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ARTICLE

Aerosolized Nanogram Quantities of Plasmid DNA Mediate Highly Efficient Gene Delivery to Mouse Airway Epithelium

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The lung is an important target of gene therapeutic interventions. In contrast to intratracheal instillation, inhalation would be the most practical route of administration in clinical applications. Here we show that aerosolized nanogram quantities of pDNA complexed to PEI (350 ng) yielded transfection levels 15-fold higher than a 140-fold higher dose (50 μg) of the same vector applied directly to the lungs of mice via intratracheal intubation. An important efficacy parameter is the osmolarity of the aerosol and not biophysical properties of the nebulized vector. Vectors formulated and nebulized in hypoosmotic distilled water yielded 57- and 185-fold higher expression levels than those in isotonic 5% glucose or Hepes-buffered saline, respectively. Pretreatment of mice with nebulized indomethacin, which prevents water-induced airway alteration, resulted in lower gene expression, whereas pretreatment with EGTA or polidocanol, which modulate tight-junction activity, had no effect. These results, together with histological analysis of regional lung deposition and gene expression, suggest that a temporary water-induced hypoosmotic shock permeabilizes the epithelium sufficiently to allow vector uptake. The so far observed inefficiency of nonviral gene delivery to the airways may be the result of an inappropriate method of vector administration.

Key Words: aerosol, gene therapy, nonviral, PEI, gene transfer, distilled water

INTRODUCTION

Due to its large surface and its accessibility, the lung is an excellent target for noninvasive localized drug delivery. The airway and the alveolar epithelium are the sites where genetic disorders such as cystic fibrosis or inherited surfactant protein B deficiency have their major fatal impacts. For these reasons, the lung has been an attractive target for gene therapy interventions. Nonviral vectors have proven safe for instillation into the lung in clinical trials but therapeutic significance is lacking. Intratracheal instillation of vectors into mouse lungs has been used for a long time as a preclinical model to study vector efficacy, toxicity, and the action of delivered transgenes. The easy detection of sensitive reporters such as the expression of the luciferase gene has somewhat obscured the actual inefficiency of the gene delivery process with nonviral vectors upon instillation. Furthermore, intratracheal instillation can hardly be envisaged to be a practical administration route for human applications. Therefore, the aerosolization of vectors to be used ultimately with inhalation devices is of great interest. Encouraged by previous work of Densmore *et al.* [1] we have recently developed and optimized a whole-body nebulization device for mouse *in vivo* studies [2] with polyethylenimine(PEI)–pDNA vectors. The device produces a "dried" aerosol from aqueous vector suspensions, which yields highly reproducible reporter gene expression in mouse lungs upon exposure of the animals to the aerosol stream. After having optimized the delivery device, the important questions were if and to what extent the solvents used for vector formulation and delivery have an impact on gene transfer efficiency. Such impact can arise from two facts: (i) the solvent influences critical biophysical vector parameters such as particle size and surface charge, which

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fundamentally affect the gene transfer efficiency *in vitro* and *in vivo* [3,4], and (ii) hypotonic solutions such as distilled water can affect the permeability of the apical membrane of airway epithelial cells [5], thereby potentially modulating vector uptake.

The colloidal stability of cationic polyelectrolyte-derived gene transfer complexes is very sensitive to the ionic strength of the surrounding solution [6]. Without effective shielding methods by protective polymers such as polyethylene glycol, physiologic salt concentrations lead to salt-induced aggregation of the complexes [6,7]. It is important to note that the resulting increase of size alters the transfection behavior of the complexes [3]. Whereas *in vitro* transfection rates correlate with increasing sizes of the complexes, the opposite has been observed *in vivo* [4]. Gene expression in the lungs of mice after intravenous application was 100-fold more efficient with small DNA complexes formulated in 5% glucose compared to large gene transfer complexes formulated under high ionic strength conditions [4]. In addition to biophysical vector parameters, the influence of the solvent itself on cellular functions has to be considered, in particular, when distilled water is used as a solvent for vector formulation. Distilled water can transiently induce airway swelling, widening the intercellular spaces [8], and can transiently permeabilize the apical membrane of airway epithelial cells, which then become leaky for proteins and other macromolecules by a hypoosmotic shock [5].

Against this background we characterized in detail solvent-dependent effects on PEI-mediated *in vivo* aerosol gene delivery in terms of both biophysical particle parameters and solvent-induced physiologic changes in the airway epithelium. We demonstrate that distilled water functions as a critical cofactor for aerosol delivery of PEI–pDNA gene vectors and that pDNA doses in the nanogram range reaching the airways upon inhalation are sufficient to yield gene transfer efficiencies highly superior to those achievable by instillation of the same vector formulations.

RESULTS

Gene Transfer Efficiency of PEI–pDNA Complexes Formulated in Various Solvents

We formulated PEI–pDNA complexes in distilled water, 5% glucose, HBS (Hepes-buffered saline), or PBS and applied them to mouse lungs via nebulization. Luciferase gene expression mediated by PEI–pDNA complexes formulated in distilled water 24 h after aerosol application was significantly ($P < 0.01$), 57- and 185-fold, higher compared with PEI–pDNA complexes formulated in 5% glucose and HBS, respectively. PEI–pDNA complexes formulated in PBS did not mediate luciferase gene expression (data not shown). Gene expression was not detected in the heart, liver, blood, stomach, intestine, colon, or kidneys.

