Optimizing Aerosol Gene Delivery and Expression in the Ovine Lung

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We have developed the sheep as a large animal model for optimizing cystic fibrosis gene therapy protocols. We administered aerosolized gene transfer agents (GTAs) to the ovine lung in order to test the delivery, efficacy, and safety of GTAs using a clinically relevant nebulizer. A preliminary study demonstrated GTA distribution and reporter gene expression throughout the lung after aerosol administration of plasmid DNA (pDNA):GL67 and pDNA:PEI complexes. A more comprehensive study examined the dose–response relationship for pDNA:PEI and assessed the influence of adjunct therapeutic agents. We found that the sheep model can differentiate between doses of GTA and that the anticholinergic, glycopyrrolate, enhanced transgene expression. Dose-related toxicity of GTA was reduced by aerosol administration compared to direct instillation. This large animal model will allow us to move toward clinical studies with greater confidence.

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INTRODUCTION

A major challenge in the field of cystic fibrosis (CF) gene therapy is to build on the progress made in the first wave of Phase 1 clinical trials. Previously, the gene transfer protocols used in clinical trials were based on data from small animal models,[1–3] but it is now clear that it is important to use a model system in which the lungs mount similar inflammatory and immune responses and have comparable size and dynamic characteristics to those in man. We have developed the sheep as a large animal model with which we can rigorously assess delivery, efficacy, toxicity, and tissue sampling.[4]

We previously used the ovine model to test the relative efficacy of different gene transfer agents (GTAs) by direct instillation into the lung.[4] An important finding of this study was dose-dependent toxicity, thought to be caused by pooling of GTA in the terminal lung units.[4,5] In mouse models[6] and in man,[7] aerosol delivery of GTAs is associated with significantly less toxicity, so we developed a system for aerosol delivery of GTAs in the sheep using clinically relevant nebulizer technology. In a preliminary study, we tested whether the system could reproducibly deliver GTA to all parts of the lung in a reasonable time and whether the doses administered resulted in detectable transgene expression. In a larger study, we obtained dose–response information and evaluated the influence of potential adjunct therapeutic agents.

The respiratory epithelium has many natural barriers to gene transfer, including airway surface fluid, the mucus layer, and ciliary beating, all of which help to clear inhaled material.[8,9] The barriers are likely to be greater in CF patients owing to increased mucus viscosity and plugging of the small airways.[10] As mucus contributes significantly to disease pathogenesis, strategies to remove it or to reduce its viscosity are already available. Nacystelyn (NAL) is a mucolytic agent that has been tested in a clinical trial in CF patients[11] and might be expected to improve gene transfer efficacy. Recombinant human DNase (rhDNAse) is used routinely in many CF patients to break down genomic DNA, which is released from dead cells or bacteria and contributes significantly to mucus viscosity. A potential drawback of rhDNase is that it might reduce efficacy by degrading exogenous plasmid DNA (pDNA). The antimuscarinic compound glycopyrrolate (GP), which reduces tracheobronchial secretions, enhances gene transfer in the mouse nose.[12] We evaluated NAL, rhDNase, and GP in the context of our large animal model. Our findings demonstrate the utility of the sheep model and highlight issues of relevance to gene therapy trials.

RESULTS

Aerosol delivery of non-viral GTAs to the sheep lung

The ventilation and nebulization system designed to aerosolize GTAs to the sheep lung is shown in **Figure 1**. The nebulizer

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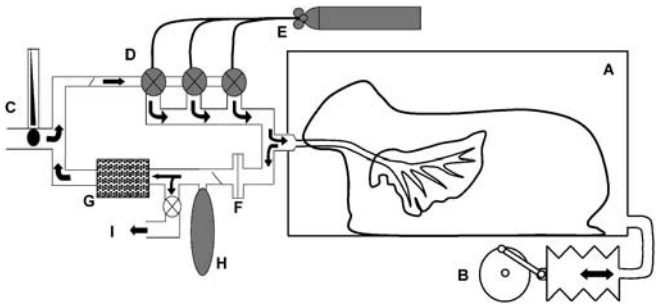

Figure 1 The ventilation and nebulization system. The anesthetized sheep is placed in a whole body respirator (volume 388 l; A) and the proximal end of the ET tube is connected through the respirator wall to a modified anesthetic circle system. Cyclical variation in pressure is facilitated by a cuirass pump (B). The frequency and amplitude of breathing is manually controlled. Anesthetic gas, delivered to the inspiratory limb of the circle system (C), reaches a nebulization manifold (D) upon which are mounted three Pari LC Plus jet nebulizers. Anesthetic is delivered in equal proportions to each nebulizer. A compressed gas source of medical air (22 p.s.i.; E) and timed solenoid valve triggered by the start of each breath is used to operate each nebulizer during the first 0.8 s of each inspiration. Exhaled air is passed through a filter (Pari filter set; F) before removal of CO_2 in soda lime (G). A rebreathing bag (H) and scavenging system (I) provide for peak inspiratory flow requirements and venting of excess gas.

