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In vivo gene delivery in the mouse lung with lactosylated polyethylenimine, questioning the relevance of *in vitro* experiments

Stéphanie Grosse ^a, Guiti Thévenot ^a, Yolande Aron ^a, Eric Duverger ^b, Mohamed Abdelkarim ^a, Annie-Claude Roche ^b, Michel Monsigny ^b, Isabelle Fajac ^{a,*}

^a Université Paris Descartes, Faculté de Médecine, AP-HP, Hôpital Cochin, EA 2511, Paris, France

^b Glycobiologie, Centre de Biophysique Moléculaire, CNRS et Université d'Orléans, Orléans, France

Polyethylenimine (PEI) is an efficient vector for *in vitro* and *in vivo* gene transfer into respiratory cells. Glycosylated PEIs were shown to enhance *in vitro* gene transfer by favoring the complex entry into the airway cells. The aim of our study was to evaluate the *in vivo* efficiency of gene transfer mediated by glycosylated PEIs in the mouse lung and to determine the transfected cell type and the intracellular trafficking of the complexes. Upon nasal instillation in mice of complexes made with various glycosylated PEIs, a high luciferase activity was observed while the green fluorescent protein (GFP) expression was similar for all the vectors tested with few cells expressing GFP. Complexes made with lactosylated PEI were then labeled and their localization studied by confocal microscopy. In the lungs, large numbers of complexes were taken up by epithelial cell which were mostly alveolar cells. In the airways, complex uptake varied greatly, depending on the area observed. Eight hours upon nasal instillation and in contrast with the *in vitro* situation, a dissociation between the plasmid DNA and the lactosylated PEI was usually observed, leading to the plasmid mostly localized in lysosomes and the Lac-PEI localized in the nucleus. These results emphasize the need to engineer a plasmid able by itself to overcome the nuclear barrier and to quickly move to *in vivo* experiments to select the best carrier.

1. Introduction

Polyethylenimine (PEI) is one of the most efficient nonviral vectors for *in vitro* and *in vivo* gene delivery [1,2]. Like other synthetic vectors, PEI provides high and easy compaction of plasmid DNA. Its chemical structure is an added advantage. Every third atom is a potentially protonable amino nitrogen that allows a "proton sponge effect" leading to an endosomal exit of plasmid/PEI complexes taken up by the cells [3,4]. PEI has been successfully tested for gene transfer into a number of organs and it was found to have limited toxic or immune effects [1,2]. It is mainly available in two forms, a linear 22 kDa and a branched 25 kDa form. They have both been studied for gene transfer into the respiratory tract. They were shown to be efficient vehicles for gene transfer into respiratory epithelial cells *in vivo* with higher efficiency than that of cationic lipids [5,6]. However, the efficiency was highly dependent of the physiochemical properties of plasmid/PEI complexes and of biological phenomena which are not yet fully

understood. Many studies were devoted to optimize the formulations of complexes made with PEI and the routes of delivery such as intravenous, aerosol or intranasal delivery [7–13]. The transfected cell types have also been studied. After intratracheal administration, linear PEI 22 kDa mainly transfected the alveolar regions and the distal tract of the bronchial tree, whereas branched 25 kDa PEI allowed gene transfer into the bronchial cells [5]. Unlike *in vitro* studies which have been largely devoted to describe the intracellular trafficking of the plasmid after its uptake by the cell [14,15], the intracellular route of the plasmid after its uptake by the cell *in vivo* is unknown.

To promote specific cellular uptake of complexes and hopefully efficient intracellular trafficking, we have substituted the branched 25 kDa PEI with sugar residues [16]. Because our ultimate goal is cystic fibrosis gene therapy, we have studied various glycosylated PEIs *in vitro* in airway epithelial cells and showed that lactosylated PEI (Lac-PEI) was the most efficient glycosylated PEI for gene transfer [16]. It was also less toxic than unsubstituted PEI [17]. Therefore, we wanted to assess the potential of glycosylated PEIs as vectors for *in vivo* gene transfer and studied their efficiency after nasal instillation in mice. To further our understanding of glycosylated PEI-mediated gene transfer *in vivo*, the plasmid and the vector were independently labeled in order to identify the cells able to take up the complexes and to study the intracellular trafficking of the complexes.