Analysis of Biophysical Parameters of PEI–pDNA Complexes Formulated in Various Solvents

Biophysical parameters of PEI–pDNA complexes are given in Table 1. Whereas PEI gene vectors formulated in distilled water and 5% glucose were kinetically stable, showing diameters of 98 ± 2 and 89 ± 10 nm, gene vectors formulated in HBS were larger (153 ± 10 nm) and slowly aggregating. PEI gene vectors formulated in PBS resulted in large diameters (848 ± 142 nm), which were kinetically unstable. We measured the ζ potentials, which describe the surface charge of PEI gene vectors. It has been previously shown that the transfection efficiency of the gene vectors correlates to a certain extent with increasing surface charge [9]. Thus, the surface charge could markedly affect gene transfer efficiency. The surface charge of the gene vectors formulated in water, 5% glucose, and HBS were 56 ± 1, 45 ± 1, and 12 ± 2 mV, respectively. In addition, we analyzed PEI–pDNA complexes by electron microscopy to identify complex morphology. Using double carbon negative staining, PEI without the addition of pDNA consisted mainly of small amorphous material independent of the solvent. Upon incubation of PEI with pDNA homogeneous populations of nearly globular particles with defined size were formed. Particle sizes varied depending on the solvent, revealing a diameter of 50 ± 8 nm for PEI–pDNA complexes in water, 54 ± 4 nm for complexes diluted in 5% glucose, and 111 ± 18 nm for those complexes in HBS (Fig. 1A, compare with Fig. 1C). To exclude hidden effects of the double carbon negative staining on particle morphology, we analyzed the same complexes by native cryoelectron microscopy. The native cryo preparation revealed particles similar in size with a nearly globular shape (Fig. 1B). However, the image contrast was lower than that of the negatively stained specimens. Size measurement of PEI–pDNA complexes in water or HBS revealed an average diameter of 57 ± 6 or 139 ± 28 nm, respectively (Fig. 1C). Thus, the sizes are comparable to those of the negatively stained samples. A similar preparation of PEI–pDNA complexes diluted in 5% glucose was not possible as the presence of glucose dramatically reduces the image contrast in native cryo preparations. The variations in particle sizes measured either by dynamic light scattering or electron microscopy could be due to the not strictly spherical particle morphology. Nonspherical particles show a lower diffusivity compared to spherical particles of the same volume-equivalent diameter. This leads to a nominally larger diameter for nonspherical particles compared to spherical particles of the same volume-equivalent diameter.

TABLE 1: Particle size (nm) and ζ potential (mV) of PEI–pDNA complexes

	Water	5% glucose	HBS	PBS
Size	98 ± 2	89 ± 10	153 ± 10	848 ± 142
ζ potential	56 ± 1	45 ± 1	12 ± 2	n.d.

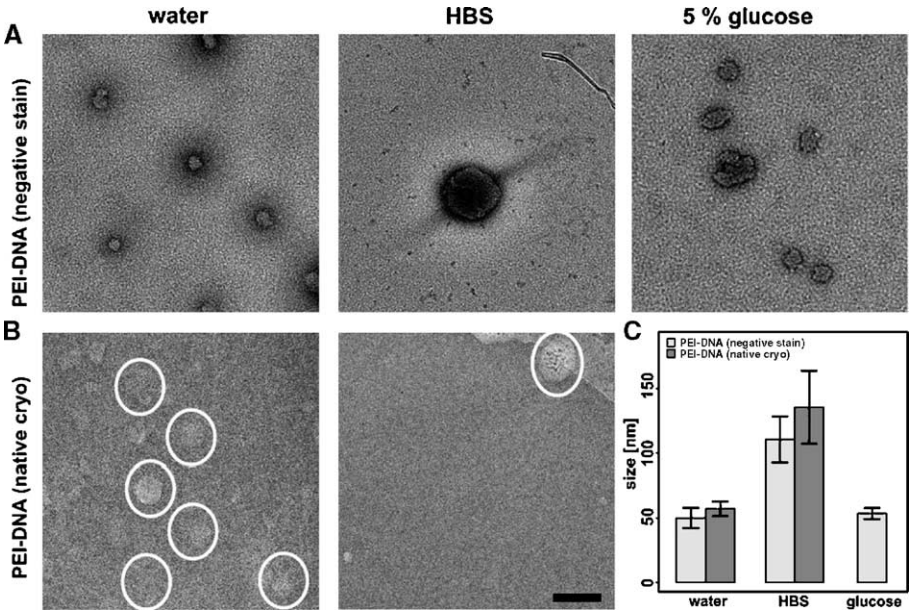

FIG. 1. Electron microscopy of PEI and PEI–pDNA complexes. (A) PEI–pDNA complexes diluted in water (left), HBS (middle), and 5% glucose (right) prepared by double carbon negative staining and (B) PEI–pDNA complexes in water (left) and HBS (right) using the native cryo preparation. Some complexes are encircled. The scale bar corresponds to 100 nm on the specimen level. (C) Size measurement of PEI–pDNA complexes visualized by negative staining and native cryoelectron microscopy. Results are presented as means ± standard deviation.

