dose) were aerosolized to the lungs of sheep ($n = 6$). Luciferase expression detected in lung tissues 24 hours after treatment was significantly (23 ± 11 fg luc/mg; $P = 0.005$) higher than background activity in untreated sheep (**Figure 5a**). In an attempt to increase the level of reporter gene expression detected, a total volume of 80 ml of pCIKLux/PEI at 0.4 mg/ml (total dose 32 mg) was also aerosolized to the lungs of sheep ($n = 7$) over a period of ~5 hours. Luciferase activity in lung tissue increased significantly (194 ± 83 fg luc/mg protein; $P = 0.008$) although aerosol delivery of this volume of PEI complexes was also associated with a significant ($P = 0.001$) inflammatory response demonstrated by a pronounced increase in the number of infiltrating neutrophils detected in BALF taken at necropsy (**Figure 5b**). In contrast, an equivalent 32-mg dose of concentrated pCIKLux/PEI at 1.6 mg/ml could be aerosolized in a total volume of 20 ml in only 75 minutes, and resulted in equivalent levels of luciferase gene expression (307 ± 105 fg luc/mg; $n = 8$) (**Figure 5a**), and was associated with a significantly lower neutrophil response ($P = 0.008$) (**Figure 5b**). Minimal inflammatory changes were observed in sheep that received either 20 or 80 ml (data not shown) aerosols of sterile water.

DISCUSSION

Nonviral GTAs possess many attributes that render them suitable for gene therapy. The relative simplicity of producing them, in conjunction with the fact that they do not produce obvious immune stimulation *in vivo*, continues to drive the development of new synthetic vectors. However, the development of nonviral formulations for clinical application may be hampered by the relatively low concentrations at which they can be prepared. In this study we have shown that pDNA/PEI complexes can be further concentrated with the use of a simple ultrafiltration process (**Figure 2**), leading to significantly increased levels of reporter gene expression after aerosol delivery to the lungs of mice (**Figure 3**) and sheep (**Figure 5**).

PEI is a cationic polymer that is thought to protect pDNA from degradation by buffering endosomal pH[29] and facilitating endosomal escape through osmotic swelling and rupture,[16] and is highly effective as a GTA both *in vitro* and *in vivo*. However, acute toxicity observed after instilling pDNA/PEI complexes into the lungs of mice[18] and rats[30] has delayed the clinical development of these complexes. The precise mechanism of PEI-mediated cellular toxicity remains unknown, but may involve adverse interactions of the highly cationic PEI molecule with anionic macromolecules within the cell. Strong associations between pDNA/PEI complexes and a number of cytoplasmic proteins[31] have been reported, and it is possible that such interactions, while possibly being a prerequisite for the liberation of pDNA from PEI complexes inside the cell, may also compromise normal cell function. Further, there is no known degradation pathway for the synthetic PEI molecule *in vivo*, and therefore any deleterious effects within the cell may well be persistent. Novel biodegradable PEI-based polymers have shown promise in terms of reduced toxicity,[32] but so far none have proven viable for aerosol delivery. It is clear, therefore, that minimizing the toxicity observed with standard PEI polymers remains an essential goal.

In this study, the concentration of PEI formulations by ultrafiltration resulted in the removal of significant quantities of "free" or uncomplexed PEI (**Figure 2**). Given that free PEI has been implicated in the acute toxicity observed with pDNA/PEI complexes,[28,33] its removal might be expected to reduce the overall inflammatory response observed *in vivo*. When the minimal toxicity observed in sheep lung after aerosol delivery of cPEI complexes is compared with the higher toxicity resulting from equivalent doses of standard pDNA/PEI complexes that retain large amounts of free PEI, the findings would appear to support this hypothesis (**Figure 5**). Again, only minimal inflammation was observed after aerosol delivery of cPEI complexes to the mouse lung (**Figure 4**). However, instillation of the same formulations resulted in severe and widespread inflammation (**Figure 4**), thereby indicating that the method of delivery is a critical factor in the lung's inflammatory response to PEI-based vectors. Similar findings have been reported after delivery of pDNA complexed with the cationic lipid Genzyme lipid 67 to the lung. Instillation to the lungs, both in mice[34] and sheep[35] resulted in a dose-dependent inflammatory response, whereas aerosol administration was associated with a significantly reduced toxicity profile.[9,25] Unlike aerosolized material that is distributed evenly throughout the airways,[36] instillation may result in localized delivery of a large bolus