* Corresponding author. Lab. Physiol. Respir., Faculté Paris Descartes, site Cochin, 24 rue du Fg St-Jacques, 75679 Paris Cedex 14, France. Tel.: +33 1 44 41 23 30; fax: +33 1 44 41 23 33.

E-mail address: ifajac@cochin.univ-paris5.fr (I. Fajac).

2. Materials and methods

2.1. Polyethylenimine derivatives and gene transfer procedure

Five percent of the amino groups of PEI (25 kDa, branched polymer; Sigma, St Louis, MO, USA) were substituted by a thiocarbonyl residue of either β -D-Lac (β -D-lactose), β -D-Glc (β -D-glucose) or α -D-Man (α -D-mannose). Briefly, to a solution of PEI dissolved in water (10 mg/ml), glycosyl phenylisothiocyanate (12 μ mol) dissolved in 1 ml of a 1:1 ethanol/water mixture was added and the solution was stirred for 30 min at room temperature. Under such conditions, the free glycosyl phenylisothiocyanate was no longer detectable upon thin-layer chromatography on silica gel plates using a 100:30:10:10:3 ethanol/water/*n*-butanol/pyridine/acetic acid mixture. Upon removal of the ethanol, the water solution was freeze-dried [16]. Fluorescein-conjugated lactosylated PEI (Lac-PEI) was prepared by using fluorescein isothiocyanate as previously described [16].

The requested amount of glycosylated PEI or unsubstituted PEI (25 kDa, branched form or 22 kDa, linear form, ExGen®) was mixed with 50, 100 or 150 μ g of the plasmid in a final volume of 150 μ l of various formulation solutions in order to reach the requested N/P ratio (from 2.5 to 15). The plasmids used were pCMVLuc (pUT 650, 5.15 kb) (Cayla, Toulouse, France) and pCMVGFP (pGFPemv-cmv; 4.80 kb) (Packard, Meriden, CT, USA) which include genes encoding the firefly luciferase and the green fluorescent protein (GFP), respectively, under the control of the human cytomegalovirus promoter.

Five-week-old female BALB/c mice (Harlan, Gannat, France) were briefly anesthetized with halothane (Belamont, Paris, France) and instilled intranasally with 150 μ l of complexes using a pipetman (P200 Gilson). At the time of the gene transfer analysis step, the animals were killed by an intraperitoneal overdose of pentobarbital. The circulation was perfused with 4% paraformaldehyde via the right ventricle and the lungs and trachea were dissected and kept on ice up to their analysis. The study was performed in accordance with the European Community Guidelines and complied with the procedures of our IFR (Institut Fédératif de Recherche Alfred Jost).

2.2. Gene expression

When luciferase expression was studied, the lungs and trachea were disrupted in a lysis buffer (8 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 15% glycerol, 1% Triton X-100, 25 mM Tris HCl at pH 7.8) using an Ultra-Turrax T25 homogenizer (Fischer Bioblock Scientific, Strasbourg, France). The cell lysate was centrifuged for 10 min at 10,000 g and luciferase gene expression was measured by luminescence according to de Wet et al. [18]. The luminescence generated upon automatic addition of 150 μ l of 167 μ M luciferin dissolved in water was recorded in duplicate samples for 10 s by using a luminometer (Lumat LB 9501, Berthold, Wildbach, Germany). The luminescence is reported as relative light units (RLU). Protein concentrations in each sample were determined using the bicinchoninic acid (BCA) colorimetric method modified according to Hill and Straka [19]. Results are expressed as RLU per mg of proteins.

When the pCMVGFP plasmid was used, the lungs and trachea were fixed in 4% paraformaldehyde and frozen in OCT compound over liquid nitrogen. Seven- μ m thick cryosections were cut, mounted in vectashield solution (Vector Laboratories, Burlingame, CA, USA) and examined for GFP expression with a Leitz epifluorescence microscope (Leica, Rueil-Malmaison, France). Lung and airway morphology was examined on adjacent sections stained with hematoxylin and eosine following standard procedures.