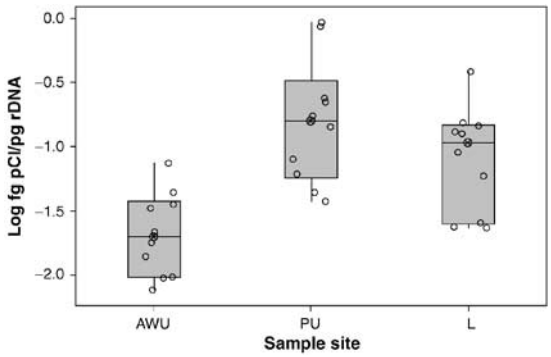

Figure 2 Boxplot showing pDNA distribution by tissue compartment immediately after delivery of 4 mg pDNA:PEI (50:50 pCIKCAT:pCIKLux). Symbols represent individual values from upper airways (AWU), upper parenchyma (PU), and lower parenchyma (L) in 10 lung segments. Upper and lower boxplot margins represent the interquartile range; middle bar indicates the median. Data are log-transformed.

delivery rate was 0.25 ml/min. In five separate experiments, gravimetric analysis of nebulizers and of filters placed in the expiratory limb and at the distal end of the endotracheal (ET) tube indicated that the system delivered 5.7 ± 2.7 and $29.4 \pm 2.8\%$ of the material that leaves the nebulizers or 4.6 ± 2.5 and $23.1 \pm 3.5\%$ of the actual nebulizer charge to these respective filters. The procedures were well tolerated with no evidence of any clinical effect in any animal.

To confirm delivery, we formulated the cationic polymer polyethyleneimine (PEI), which delivers pDNA effectively to the mouse lung,[13] with empty pCI plasmid and aerosolized this complex to the sheep lung (4 mg pDNA in 20 ml; $n = 1$). Immediately after treatment, the amount of pDNA (fg per 100 ng sample DNA) was measured by quantitative polymerase chain reaction, then converted to the amount of plasmid extracted per 25 mg tissue and extrapolated to the post-mortem wet weight of the lungs. About 2% of the nebulizer dose was detected in the lung. This figure probably underestimates actual lung deposition because recovery of pDNA in its condensed state, when complexed with polycationic PEI, is reduced in murine lung tissue (L Davies, unpublished data). From our results and published data,[14–16] we estimate that although the actual amount of pDNA deposited is probably higher than 2% it is unlikely to be more than 10% of the dose in the nebulizers (see Discussion). Importantly, pDNA was detected in all 10 different lung segments (**Figure 2**). Within lung segments, less pDNA was found in the larger airways (upper airway (AWU)) in the upper (proximal) portion of the segment compared with samples from the upper portion containing only parenchyma (PU) or compared with samples from the lower portion containing small airways and parenchyma (L) (**Figure 2**; $P = 0.006$ and $P = 0.014$, respectively; Wilcoxon's signed rank test).

Preliminary study

A preliminary study involving six sheep was conducted to test whole lung aerosol delivery of two non-viral GTAs that were available in the required quantities. We measured transgene expression by quantitative reverse transcriptase–polymerase chain reaction 24 h after delivery of a 50:50 mix of pCIKCAT and pCIKLux pDNA complexed either with PEI ($n = 2$) or cationic lipid GL67 ($n = 2$). The GL67 dose (52.8 mg pDNA in 20 ml) was similar to that previously given to CF patients.[7] Control animals ($n = 2$) were treated with vehicle alone. The delivery time was 75–90 min and the actual volume delivered was 15.6–17.5 ml. The mass median aerodynamic diameters (MMADs) of pDNA:PEI and pDNA:GL67 aerosols were $3.78 \pm 0.32\,\mu$m ($n = 5$ replicates) and $5.61 \pm 0.14\,\mu$m ($n = 6$ replicates), respectively.

Reporter expression. Although the low numbers preclude statistical comparison across treatments, mRNA was clearly detectable in all 10 lung segments 24 h after aerosol delivery of either GTA. The majority of samples were above the range of levels observed in the vehicle controls (**Figure 3a**). mRNA levels following delivery of pDNA:GL67 appeared to exceed those seen after delivery of pDNA:PEI. The inclusion of pDNA:GL67 allowed comparison with our previous study in which pDNA:GL67 was directly instilled into individual lung segments.[4] Aerosol delivery of pDNA:GL67 gave similar mRNA levels to 5 mg instillation (**Figure 3a**). The highest actual dose aerosolized was 46 mg DNA complexed to GL67 and we estimated that up to 10% of this ($\sim$5 mg pDNA) was deposited in the lung compared to 5 mg deposited in a single segment ($\sim$7% of the lung) by direct instillation.