We analyzed the binding strength of PEI to pDNA in each of the solvents by a poly-L-glutamic (pLG) acid competition assay based on polyanion-induced restoration of the fluorescence signal intensity of bisintercalated TOTO-1, which is quenched after pDNA condensation with polycations. The degree of restoration of the fluorescence signal intensity of condensed pDNA in the presence of a polyanionic polymer such as pLG can be used as a measure for the PEI–pDNA binding strength [10]. As shown in Fig. 2, increasing the concentrations of pLG resulted in disassembly of the PEI–pDNA complexes in water, 5% glucose, and HBS, but this effect was more pronounced under high ionic strength conditions. We observed no differences in the pDNA binding behavior between complexes formulated in distilled water or 5% glucose.

The Effects of Pretreatment of Mice with Indomethacin

We pretreated BALB/c mice by nebulization of 3.0 ml of an indomethacin solution (5 mg/ml) as described by Mochizuki *et al.* [11]. Immediately afterward we nebulized PEI gene vectors formulated in distilled water to the mice and measured luciferase expression 24 h later. Indomethacin pretreatment of BALB/c mice resulted in significantly, 2.9-fold, reduced gene expression compared to untreated mice ($P < 0.01$, data not shown).

The Effects of Tight-Junction Modulation on Aerosol Gene Transfer Efficiency

We pretreated BALB/c mice by nebulization of distilled water, EGTA (400 mM), or polidocanol (0.1%) for 30 min before aerosolization of PEI gene vectors formulated in either distilled water or 5% glucose (Fig. 3). Pretreatment of mice with distilled water, EGTA, or polidocanol did not significantly influence gene transfer efficiency of PEI–pDNA complexes formulated in 5% glucose. Gene transfer efficiency mediated by PEI gene vectors formulated in distilled water was significantly reduced only after pretreatment of mice with EGTA (sevenfold, $P < 0.01$).

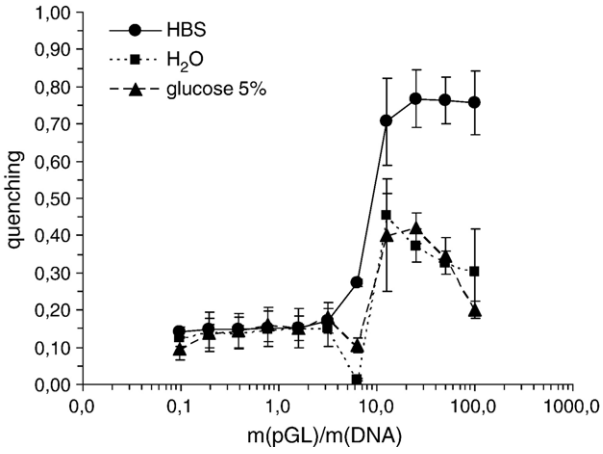

FIG. 2. The effects of the solvent used for formulation of PEI–pDNA gene vectors on PEI–pDNA binding. pDNA was labeled with the bisintercalating dye TOTO-1 and formulated with PEI at an *N/P* ratio of 10 in the indicated solvents. Increasing amounts of poly-L-glutamic acid were added to the complexes and the fluorescence emitted at 530 nm was measured. Results are expressed as the fluorescence intensity ratio of complexed pDNA to uncomplexed pDNA. The experiments were performed in triplicate and the means ± standard deviations of three independently performed experiments are shown.

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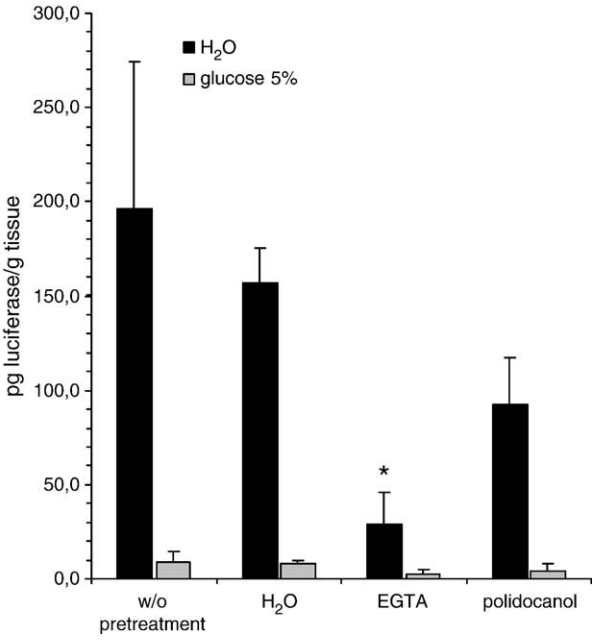

FIG. 3. The effects of pretreatment of mice with aerosolized tight-junction active compounds. BALB/c mice were pretreated by nebulization of distilled water, EGTA (400 mM), or polidocanol (0.1%) for 30 min before aerosolization of PEI gene vectors formulated in either distilled water or 5% glucose. Luciferase expression was examined 24 h after gene vector application. Results are reported as means ± standard deviation of the mean ($n = 4$). *Significant differences between pretreated and not pretreated ($P < 0.01$).