2.3. Complex localization in airways and lungs

Mice were instilled with 150 μ l of complexes made of pCMVLuc (100 μ g) and fluoresceinylated Lac-PEI (N/P=12.5) in a 5% glucose

solution. Mice were killed 48 h later and the dissected lungs and trachea fixed in 4% paraformaldehyde. Tissue cryosectioned 7- μ m thick slides were mounted in Vectashield mounting medium with propidium iodide (Vector laboratories) and examined with a Leitz epifluorescence microscope (Leica). In order to determine in which cells complexes were localized, tissue cryosectioned 7- μ m thick slides were incubated for 10 min with 0.1 M glycine in phosphate-buffered saline (PBS), for 15 min with 0.2% mouse serum albumin and 0.2% tween in PBS and then stained using antibodies raised against mouse surfactant protein B (Chemicon, Temecula, CA, USA) or F4/80 antigen (Abcam, Cambridge, UK) for alveolar type II cells or alveolar macrophages, respectively. Sections were then incubated with Cy5-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), mounted in Vectashield solution and examined with a TCS SP2 AOBs Leica confocal system mounted on a Leica DMIRE2 inverted microscope.

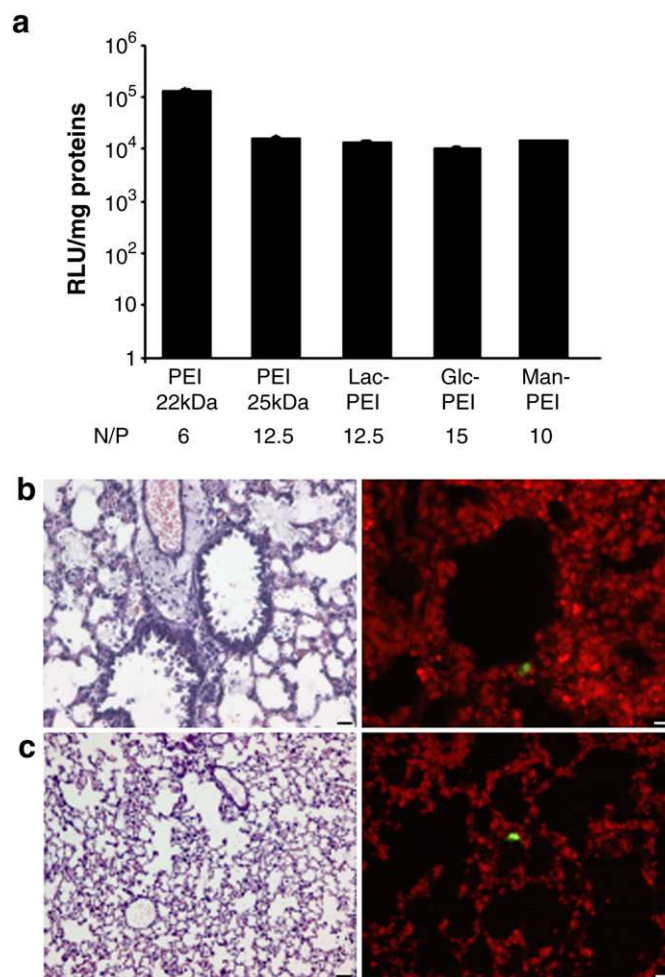


Fig. 1. Gene transfer with cationic polymers into the mouse lung. a: Optimal PEI-mediated luciferase expression in the mouse lung. The requested amount of cationic polymer, i.e. 22 kDa linear PEI, 25 kDa branched PEI, lactosylated PEI (Lac-PEI), glycosylated PEI (Glc-PEI) and mannosylated PEI (Man-PEI), was mixed with 100 μ g of a plasmid encoding the firefly luciferase protein in a 5% glucose