Chloramphenicol acetyl transferase (CAT) levels in 41/60 and 3/60 tissue samples from animals treated with pDNA:GL67 and pDNA:PEI, respectively, were above the background range observed in control animals (**Figure 3b**). CAT levels following aerosol delivery were similar to those observed after 1 and 5 mg instillation in our previous study[4] (**Figure 3b**). We used a 50:50

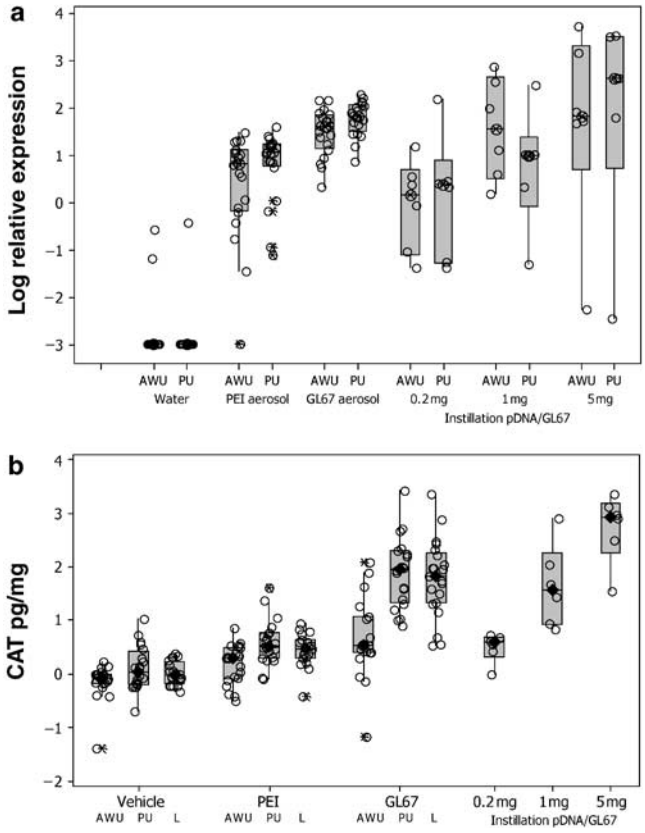

Figure 3 Preliminary study: mRNA and protein expression. Boxplots showing (**a**) mRNA and (**b**) CAT protein levels by tissue compartment after delivery of pDNA:GL67 (GL67), pDNA:PEI (PEI), and vehicle (water). pDNA was 50:50 pCIKCAT:pCIKLux. Symbols represent individual **a** mRNA or **b** CAT protein values from each of 10 lung segments (two sheep per treatment). For comparison, data to the right of each graph show **a** mRNA or **b** CAT protein expression after instillation to individual lung segments of 0.2, 1, or 5 mg of pCIKCAT complexed with GL67.[4]

mix of pCIKCAT and pCIKLux, so only 50% of the aerosolized pDNA was pCIKCAT. Luciferase activity in pDNA:GL67- or pDNA:PEI-treated animals overlapped that observed in vehicle-only animals and generally failed to indicate any evidence of a treatment effect (data not shown).

Toxicity. At post-mortem examination 24 h after treatment, the lungs from all the sheep under study were judged to be free from past or current natural ovine pulmonary disease. After histopathology, only 50% of water-treated animals were graded above zero in comparison to 85% for GL67 and 95% for PEI. Pathology included foci with goblet cell hyperplasia and mucus within the airway lumen, foci of interstitial thickening with neutrophils in more severely affected areas, and areas of intra-alveolar edema and neutrophil influx with perivascular lymphocyte aggregates. In our previous study,[4] instillation of just 1 mg pDNA:GL67 resulted in extremely severe lesions (grade 4). No such lesions were seen after aerosol delivery of 55 mg pDNA:GL67. Overall, the pathology observed after aerosol delivery of pDNA:GL67 or pDNA:PEI was limited to small foci within the lung.