of material to a relatively small area of the lung,[37] and this may overwhelm regional clearance mechanisms and act as a potent stimulus for inflammation.

Aerosol delivery of larger volumes could also result in localized "pooling" of deposited material within the lung that may stimulate inflammation in a manner similar to bolus delivery. This pooling effect may have contributed to the high levels of inflammation seen after aerosol delivery of 32 mg of standard PEI complexes in a large 80 ml volume when compared with the inflammation levels after 32 mg of cPEI formulations in a volume of only 20 ml (**Figure 5**). Scattered foci of neutrophils were found in the histological analysis of sections taken from 5 of the 7 sheep that had been treated with the 80 ml volume. Such foci are rarely observed after delivery of 20 ml (data not shown), and this further supports the hypothesis regarding the pooling effect associated with larger volumes. Consequently, cPEI formulations may be advantageous in reducing both the amount of toxic free PEI in the formulation and the total volume of formulation required to be administered to the lung. While these benefits clearly enhance the viability of PEI for lung gene therapy applications, it remains to be seen whether the improved levels of gene expression associated with cPEI formulations can be translated into clinical benefit. A final assessment will be possible only after actual aerosol delivery of a therapeutic transgene to the lungs of patients.

The identification of appropriate lung disease models will, of course, depend upon the cell types transfected after aerosol delivery, and this in turn will be influenced to some extent by the regional deposition of cPEI complexes in the lung. Aerosol studies utilizing fluorescent PEI derivatives have demonstrated delivery to both bronchial and alveolar regions of the mouse lung,[36] and this pattern correlates well with our observed GFP expression profile mediated by cPEI aerosols. Although significant gene expression was detected in ciliated epithelial cells, the majority of the GFP-positive cells were identified as alveolar type I pneumocytes. It is possible that these results could be explained simply by the fact that the cytoplasm of type I pneumocytes makes up the vast majority of the alveolar surface area and, that these cells are therefore far more likely to come into contact with GTAs delivered by aerosol. Alternatively, it is also possible that pDNA/PEI complexes possess properties that result in preferential transfection of, or expression in, these cells. This expression profile would suggest alveolar diseases such as α-1–antitrypsin deficiency or acute respiratory distress syndrome as being suitable targets for cPEI aerosol gene therapy. However, extrapolation of mouse aerosol studies toward the treatment of human disease requires caution, given the very different deposition profiles in the lungs of mice and humans.[38] In addition, the deposition pattern can be manipulated by careful selection of the nebulization device; therefore aerosol studies in the larger sheep model may provide a more valid comparison. Deposition studies in the sheep lung are technically challenging, but the detection of significant transgene messenger RNA expression in epithelial cells from the conducting airways after PEI aerosol delivery (data not shown) suggests a more proximal expression profile that might prove suitable for application in alternative lung gene therapies in humans, including for cystic fibrosis.

These studies show that the concentration of pDNA/PEI formulations significantly improves the potential of PEI lung gene therapy by reducing the time taken to deliver a clinically relevant pDNA dose as well as by minimizing subsequent lung toxicity. The ultrafiltration process used for concentrating the GTA is relatively quick, simple, and scalable for pharmaceutical manufacture. This process could be equally applicable for the concentration of a range of synthetic nonviral vectors; as such, it represents an important step forward in the development of formulations for clinical application.