solution. Complexes were administered intranasally to BALB/c mice and 48 h later, the animals were killed, the lungs and trachea dissected and luciferase expression studied. (the optimal N/P ratio is indicated). b and c: Lac-PEI-mediated GFP expression in the mouse bronchi and lung, respectively. A plasmid encoding the GFP protein was complexed with Lac-PEI (N/P=6) and administered intranasally to mice. Forty-eight hours later, mice were killed, the lungs and trachea dissected, fixed and cryosectioned. GFP expression appears green and the cell nuclei stained with propidium iodide, red in the right-hand panel (b: bar = 10 μ m; c: bar = 20 μ m). Adjacent sections stained with hematoxylin and eosine are shown on the left-hand panel (b: bar = 20 μ m; c: bar = 100 μ m).

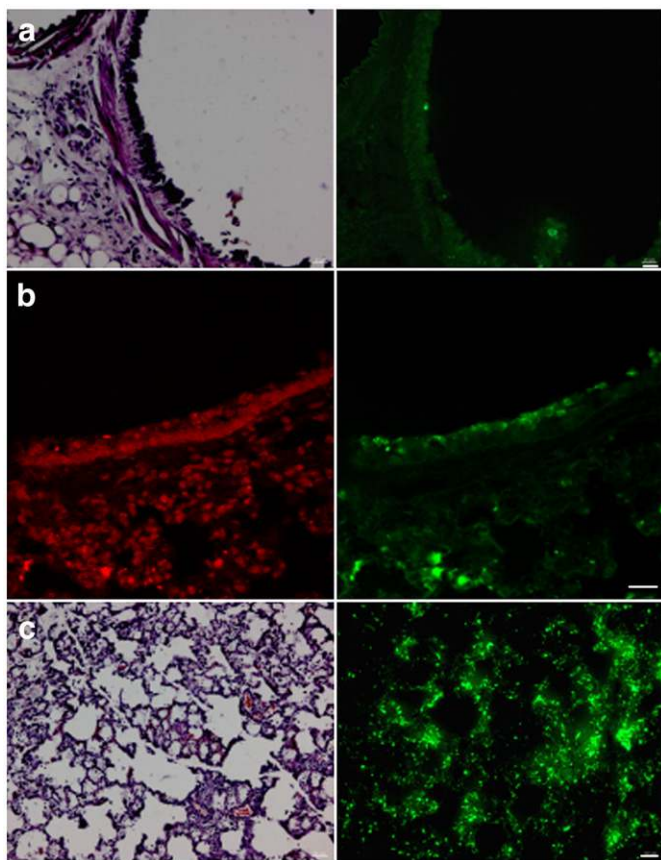


Fig. 2. Distribution of Lac-PEI complexes in the mouse bronchi (a and b) and lung (c). Complexes were made with fluoresceinylated Lac-PEI and a plasmid encoding the firefly luciferase protein and instilled intranasally into mice. Animals were killed 48 h later and the biodistribution of complexes was analyzed on cryosectioned slides. The left-hand panels show tissue sections stained with hematoxylin and eosin (a and c) or with propidium iodide (b). The right-hand panels show the same area of an adjacent section and the fluoresceinylated complexes which appear green: distribution in the airway epithelium was uneven, from very few (a) to numerous complexes (b). In contrast, numerous Lac-PEI complexes were seen inside the lungs (c). (a and b: bar=20 μ m; c: bar=100 μ m).