Main study

Having achieved gene transfer levels above the threshold of detection with doses that could be delivered within a practical time period, we initiated a further study with six animals in each of seven treatment groups (42 animals in total) to obtain dose–response information and to assess the effect of adjunct therapies. PEI was used as the GTA based on cost and availability. In the preliminary study, we failed to demonstrate luciferase activity above baseline with pDNA:GL67 or pDNA:PEI, but we subsequently improved the sensitivity of the luciferase (LUX) assay by perfusing the lungs with phosphate-buffered saline to remove contaminating hemoglobin and using a more suitable lysis buffer (see Materials and Methods). Consequently, we used pCIKLux in the main study. In the preliminary study, we used 4 mg pDNA complexed with PEI in 20 ml based on data suggesting that the highest achievable concentration was 0.2 mg DNA/ml.[17] Subsequent improvements generated formulations of 0.4 mg/ml (8 mg pDNA in 20 ml) at the same ratio of pDNA:PEI with no loss of efficacy. For doses above 8 mg (20 ml), it was necessary to increase the volume and therefore the delivery time. A delivery time of 5 h was chosen for practical and ethical reasons. The maximum dose delivered was ~32 mg of pDNA complexed to PEI. The mean delivery time was ~75 min for 20 ml and 5 h for 80 ml.

Dose–response effect. Following aerosol delivery of pCIKLux/PEI, lung tissue samples were analyzed for vector-derived mRNA and luciferase activity (**Figure 4**). Within each treatment group, there was no significant difference between luciferase activity in PU, M, and L samples. As these samples are biologically similar, consisting of parenchyma and small airways, the data were combined for analysis. Significant levels of mRNA expression were observed in AWU or combined PU, M, and L (both $P < 0.006$; Mann–Whitney (MW) following significant Kruskal–Wallis (KW)) for all the treatment groups compared to controls (**Figure 4a**). For combined PU, M, and L, there was a dose response: the 32 mg group was significantly higher than the 4 mg or the 8 mg groups ($P = 0.005$; MW following significant KW). We also observed dose-related luciferase activity in animals treated with 4, 8, or 32 mg pDNA (**Figure 4b**). Significant LUX activity was seen in combined PU, M, and L samples in the group receiving 32 mg pDNA ($P = 0.005$ compared to controls and $P = 0.02$ compared to 4 mg and $P = 0.04$ compared to 8 mg pDNA-treated groups; MW following significant KW). Luciferase activity in airway (AWU) samples from all of the groups was no different for untreated animals despite the presence of mRNA (**Figure 4a**). This probably reflects the technical difficulties in homogenizing and lysing the cartilaginous AWU tissues for LUX analysis.

Adjunct treatments. We tested the effect of three adjunct therapeutic agents on transgene expression: NAL, a mucolytic agent; rhDNase/Pulmozyme; and GP, an antimuscarinic that reduces oral and bronchial secretions. These adjuncts were tested with 8 mg pDNA:PEI to avoid the possibility of saturation at the 32 mg dose. NAL and rhDNase were administered by aerosol, whereas GP was given by intramuscular injection. To ensure that

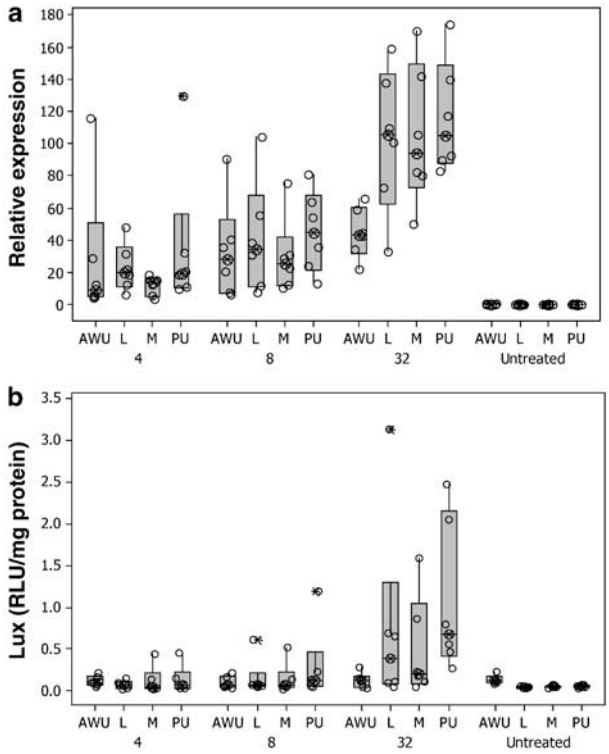

Figure 4 Main study: dose–response measurements. Boxplots showing (**a**) mRNA and (**b**) luciferase levels in samples from upper (AWU and PU), middle (M), and lower (L) regions dissected from lung segments. Groups of six animals received 4, 8, or 32 mg pCIKLux pDNA complexed to PEI or no treatment. Upper and lower boxplot margins represent the interquartile range and the middle bar the median. Individual symbols represent the mean **a** plasmid-derived mRNA or **b** luciferase activity from three different lung segments (right apical, right cardiac, RVD1) for each of the six animals.