MATERIALS AND METHODS

Plasmid expression vectors. The plasmid vector pCIKLux (5.6 kilobase) contains the firefly luciferase gene under the control of the human cytomegalovirus virus immediate/early promoter/enhancer.[39] Plasmid pEGFP-N1 encodes enhanced GFP (Clontech, Oxford, UK). Endotoxin-free pDNA was prepared by Bayou Biolabs (Harahan, LA) and all plasmids were resuspended in endotoxin-free water for injection (B. Braun Medical, Sheffield, UK).

Formulation of pDNA/PEI complexes. The 25 kd branched polymer (Sigma-Aldrich, Poole, UK) was prepared at a concentration of 4.3 mg/ml in sterile water at pH 7.4. Unless otherwise stated, pDNA/PEI formulations were prepared at a PEI nitrogen to DNA phosphate (N:P) molar ratio of 10:1 by slowly adding equal volumes of appropriately diluted aqueous pDNA to aqueous PEI while vortexing. The complexes were allowed to stand at room temperature for 15–20 minutes before use. The DNA concentration in the pDNA/PEI complexes was quantified by absorbance measurements at 260 nm, using 1 OD_{260} = 45 μg/ml for DNA bound to 25 kd branched PEI.[40] PEI concentration was determined using a modified trinitrobenzene sulfonic acid assay.[41]

Vector administration to the mouse airways. All research involving the use of animals adhered to the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. Nasal instillation studies were conducted using female BALB/c mice (8–16 weeks old), essentially as described earlier.[42] pDNA/PEI complexes were instilled under isoflurane general anaesthesia in a total volume of 100 μl of formulation per mouse. Aerosol delivery of pDNA/PEI formulations was performed using a continuous, unrestrained whole body exposure protocol. Mice (n = 6–36) were placed in an 8-l Perspex exposure chamber and exposed to aerosol generated using an Aerotech II nebulizer (CIS-US, Bedford, MA) operating at 40 psi, with 5% CO_2 in air as the driving gas (BOC, Bristol, UK).

Analysis of luciferase gene expression. Total lung luciferase expression was determined as described earlier,[43] using a Turner Designs 20/20 luminometer (Turner Designs, Sunnyvale, CA). The expression was quantified using standard curves prepared from recombinant luciferase protein (Promega, Southampton, UK), and the data were expressed as picogram of luciferase per milligram of total protein.

Concentration of DNA/PEI complexes. The concentration of pDNA/PEI complexes (<10 ml) was carried out using Centriplus YM-100 centrifugal filter units (Millipore, Watford, UK) incorporating a regenerated cellulose filter with a nominal molecular weight limit of 100 kd. Unless otherwise stated, 10 ml pDNA/PEI complexes were placed into the filter reservoir and concentrated by centrifugation at 500*g*. Concentration of larger volumes of pDNA/DNA complexes (<400 ml) was performed using a model 8400 stirred ultrafiltration cell (Millipore, Watford, UK) fitted with an equivalent 100 kd filter and operating under an applied pressure of 10 psi nitrogen gas.

Particle size and ζ potential measurements. The particle sizes of pDNA/PEI complexes was measured by laser light scattering using a Malvern Zetasizer 3000HS (Malvern Instruments, Malvern, UK). A total of 10 measurements per sample were performed at a fixed temperature of 25 °C, with the viscosity of water assumed as 0.89 cP. In order to enable measurement of surface

charge, pDNA/PEI complexes were diluted 50-fold in 10 mmol/l HEPES buffer pH 7.4 before the measurement of ζ potential. A total of five measurements per sample were performed at a fixed temperature of 25 °C.

Electron microscopy. Samples were examined using a routine negative staining technique.[44] Carbon/formvar-coated copper grids were floated on drops of the various suspensions for 30 seconds. The excess fluid was removed and the grids were floated on drops of 1% methyl tungstenate, and then dried and examined using a Jeol 1200EX transmission electron microscope.