2.4. Intracellular trafficking of complexes

The pCMVLuc plasmid was biotinylated using the FastTag nucleic acid labeling system (Vector Laboratories). Mice were instilled with 150 μ l of complexes made of biotinylated pCMVLuc and fluoresceinylated Lac-PEI (N/P=12.5 in a 5% glucose solution). To verify if the trafficking was modified by the labeling of plasmid or Lac-PEI, some mice were also instilled with complexes made with unlabeled pCMVLuc and fluoresceinylated Lac-PEI or with complexes made with biotinylated pCMVLuc and unlabeled Lac-PEI. Three, 8 or 24 h later, the animals were killed and the lungs were excised, fixed in 4% paraformaldehyde and cryosectioned in 7- μ m thick sections in order to study the intracellular localization of complexes. Slides were incubated for 10 min with 0.1 M glycine in PBS, for 15 min with 0.2% mouse serum albumin and 0.2% tween in PBS and then the biotinylated plasmid DNA was labeled with a streptavidin conjugate (Molecular Probes, Eugene, OR, USA). Lysosomes were immunolabeled with rat antibodies raised against the lysosomal-associated membrane protein 1 (LAMP-1) (BD Biosciences, San Jose, CA, USA) followed by Qdot® 605 anti-rat antibodies (Molecular Probes). The nuclear membrane was immunolabeled with rabbit antibodies directed against lamin A/C (Cell Signaling Technology, Danvers, MA, USA) followed by Cy5-conjugated anti-rabbit antibodies (Jackson Immuno

Research). The localizations of plasmid DNA and Lac-PEI were then analyzed by confocal microscopy. Serial sections collected at increments of 0.5- μ m thick were used to define the intracellular localization of plasmid DNA/lactosylated PEI complexes. The images obtained were processed with ImageJ, 1.34s software.

3. Results

3.1. Gene transfer efficiency

The efficiency of gene transfer into the mouse lungs and airways was studied for five vectors: 22 kDa PEI (linear form, ExGen®), 25 kDa PEI (branched from) and three glycosylated branched PEI: lactosylated PEI (Lac-PEI), glucosylated PEI (Glc-PEI) and mannosylated PEI (Man-PEI). These sugar residues were chosen on the basis of their efficiency for *in vitro* gene transfer into airway epithelial cells (Lac-PEI and Glc-PEI) or of their high uptake by these cells (Man-PEI) [16].

Various conditions of gene transfer were studied for each vector: the plasmid dose varied from 50 to 150 μ g, the N/P ratio from 2.5 to 15, the formulation solutions were water, 9% NaCl or 5% glucose and the time of study 24 or 48 h after nasal instillation. For all the vectors tested, no luciferase activity was observed in the trachea. In the lungs, for all 25 kDa PEI derivatives, the highest luciferase activity was observed 48 h after complex instillation and with complexes generated in a 5% glucose solution, using 100 μ g of plasmid (Fig. 1a). For each PEI derivative, the optimal N/P ratio was in the 10 to 15 range. A 10-fold higher luciferase activity was observed with complexes made with linear 22 kDa PEI (100 μ g of plasmid, N/P=6 in 5% glucose solution), as compared with that obtained with branched 25 kDa PEI derivatives ($p < 0.01$, Mann-Whitney *U*-test). However, when GFP expression was studied, it was similar for all