Biodistribution of pDNA after Aerosol Application in BALB/c Mice of PEI–pDNA Complexes Formulated in Distilled Water, 5% Glucose, or HBS

We formulated radioactively labeled plasmid pDNA (^{123}I) with PEI in distilled water, 5% glucose, or HBS and nebulized it to BALB/c mice (Fig. 4). Immediately after aerosolization was complete, we sacrificed the mice and measured the radioactivity in the indicated organs. The pDNA doses delivered to the lungs of mice (referring to the tissue weight) formulated either in distilled water or in 5% glucose were not significantly different but were significantly, threefold, higher compared with gene vectors formulated in HBS ($P < 0.01$). The absolute pDNA doses delivered to the lungs of BALB/c mice varied from 320 ± 10 and 310 ± 30 to 82 ± 5 ng for complexes formulated in distilled water and 5% glucose or HBS, respectively.

We found the highest pDNA doses in the stomach for each of the solvents used for complex formulation (not examined for complexes formulated in distilled water). The pDNA doses per tissue weight delivered to the stomach of the mice were approximately 4-fold higher compared with the lungs. The absolute pDNA doses recovered from the stomach were approximately 5- to 10-fold higher compared with the lungs (0.34–3.77 μg). We found significant doses of pDNA in the small intestine (0.27–1.68 μg) and the colon (0.02–0.10 μg). We found high doses of pDNA on the fur of the carcasses (C1–C3) of the mice with C1 (head) showing the highest pDNA deposition. (The absolute pDNA dose was 3- to 10-fold higher compared with the lungs.) We found very low but detectable doses of pDNA in the blood (7–38 ng), heart (3–20 ng), liver (25–63 ng), spleen (3–6 ng), and kidneys (11–28 ng).

Regional Lung Deposition of PEI–pDNA Complexes after Aerosol Application

We formulated PEI–pDNA complexes in distilled water with fluorescently labeled PEI (FITC–PEI) and nebulized them to the lungs of BALB/c mice. One hour after application, we investigated gene vector deposition in the lungs by fluorescence microscopy. At low magnifica-

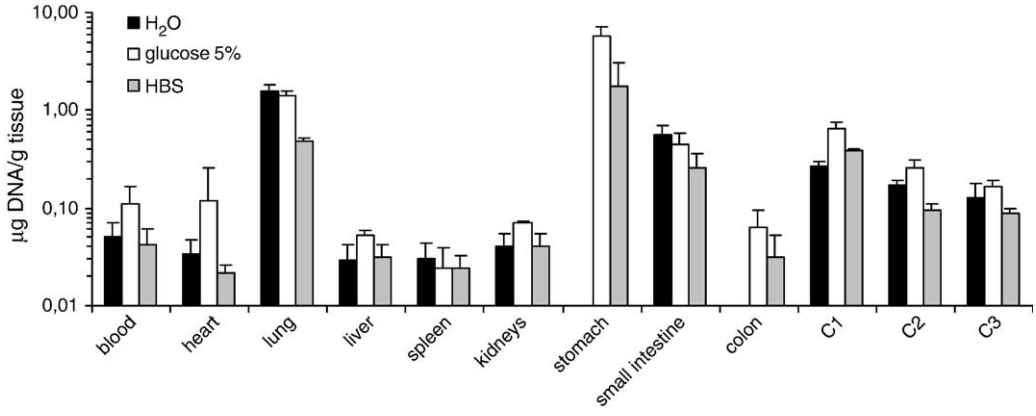

FIG. 4. Biodistribution of aerosolized radioactively labeled PEI–pDNA complexes. A mixture of unlabeled and iodide 123-labeled pDNA (total of 1 mg) was formulated with PEI at an *N/P* ratio of 10 in distilled water, 5% glucose, or HBS and nebulized to BALB/c mice ($n = 3$). After nebulization was complete, mice were killed and the indicated organs were immediately analyzed for radioactivity. Results are reported as means ± standard deviation of the mean.

tion fluorescent spots of small PEI–pDNA complexes were detectable homogeneously distributed in the lungs in the alveolar (Fig. 5A) but not in the bronchial epithelium (Fig. 5B). At high magnification complexes were detectable in both the alveolar (Fig. 5C) and the bronchiolar epithelium (Fig. 5D). Some complexes detected in the bronchial epithelium appeared to be endocytosed by bronchial epithelial cells (Fig. 5D). A large fraction of the PEI–pDNA complexes that were deposited in the alveolar region were phagocytosed by alveolar macrophages (Figs. 5A and 5E; arrowheads). This was further evidenced by double immunostaining using the macrophage-specific antibody F4/80 (data not shown).

Detection of Cells Expressing the Transgene

We formulated a plasmid that codes for a fusion of a nuclear localization sequence (NLS) with β-galactosidase with PEI in distilled water and nebulized it to BALB/c mice. Gene expression was found primarily in the ciliated cells of the bronchial epithelium of the large airways (Figs. 6A and 6B). It has to be mentioned that only the nuclei of the bronchial cells stained positive for β-galactosidase expression due to the NLS function. In addition, few alveolar macrophages expressing β-galactosidase were detected (Fig. 6C). β-Galactosidase expression was not found on control sections of untreated animals.