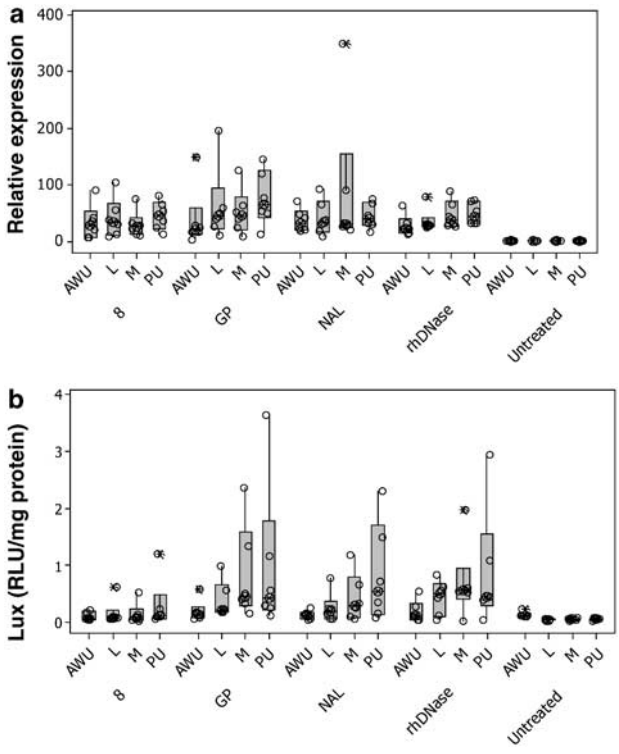

Figure 5 Main study: effect of adjunct agents. Boxplots showing (**a**) mRNA and (**b**) luciferase levels after delivery of pDNA:PEI and adjunct agents. Groups of six animals received 8 mg pCIKLux/PEI alone, 8 mg pCIKLux/PEI plus GP, NAL or rhDNase, or no treatment. Individual symbols represent the mean values from three different lung segments (right apical, right cardiac, RVD1) for each of the six animals. Upper and lower boxplot margins represent the interquartile range and the middle bar the median.

GTA delivery coincided with the peak activity of each agent, NAL, rhDNase, and GP were administered 4 h, 30 min, and immediately before GTA delivery, respectively.

Significant levels of mRNA expression were observed in combined PU, M, and L samples from all the treatment groups compared to controls (**Figure 5a**; $P < 0.006$; MW following significant KW). There was a nonsignificant trend toward higher mRNA expression in the 8 mg plus GP group compared to the group receiving 8 mg alone. Importantly, RNA levels in the 8 mg plus GP group were significantly higher than in the group receiving 4 mg alone (**Figure 4a**; $P = 0.005$; MW following significant KW). Luciferase activity was significantly higher in combined PU, M, and L samples in the group receiving 8 mg pDNA:PEI plus GP compared to the group receiving 8 mg pDNA:PEI alone (**Figure 5b**; $P = 0.04$, MW following significant KW). Treatment with rhDNase and NAL tended to show similar increases, although these did not reach significant levels. Importantly, LUX levels in all three groups receiving adjuncts before delivery of 8 mg pDNA:PEI were not significantly different to levels after delivery of 32 mg pDNA:PEI without adjuncts (**Figure 4b**). Moreover, a linear relationship was observed between log-transformed levels of mRNA and luciferase activity (**Figure 6**; $P < 0.001$; $r_s = 0.363$ Spearman's rank procedure).

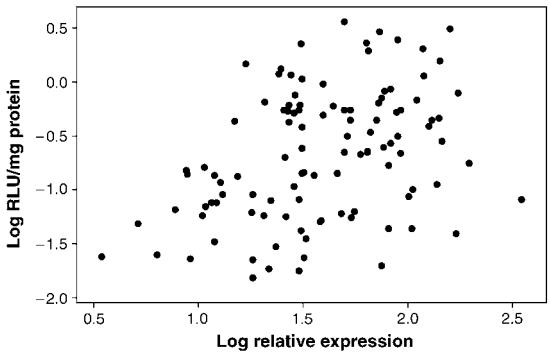

Figure 6 Scatterplot illustrating the linear relationship between log-transformed mRNA levels and log-transformed luciferase activity in PU, M, and L samples from all the animals in the six treatment groups.