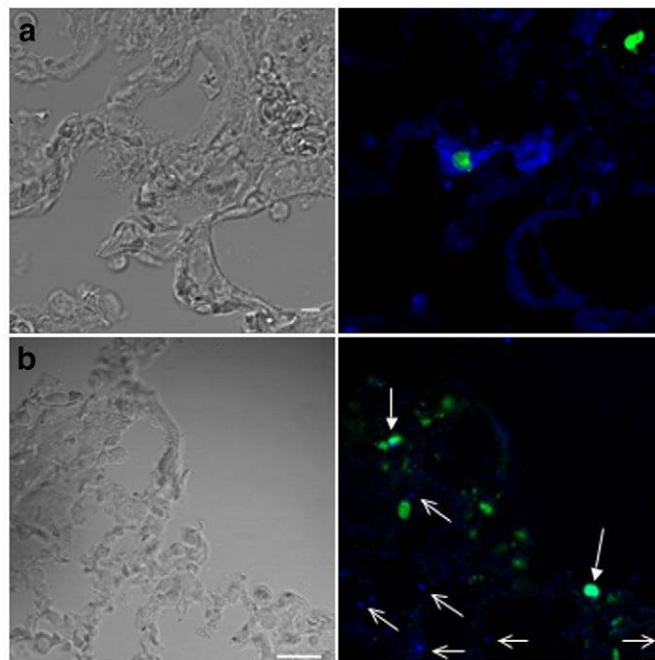


Fig. 3. Determination of cells that have taken up Lac-PEI complexes. Complexes were made with fluoresceinylated Lac-PEI and a plasmid encoding the firefly luciferase protein and instilled intranasally into mice. Animals were killed 48 h later and alveolar macrophages (a) or alveolar type II cells (b) were immunolabeled on lung cryosectioned slides. a: On the left-hand panel, phase-contrast image of cells; on the right-hand panel, merged image of cells with two alveolar macrophages (in blue) and of fluoresceinylated complexes (in green): complexes were occasionally observed in alveolar macrophages (in the middle), but some were outside macrophages (in the upper right corner). (bar=5 μ m) (b) On the left-hand panel, phase-contrast image of cells; on the right-hand panel, merged image of alveolar type II cells (in blue, open arrows) and of fluoresceinylated complexes (in green). Closed arrows show complexes in alveolar type II cells. (bar=20 μ m).

the vectors tested: few cells expressed GFP and they were more often observed in the lungs than in the airway epithelium (Fig. 1b and c).

When mice received only 5% glucose, no death was registered. Depending on the vectors, the number of mice studied varied from 20 for Man-PEI to 40 for 22 kDa PEI and 115 for Lac-PEI. Mice were occasionally found dead in the 24 h following nasal instillation. The mortality rate registered 24 h after administration was 7.1% for 22 kDa PEI, 8.6% for 25 kDa PEI, 0.8% for Lac-PEI, 2.7% for Glc-PEI and 10.5% for Man-PEI. Deaths were usually observed when complexes at the highest N/P ratios have been administered. With all the investigated vectors at the most efficient complex formulation, the lung morphology was found to be normal: specially, neither oedema nor inflammatory cell recruitment could be observed (Fig. 1b and c).

3.2. Complex localization in airways and lungs

In order to further our understanding of glycosylated PEI-mediated gene transfer *in vivo*, we labeled one glycosylated PEI and studied the localization of the complexes and their intracellular trafficking. Since no glycosylated PEI was clearly more efficient than the others, we chose to study Lac-PEI because it was the most efficient one *in vitro* and we had studied its *in vitro* intracellular trafficking [17]. Complex distribution into the epithelium of large airways was uneven (Fig. 2a and b). This was not dependent on the mouse studied, nor on some localization in the airways. In contrast, numerous Lac-PEI complexes were always observed in alveolar spaces (Fig. 2c). To identify cells in