Comparison of Gene Transfer Efficiency to the Lungs of Mice either by Nebulization or by Intratracheal Instillation

We formulated pDNA doses of 0.35 μg, as this amount was found to be delivered to the lungs of mice via aerosol application (see above), with PEI at an *N/P* ratio of 10 in distilled water, 5% glucose, or HBS and instilled them directly to the lungs of BALB/c mice. At such a low gene vector dose luciferase expression was not detected for PEI–pDNA complexes formulated in distilled water, 5% glucose, nor HBS 24 h after treatment (data not shown).

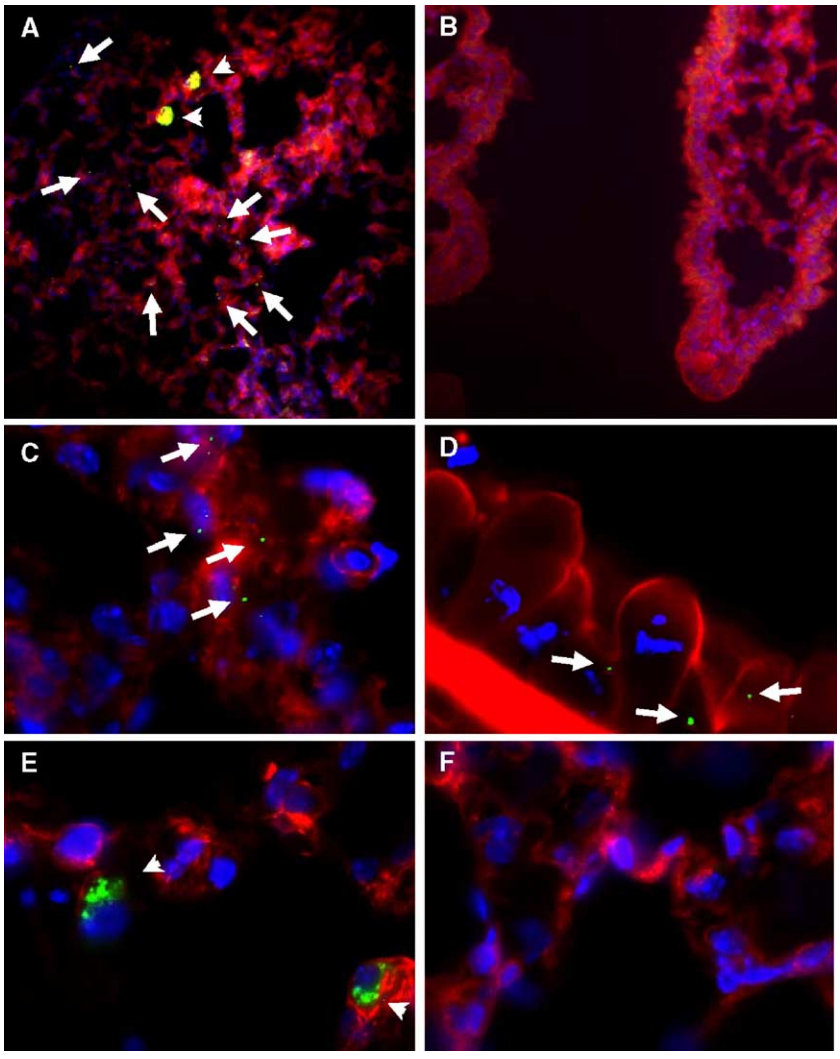

FIG. 5. Regional lung deposition of aerosolized PEI–pDNA gene vectors. FITC-labeled PEI gene vectors (PEI 25 kDa, *N/P* = 10, 1 mg pCMVLuc) formulated in distilled water were nebulized into a plastic box that housed the mice (BALB/c). One hour after nebulization was complete, mice were killed and cryosections of the lungs were analyzed for deposition of FITC–PEI–pDNA complexes. At lower magnifications PEI–pDNA complexes (green, marked by arrows) were detectable (A) within the alveolar epithelium but not (B) within the bronchiolar epithelium. At higher magnification complexes were detected (C) within the alveolar epithelium and (D) the bronchiolar epithelium. The complexes seemed to be endocytosed by the bronchiolar epithelium. Phagocytosis of FITC–PEI–pDNA complexes by alveolar macrophages was observed (A, E; arrowheads). The actin filaments were stained red with Alexa 568–phalloidin (A–F). Control sections receiving unlabeled FITC–pDNA complexes are shown in (F). Nuclei were stained with DAPI (blue). (Original magnifications: A and B, 20×; C, E, and -F, 63×; D, 100×).