Toxicity. Five slides from each animal were investigated for histopathological changes (30 slides in total for each treatment group of $n = 6$; **Figure 7**). In all seven treatment groups the level of inflammation was generally mild, with some mild to moderate changes seen particularly in the 32 mg pDNA:PEI group and the NAL-treated group. Animals in these two groups appeared to have a slightly higher grading of pathological change (**Figure 7**). The 32 mg pDNA group showed signs of airway-centered acute

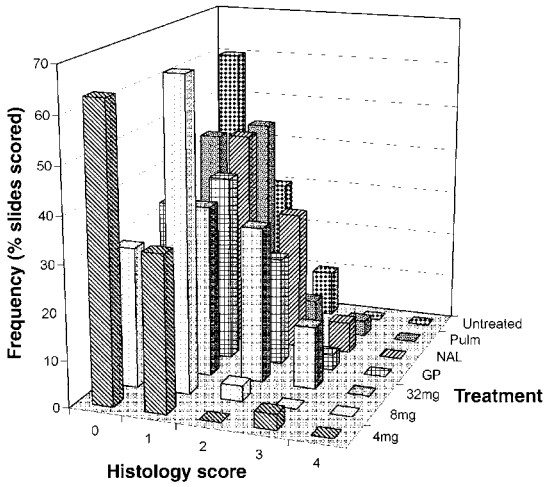

Figure 7 Histological assessment of slides from animals treated with 4, 8, or 32 mg pDNA:PEI or 8 mg pDNA:PEI following pre-treatment with GP, NAL, or rhDNase (Pulm). Slides were graded on a scale of 0–4 with 4 the most severely affected tissue. Bars show the % of slides scored at each grade. For each animal, one slide from each of the five lung segments sampled was graded, giving a total of 30 scores for each treatment.

inflammation with varying degrees of interstitial thickening and occasional consolidation. Within this group, the majority of sections showed evidence of mild inflammation with a few scattered areas of moderate inflammation.

DISCUSSION

The negative pressure ventilation system is of particular value in relation to nebulization because aerosol delivery occurs with the airway at, or very close to, atmospheric pressure. This simplifies the provision of gaseous anesthesia. Using multiple nebulizers and inspiratory gating to maximize delivery, ~23% of the nebulizer charge reached the distal end of the ET tube, and therefore should reach the trachea when an animal is in place. However, other factors can influence how much material from the nebulizer is actually deposited in the lung. In healthy human volunteers, with a Pari LC nebulizer operating intermittently to simulate breath actuation (inspiratory flow rate 20 l/min, MMAD 4.0 μm), actual pulmonary deposition, measured by gamma-scintigraphy, was 15.3%.[14] The amount remaining in the nebulizer was 61.5%. In other studies, lung depositions of 13.8–15% were reported, based on predictions from measurement of nebulizer output and respirable fraction (proportion of particles $<5\,\mu$m).[15,18] The respirable fraction is particularly relevant when patients are inhaling aerosol from a nebulizer mouthpiece because particles $>5\,\mu$m are unlikely to get beyond the mouth, oropharynx, and trachea. In our system, pDNA:PEI particles (MMAD 3.8 μm) are within the respirable range, whereas pDNA:GL67 particles (MMAD 5.6 μm) are at the upper limit for airway deposition. The impact of particle size difference remains largely speculative at present but would most likely predispose toward pDNA:GL67 being deposited more proximally in the airway tree than pDNA:PEI.

In our system, the aerosol enters the trachea via an ET tube, bypassing the oral cavity. Only a few studies have investigated aerosol delivery to the lungs under these conditions, and none of these used the Pari LC plus nebulizer. In one such study, using a System 22 Acorn nebulizer and looking at aerosol deposition in the lungs of patients ventilated by ET tube, the total pulmonary deposition, measured by gamma-scintigraphy, was just $2.2 \pm 0.8\%$.[16] The same system delivered 5% to the lungs of spontaneously breathing patients. Therefore, the addition of the ET tube decreased lung deposition by about 50%. It seems reasonable to assume that the ET tube in our system would have a similar effect. As discussed above, about 15% of the nebulizer charge from the Pari LC plus was deposited in the lungs of spontaneously breathing patients.[14,15,18] Therefore, pulmonary deposition in our system may be as low as 7% of the nebulizer charge. Nevertheless, GTA clearly reached all parts of the lung in the animal examined for pDNA deposition immediately after delivery.

In our preliminary study, we opted for intensive sampling throughout the lungs of a small number of animals per treatment. Statistical analysis was therefore limited and the data were interpreted with caution. At 24 h, we saw widespread RNA levels above the range observed in controls. We also detected widespread CAT expression consistently above background but only in the GL67-treated animals. It should be remembered that in the preliminary study 20 ml of pDNA:GL67 contains more than 10 times as much pDNA as 20 ml of pDNA:PEI. In the main study, significant mRNA expression was detected in multiple segments of all treated animals and a dose–response effect was observed. It will be important to determine if the increased expression in the 32 mg pDNA group relative to the 8 mg pDNA group is due to the higher dose alone or a combination of higher dose and extended delivery time, which would increase the contact time with the target cells. This information could impact on the design of clinical trials in CF patients.