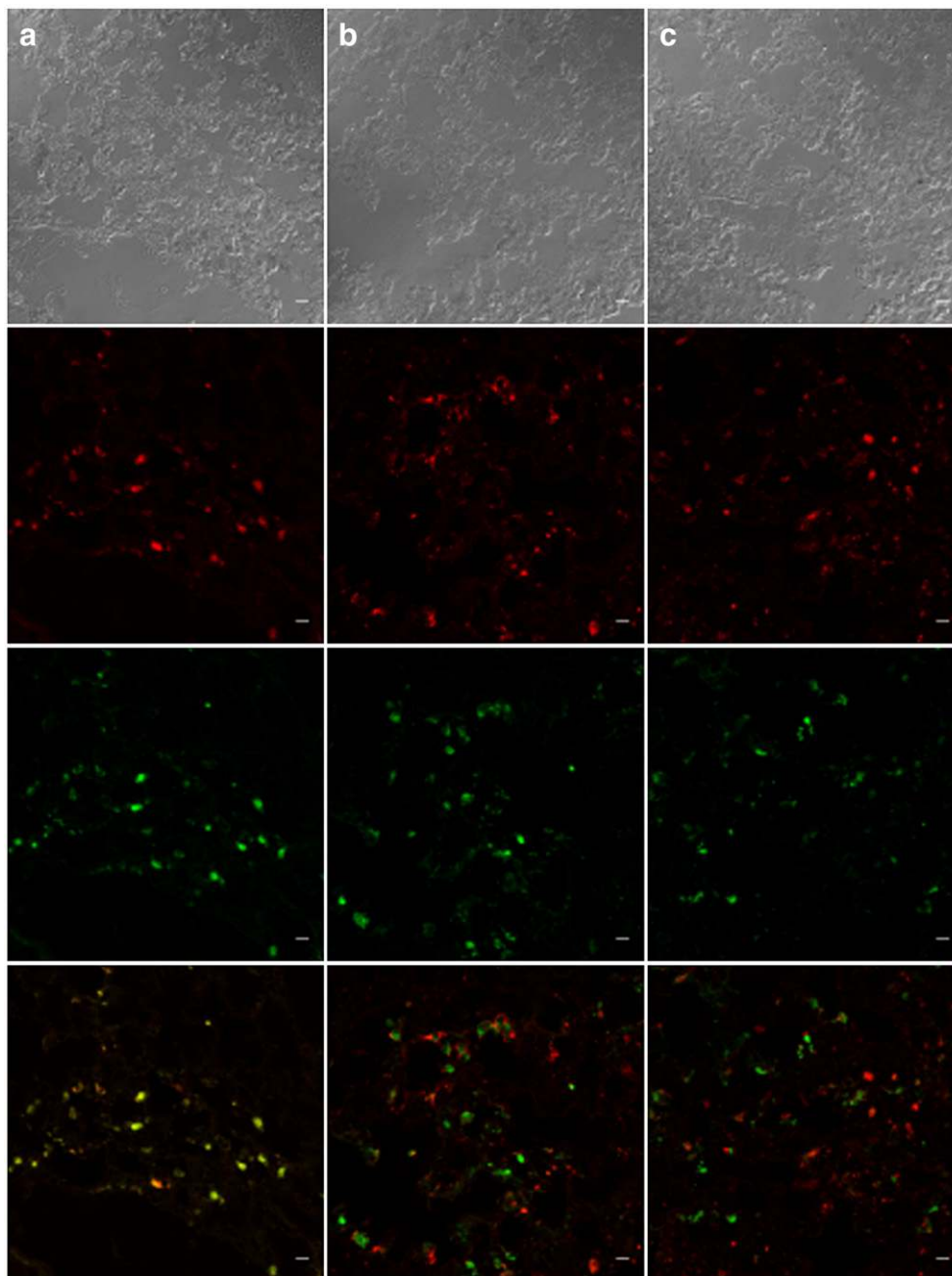


Fig. 4. Dissociation of plasmid DNA/Lac-PEI complexes. Complexes were made with fluoresceinylated Lac-PEI and a biotinylated plasmid and instilled intranasally into mice. Three (a), 8 (b) and 24 (c) hours later, animals were killed, and pulmonary tissues were analyzed by confocal microscopy after labeling of the biotinylated plasmid DNA with Qdot 655®-coupled streptavidin. From top to bottom: phase-contrast image of cells; the plasmid DNA which appears red; the fluoresceinylated Lac-PEI which appears green and merged images with complexes that appear yellow (bar = 10 μ m).