Collection and analysis of BALF from mice. BALF was collected manually by gentle aspiration of fluid after intubation of the trachea and infusion of the lungs with 3 × 1 ml of BALF solution (phosphate-buffered saline containing 1 mg/ml bovine serum albumin and 50 μmol/l EDTA). Total cell counts in BALF were performed by staining nucleated cells with Turk solution (0.01% wt/vol crystal violet and 3% vol/vol acetic acid) and counting using a hemocytometer. The quantification of BALF neutrophils was performed using fluorescence microscopy on cytospin preparations after staining the nuclei with 4′,6-diamidino-2′-phenylindole dihydrochloride (Roche Diagnostics GmbH, Mannheim, Germany). Neutrophils were clearly identified by their multilobar nuclei, and the data were expressed as a percentage of the total cells present. A minimum of 300 nucleated cells were assessed for each BALF sample.

Lung histology and visualization of GFP expression. Lungs from treated mice were intubated through the trachea, inflated, and fixed as described earlier.[43] For histological analysis the left lobe from each mouse was embedded in paraffin and 7-μm sections were prepared using a Leica RM2125 microtome (Leica Instruments GmbH, Nussloch, Germany). The sections were stained with hematoxylin and eosin, and lung histology was assessed by an independent pathologist using the following scoring system: 0 = normal histology; 1 = few foci of inflammation limited to bronchial or vascular wall; 2 = some foci of inflammation and necrosis affecting <1/3 of lung tissue; 3 = many foci of inflammation and necrosis affecting 1/3 to 2/3 of lung tissue; 4 = severe and diffuse inflammation in walls and lumen of bronchi and alveoli affecting >2/3 of lung tissue. For visualization of GFP expression, 7-μm cryosections were prepared, examined, and quantified as described earlier.[43,45]

Aerosol delivery to sheep. Aerosol delivery of pDNA/PEI formulations to Suffolk Cross ewes (35–60 kg) was carried out essentially as described earlier.[9] After intramuscular administration of the antimuscarinic glycopyrrolate (Robinul Injection; Antigen Pharmaceuticals, Dublin, Ireland) at 0.02 mg/kg body weight (30 minutes before gene delivery), general anaesthesia was induced and maintained with intravenous propofol (Fresenius propofol, 1%; Fresenius Kabi, Runcorn, UK) (6–8 mg/kg). Individual sheep were placed in sternal recumbency inside a negative pressure ventilation system (described earlier)[35] and intubated with an endotracheal tube. The ventilating air was entrained through three jet nebulizer devices (Pari LC Plus; Pari GmbH, Starnberg, Germany). A compressed gas source of medical air (29 psi) and timer-controlled solenoid valve triggered by the start of each breath were used for operating the nebulizers during the first 70% of each inspiration.

Sheep tissue sampling. One to three weeks before the treatment, BALF was collected by bronchoscopy from the right apical lung segment of each animal by instillation and recovery of 2 × 20 ml of sterile saline. BALF was filtered through gauze to remove mucus and large particulates, and total and differential cell counts were performed on BALF cell pellets. After treatment, BALF samples were collected from the right caudal diaphragmatic lobe of each animal for comparison. Necropsy and tissue sampling for reporter gene expression were performed essentially as described earlier.[9] After the animal was killed and exsanguinated, three lung segments (the right apical, the right cardiac, and the right ventral diaphragmatic 1) were removed and repeatedly sectioned transversely into 1-cm thick slices. Representative samples from proximal, middle, and distal regions of each lobe were assayed for luciferase gene expression as described earlier.[9] The mean expression value from all the tissue samples (n = 12) taken from an individual sheep was used in subsequent analyses.

Statistical analysis. All results are expressed in terms of mean value ± SEM for a minimum of three repeated experiments. Statistical analysis between data sets in the mouse studies was carried out using analysis of variance with Fisher's protected least square difference post-hoc analysis. Data sets in the sheep studies were analyzed using nonparametric analysis, because the data were not normally distributed. Pairwise comparisons between treatments were made using Mann–Whitney, only when a positive Kruskal–Wallis test indicated that differences existed. Results were regarded as significant when $P < 0.05$.

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