We also tested the effects of adjunct agents. The absence of any adverse effect of rhDNase (Pulmozyme) indicates that pDNA:PEI, as previously reported for cationic liposome complexes,[19] is at least partially resistant to DNase degradation. An important consequence of this may be that patients using Pulmozyme to reduce mucus viscosity would not have to be excluded from a clinical trial or have to change their prescribed medicine. Although the mucolytic agent NAL is not used routinely in CF patients, it has been tested in clinical trials. Our data suggest that it may not be very effective, although a bigger effect may be seen in the diseased lungs of CF patients. Oral mucolytics might be a better option, as they would reduce the possibility of interference with nebulized GTA. The enhancement of gene expression following pre-treatment with GP is consistent with the hypothesis that extracellular barriers contribute to the inefficiency of airway gene transfer. From a clinical perspective, GP may not be relevant to CF patients owing to pre-existing dehydration of their airway surface liquid. However, it may be a valuable pre-treatment in our future studies in the sheep, and the systemic route of administration makes an adverse interaction with GTAs unlikely. Luciferase activity in animals receiving all three adjuncts ($n=3$) appeared to be reduced compared to

pDNA:PEI alone (data not shown). It is possible that there was adverse interaction between the agents themselves or between the agents and the GTA.

A major difference between aerosol and instillation delivery of pDNA:GL67 relates to toxicity at 24 h. Instillation caused a severe airway-centric acute inflammatory response, which often extended to the peribronchiolar interstitial tissue leading to consolidation, bronchiolar destruction, extensive alveolitis, and alveolar neutrophil exudates.[4] Bacterial CpG dinucleotides in the plasmids are likely to be a major factor in this response.[20] Aerosol delivery resulted in consistently less severe pathological changes than instillation, as previously observed in mice.[6] This is probably due to improved distribution and absence of pooling effects.

Our data show that the sheep complements existing small animal systems for pre-clinical product evaluation studies. Data from this large animal model on delivery, toxicity, and spatial distribution of transgene expression will directly inform future selection and implementation of protocols to determine the optimal treatments to take forward to clinical trials.

MATERIALS AND METHODS

Animals and anesthesia. All research adhered to the UK Home Office Animals (Scientific Procedures) Act 1986. The preliminary study used seven commercially sourced crossbred sheep (four female, two male neutered, one male; body weight 34–60 kg). The main study used Suffolk cross ewes (body weight 35–60 kg). Animals were treated with anthelminthic and were judged free from significant pulmonary disease by clinical examination >1 week before treatment. Anesthesia was induced and maintained as described[4] using the system shown in **Figure 1**.

GTAs and pDNA. Plasmids pCIKCAT[4] and pCIKLux[21] contain the CAT and luciferase (LUX) complementary DNAs in the mammalian expression vector pCI.[21] Endotoxin-free plasmids were prepared by Bayou BioLabs (Harahan, LA). GL67:pDNA complexes were prepared as before[4] but with a molar ratio of 6:8. pDNA was complexed with PEI (25 kDa branched; Sigma, Poole, Dorset, UK) as before[21] with a final DNA concentration of 0.2 or 0.4 mg/ml at a nitrogen:phosphate ratio of 10:1.[13]

In the preliminary study, animals received pDNA complexed with PEI (pDNA:PEI; $n=2$), pDNA complexed with cationic lipid (pDNA:GL67; $n=2$), or vehicle alone (pyrogen-free water; $n=2$). For pDNA:PEI, nebulizers were charged with 20 ml complex (2 mg pCIKLux and 2 mg pCIKCAT; $\sim$0.61 mM DNA). For pDNA:GL67, nebulizers were charged with 20 ml complex (26.4 mg pCIKLux and 26.4 mg pCIKCAT; $\sim$8 mM DNA). For vehicle, nebulizers were charged with 20 ml pyrogen-free water. One animal received empty pCI vector complexed with PEI and was killed immediately afterward to measure pDNA distribution. The remaining sheep were killed 24 h after treatment for necropsy. In the main study, animals received pDNA:PEI at pDNA doses of either 4 or 8 mg in 20 ml. A further group received 32 mg pDNA in 80 ml (0.4 mg/ml) delivered over $\sim$5 h.