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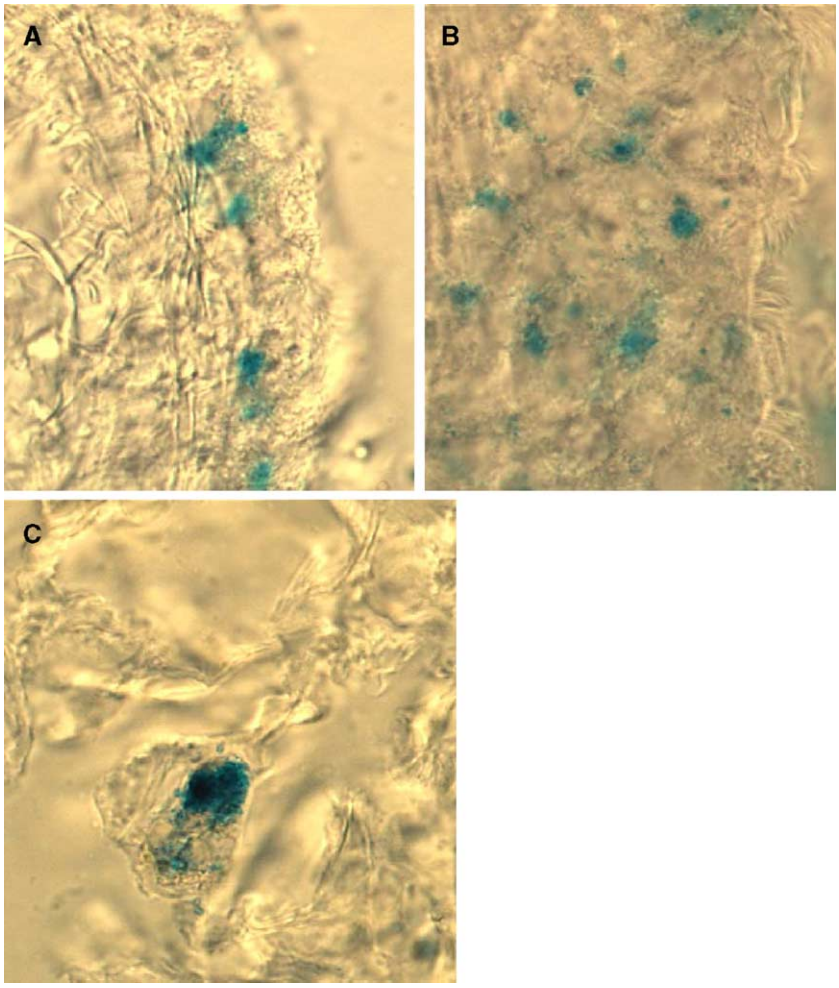

FIG. 6. Regional gene expression in the lungs of mice upon aerosol gene delivery. PEI gene vectors (PEI 25 kDa, *N/P* = 10, 1 mg pVR-1411) were nebulized into a plastic box that housed the mice (BALB/c). The nebulizer was operated with synthetic air containing 5% CO_2. β-Galactosidase expression was examined 72 h after gene vector application. (A, B) Gene expression was found primarily in the ciliated cells of the bronchial epithelium of the large airways. Only the nuclei of the bronchial cells stained positive for β-galactosidase expression due to the NLS function. (C) In addition, a few alveolar macrophages expressing β-galactosidase were detected.

To compare the gene transfer efficiency of the different routes of application, we instilled a higher dose of pDNA (50 μg) complexed to PEI (in distilled water, *N/P* = 10) directly to the lungs of the mice and compared it with gene expression mediated by aerosol application. Gene expression mediated by pDNA formulated with PEI after nebulization was significantly, 2280-fold, higher compared with direct instillation of PEI–pDNA (50 μg) complexes to the lungs of BALB/c mice when normalized to the amount of pDNA applied to the lungs (0.32 μg vs 50 μg, $P < 0.01$, data not shown).

DISCUSSION

In this study we demonstrate that aerosolized nanogram quantities of pDNA complexed to PEI (350 ng) yielded transfection levels 1 order of magnitude higher than microgram quantities of pDNA (50 μg), which were applied directly to the lungs of mice via intratracheal intubation. In terms of dose–effect relationship these results demonstrate that aerosol delivery is 3 orders of magnitude more efficient compared with direct instillation. As a consequence, the results of this study reveal that the mode of gene vector application to the lung, i.e., aerosol delivery vs direct instillation, exerts a pronounced effect on the gene transfer efficiency. To our best knowledge this is the first time that various modes of nonviral gene vector application to the lung were compared directly.

In addition to these important findings, we demonstrate that aerosolized PEI–pDNA complexes formulated in distilled water resulted in up to 2 orders of magnitude higher gene expression in the lungs of mice compared to aerosolized PEI–pDNA complexes formulated in the same manner but using 5% glucose or HBS as solvent. Although the solvent, in particular the ionic strength of the solvent, influenced critical biophysical complex parameters, we suggest that these parameters were not the decisive reason to explain this observation. PEI–pDNA complexes formulated in either distilled water or 5% glucose were indistinguishable from each other both by their external structure, i.e., size, shape, and surface

charge, and by their internal structure, i.e., pDNA binding affinity. Therefore, we suggest that the solvent-dependent effect resulted from a physiologic effect of the solvent on the airway epithelium. It has been reported previously that distilled water could be used for a hypoosmotic shock procedure whereby the apical membrane of the airway epithelium can be made transiently leaky to proteins and macromolecules [5]. This effect, which can be inhibited by indomethacin pretreatment [11], has been successfully used to increase gene expression of naked plasmid pDNA *in vivo* [12]. Indeed, gene expression of indomethacin-pretreated mice was significantly lower than that of not pretreated mice. It has been previously reported [13] that pretreatment of mice with indomethacin did not affect macrophage phagocytic activity, which could affect gene expression due to different particle clearance by phagocytosis. In addition to distilled water-induced permeabilization of the apical membrane, airway swelling is observed [8], which could principally allow paracellular gene vector passage leading to transfection of the airway epithelium from the basolateral side. Such an approach has been successfully used to target viral vectors to the basolateral side of the airway epithelium by pretreatment of mice with compounds affecting tight-junction permeability of the airway epithelium, such as EGTA and polidocanol [14–16]. However, gene expression mediated by gene vectors formulated in either 5% glucose or distilled water did not increase after pretreatment of mice with EGTA or polidocanol, suggesting that paracellular delivery was not the main transfection mechanism.