GTA and adjunct delivery. Aerosol delivery was performed as described[21] using the system shown in **Figure 1**. For the 20 ml dose, each nebulizer (max. vol. 8 ml) was charged with 6.7 ml GTA and weighed before and after delivery to determine the residual volume. For the 80 ml dose, each nebulizer was charged with 8 ml GTA and weighed. The residual volume was determined hourly by weighing, and the nebulizers recharged to 8 ml until all 80 ml was delivered.

NAL (10% solution; Galephar SMB, Brussels, Belgium) was delivered by nebulizer 4 h before GTA delivery. A mean dose of 3.58 ml (358 mg) was aerosolized for 10 min at $\sim$0.25 ml/min. A 1 mg/ml solution of rhDNAse (Pulmozyme, Roche Products Ltd., Welwyn Garden City, UK) was delivered by nebulizer immediately before GTA delivery (mean aerosolized dose 2.07 ml). GP (Robinul injection 0.2 mg/ml, Antigen Pharmaceuticals, Southport, UK) was administered via intramuscular injection at a dose of 0.02 mg/kg body weight 30 min before GTA delivery.

Necropsy and tissue sampling. For the preliminary study, 10 lung segments were harvested: right apical, right intermediate, right cardiac, right ventral diaphragmatic 1 and 2 (RVD1, RVD2), right caudal diaphragmatic, left cardiac, left ventral diaphragmatic 1 and 2 , and left caudal diaphragmatic. For the main study, the pulmonary circulation was flushed out via the pulmonary artery with 2–3 l phosphate-buffered saline before sampling to minimize interference of hemoglobin with the luciferase assay and five segments were harvested (right apical, right cardiac, RVD1, left cardiac, left ventral diaphragmatic 1). Each segment was cut transversely into $\sim$1 cm thick slices representing upper, middle, and lower regions. Individual airways (>2 mm diameter) were dissected from the upper region (AWU). Parenchyma samples (containing airways too small to dissect, <2 mm diameter) were derived from the upper (PU), middle (M), and lower (L) regions.

Assays. Histopathological analysis was carried out on lung slices from the middle region as described.[4] Slides were graded according to severity on a scale of 0–4 with each slide scored according to the most severe lesion present. Photomicrographs of each histological grade are shown in ref. 4.

To assay plasmid levels, total DNA was prepared from 25 mg tissue samples (DNeasy Kit, Qiagen, Crawley, UK) and pDNA was measured by TaqMan quantitative polymerase chain reaction as described in the **Supplementary Material**. Plasmid-derived mRNA expression was measured by real-time quantitative multiplex TaqMan reverse transcriptase–polymerase chain reaction as described previously[22] and in the **Supplementary Material**. For reporter gene protein assays, samples (AWU, PU, M, and L) were submerged in either lysis buffer (1 ml/g tissue; CAT enzyme-linked immunosorbent assay, Roche Diagnostics, East Sussex, UK) for the preliminary study or reporter lysis buffer (RLB; Promega Corp., Madison, WI) for the main study and stored at -80°C. CAT activity was measured by enzyme-linked immunosorbent assay.[4] LUX activity was measured on undiluted lysates (Luciferase Assay System; Promega). Values were calculated as a proportion of total protein measured with a Pierce BCA Assay reagent kit (Perbio Science, Cheshire, UK) on 10 μl lysate diluted with 390 μl phosphate-buffered saline.

MMAD analysis. To measure pDNA:PEI and pDNA:GL67 particle size, aerosols were drawn through a Next Generation Pharmaceutical Impactor (NGI; MSP Corporation, Shoreview, MN) with an applied airflow of 20 l/min at 4°C to prevent water evaporation.[23] Aerosol characteristics were determined after quantification of deposition on the NGI collection stages. Deposition of the PEI component of pDNA:PEI aerosols was measured using a modified trinitrobenzenesulfonic acid assay[24] and deposition of the phospholipid component of pDNA:GL67 aerosols was measured as described.[25] Data were analyzed with CITDAS version 2.0 software (Copley Scientific, Nottingham, UK) and results were expressed as MMAD.

Statistical analysis. Statistical techniques used are indicated in the Results section. None of the data were normally distributed (Kolmogorov–Smirnov), so non-parametric methods were used. To compare regional deposition and expression in the preliminary study, we used the

Wilcoxon's signed rank test on data from each individual animal. For comparisons across the multiple treatment groups in the main study, the Kruskal–Wallis test was used to determine if there were overall differences between treatment groups. This analysis was performed on the mean value from regions PU, M, and L combined. Where differences were observed, pairwise Mann–Whitney tests were performed to identify where those differences lay. The null hypothesis was rejected at $P < 0.05$.

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SUPPLEMENTARY MATERIAL

Materials and Methods.

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