Although aerosol delivery of PEI–pDNA complexes led to deposition in the lungs of mice, the highest doses were found in the stomach and high doses of pDNA were found even in the small intestine and colon due to swallowing of the complexes. In addition, pDNA was detected in the blood, heart, liver, spleen, and kidneys. Because gene expression was detected only in the lungs, we presume that radioactivity measured in other organs was not caused by intact pDNA but by pDNA metabolites that arose from degradation by the harsh conditions in the stomach. This observation is further supported by the results of Koshkina *et al.,* who did not detect levels of intact pDNA distinguishable from untreated controls in the liver, blood, kidney, spleen, or heart after aerosol treatment with PEI–pDNA gene vectors under comparable conditions by TaqMan-PCR [17].

Gene expression was primarily observed in the bronchial epithelium of the large airways and in a few alveolar macrophages. This gene expression pattern presumably correlated with the biodistribution pattern of the complexes. We suggest that the PEI–pDNA complexes were first homogeneously distributed in the lungs, including the alveolar epithelium, after aerosol application but the majority of the complexes were cleared by the alveolar macrophages, thus not being capable of transfecting the alveolar epithelium with high yields. Since the number of alveolar macrophages is lower in the bronchial epithelium, presumably reduced complex clearance led to higher transfection yields.

To summarize, aerosol gene delivery is more than 3 orders of magnitude more efficient compared with direct intratracheal application. Distilled water represents a critical cofactor for aerosol gene delivery of PEI–pDNA complexes, which is presumably based on distilled water-induced permeabilization of the apical membrane by hypoosmotic shock. With respect to future applications it has to be noted that inhaled nebulized distilled water does not alter lung function parameters in healthy nonasthmatic patients or patients suffering from various diseases, e.g., cystic fibrosis [18], offering its potential for use in human patients. However, increased cellular access of inflammatory mediators, bacterial products, etc., could be limiting, which has to be considered carefully in each case. The findings of this study could be of importance for further preclinical aerosol gene delivery studies using synthetic gene carriers in the future.

Materials and Methods

Chemicals. Branched PEI (average MW 25 kDa) was obtained from Aldrich (Deisenhofen, Germany), dialyzed in water (12–14 kDa MW cutoff), and adjusted to pH 7. Double-distilled endotoxin-free water and 5% glucose solution were purchased from Delta Pharma (Boehringer Ingelheim, Germany). Poly-L-glutamic acid MW 31 kDa (P-4761) was purchased from Sigma (Deisenhofen, Germany).

Plasmid. pCMV-Luc containing the firefly luciferase cDNA driven by the CMV promoter was generously provided by Dr. E. Wagner (Department of Pharmacy, University of Munich, Germany). pVR-1411 containing the β-galactosidase–NLS fusion cDNA driven by the CMV promoter was generously provided by Vical, Inc. (San Diego, CA, USA). Plasmids were amplified and purified by PlasmidFactory (Bielefeld, Germany). The purity (LPS) of this plasmid is ≤0.1 EU/μg pDNA, the amount of supercoiled pDNA ≥ 90% ccc.

Animals. Six- to eight-week-old female BALB/c mice (Charles River Laboratories, Sulzfeld, Germany) were maintained under specific-pathogen-free conditions. Animals were acclimatized for at least 7 days prior to the start of the experiments. All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life.

Aerosol application of PEI–pDNA polyplexes. Formulation of polyplexes, aerosol application, and luciferase measurements were performed as reported previously [2].

Application of PEI–pDNA polyplexes by intratracheal instillation. Gene vector complexes used for *in vivo* experiments were generated as described above. For one mouse, the appropriate amounts of PEI (corresponding to $N/P = 10$) were diluted to 25 μl with the solvent indicated and added to 25 μl of the same solvent containing either 0.35 or 50 μg of pCMVLuc and incubated for 10 min. Intratracheal gene vector application was performed as described by Rudolph *et al.* [19].

Size and ζ potential measurement of PEI–pDNA complexes. The particle size was determined by dynamic light scattering and the ζ potential was determined electrophoretically (ZetaPals; Brookhaven Instruments Corp., Holtville, NY, USA).

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Negative staining of PEI and PEI–pDNA complexes. For electron microscopy, PEI–pDNA complexes were prepared freshly by rapidly mixing equal volumes (20 μl) of PEI (130 μg/ml) and pDNA (100 μg/ml) diluted in water, HBS, or 5% glucose. PEI in water, HBS, or 5% glucose was used as controls. After incubating for 15 min at room temperature, the samples were placed into a well of a Teflon container. Particles were adsorbed for 90 s onto a small piece of carbon film, which was previously evaporated onto freshly cleaved mica, and transferred to a second well filled with 2% uranyl formate (in water) for 2 min [20]. The carbon film with absorbed particles was picked up from the staining solution by putting an EM grid covered with a perforated carbon film on top of the floating carbon film. A second small piece of carbon film was floated into another well filled with 2% uranyl formate and placed on top of the particles by submerging the EM grid under the second carbon film [21]. Thus the particles were embedded in a stain-buffer layer sandwiched between two carbon films. After blotting, the grid was dried at room temperature. For imaging, a Philips CM200 FEG electron microscope operated at 200 kV at a magnification of 38,000× and a defocus of about 2–3 μm was used. Images were taken at room temperature on a 1 k × 1 k CCD (charge-coupled device) camera or 4 k × 4 k CCD camera (TVPIS, Gauting, Germany) with fourfold binning. Particle sizes were determined using the image software of TVIPS.

Native cryo preparation of vitrified specimens. PEI–pDNA complexes diluted in water or HBS were prepared as described above. Five microliters of the solutions was pipetted onto an EM grid covered with a perforated carbon film, which was glow-discharged prior to usage. After excess liquid was blotted, the EM grid was plunged into liquid ethane and stored in liquid nitrogen until EM analysis [22]. The samples were analyzed at liquid nitrogen conditions by taking images of PEI–pDNA complexes embedded in amorphous ice using the Philips CM200 FEG electron microscope. Images were taken on both the 1 k × 1 k CCD camera and Kodak SO 163 film. Negatives were scanned with a rotating-drum scanner (Tango, Heidelberger Druckmaschinen, Germany) at 1200 dpi.

Fluorescence quenching assay. Plasmid pDNA (pCMVLuc) was labeled with the bisintercalating dye TOTO-1 at a base-pair-to-dye ratio of 20:1. The experiments were performed in 96-well plates. Equal volumes of TOTO-1-labeled pDNA (0.25 μg in 50 μl of solvent per well) and PEI (0.33 μg in 50 μl of solvent per well) were diluted in each of the solvents and mixed by pipetting the TOTO-1-labeled pDNA into the PEI dilution, resulting in PEI–pDNA complexes at an *N/P* ratio of 10. The complexes were incubated for 10 min at room temperature. Subsequently, serial diluted poly-L-glutamic acid (25–0.1 μg in 50 μl of solvent per well) was added to the complexes and the fluorescence intensity was measured using a Wallac Victor^2/1420 Multilabel Counter (Perkin–Elmer, Rodgau-Jügesheim, Germany).

Histology. PEI (15 mg dialyzed against distilled water) was diluted to 300 μl with Na_2CO_3 (pH 9) and reacted with a 10-fold molar excess of 5(6)-carboxyfluorescein (Sigma) for 1.5 h and purified using disposable PD-10 Sephadex G-25 columns (Amersham Biosciences Europe GmbH, Freiburg, Germany). The degree of modification was found to be ~3 based on photometric measurements of FITC and quantitation of PEI using a copper-based assay [23].

FITC–PEI–pDNA complexes were generated as described above and nebulized to the mice. One hour after application mice were anesthetized intraperitoneally with pentobarbital and the peritoneum was opened by a midline incision. After cardiac perfusion with 10–15 ml of a heparinized isotonic sodium chloride solution (25,000 IE heparin/1000 ml), lungs of mice were fixed by intratracheal application with 4% paraformaldehyde in PBS and were kept inflated for 1 h at room temperature using fine cotton tied around the trachea. The lungs were removed *en bloc* and after 5 h incubation in fixative at 4°C, the tissue was rinsed in PBS and stored overnight in 30% sucrose in PBS at 4°C. The lungs were frozen the next day in OCT compound (Tissue Tek, Sakura Finetek Europa) on dry ice and stored at −80°C. Cryosections (6 μm) were cut, stained briefly with Alexa 468-phallotoxin (1:200 in PBS containing 1% BSA, 20 min at room temperature), and mounted (Vectashield; Vector Laboratories Inc., Burlingame, CA, USA). An epifluorescence Axiovert 135 microscope (Zeiss, Jena, Germany) with a 63× or 100×/1.30 oil plan-neofluor objective (Zeiss) was used for microscopy. For β-galactosidase histochemistry, cryosections were incubated in X-gal staining solution (1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM K-ferricyanide, 5 mM K-ferrocyanide in PBS, pH 7.4) at 37°C overnight.

Radioactive labeling of pDNA with ^{123}I and analysis of biodistribution. Plasmid DNA (pCMVLuc, 150 μg) was radioactively labeled with ^{123}I according to the method of Commerford as described in detail by Terebesi *et al*. [24]. The labeling mixture was separated using a PD-10 gel filtration column (Amersham Biosciences) with water as eluent. The iodinated pDNA was mixed with unlabeled pDNA resulting in a total of 1 mg of pDNA, which was formulated with PEI as described above and nebulized to the mice. Directly after nebulization was completed, mice were killed and radioactivity of the organs as indicated was measured using a gamma counter (Wallac 1480 Wizard 3″; Perkin–Elmer Wallac, Freiburg, Germany).

Statistical analysis. Results are reported as means ± standard deviations. The statistical analyses between different groups were determined with an unpaired *t* test. Probability (P) ≤ 0.01 was considered significant. All statistical analyses were performed using the program StatView 5.0 (SAS Institute, Inc., Cary, NC, USA).

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