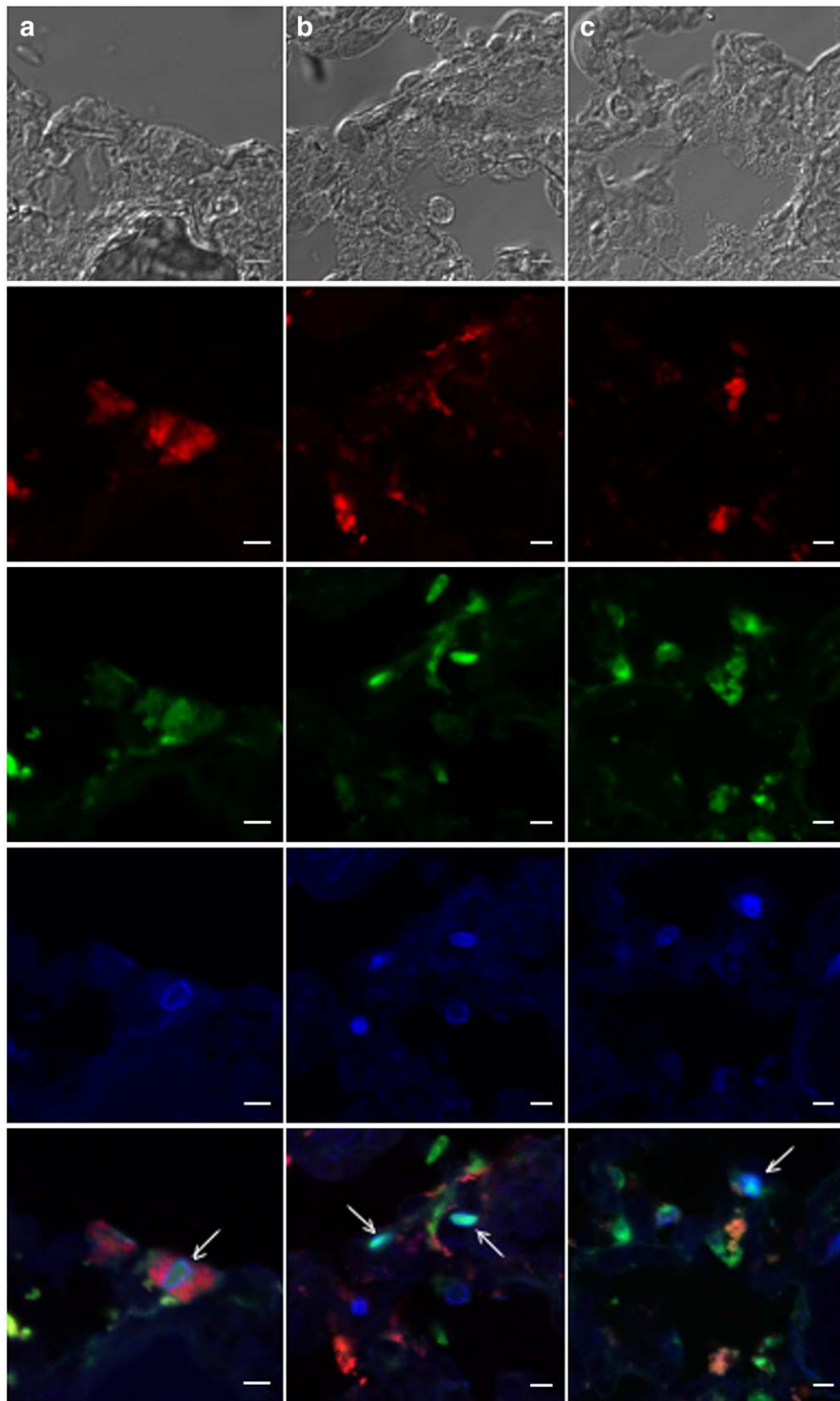


Fig. 5. Nuclear localization of plasmid DNA/Lac-PEI complexes. Complexes were made with fluoresceinylated Lac-PEI and a biotinylated plasmid and instilled intranasally into mice. Three (a), 8 (b) and 24 (c) hours later, animals were killed, and pulmonary tissues were analyzed by confocal microscopy after labeling of the biotinylated plasmid DNA with alexa fluor 555-coupled streptavidin and of the nuclear membrane, with anti-lamin A/C antibodies. From top to bottom: phase-contrast image of cells; the plasmid DNA which appears red; the fluoresceinylated Lac-PEI which appears green, the labeling of the nuclei which appear blue and merged images of the plasmid DNA, the Lac-PEI and the labeled nuclei. The arrows show the nuclear localization of Lac-PEI (bar=5 μ m).

which complexes were present, alveolar type II cells and alveolar macrophages were labeled. Complexes were only occasionally observed in alveolar macrophages (Fig. 3a) and in alveolar type II cells (Fig. 3b).

3.3. Intracellular trafficking of complexes

Despite numerous complexes in alveolar cells, the efficiency of gene transfer was quite low, with only a limited number of cells expressing GFP. This suggested that the intracellular trafficking of complexes was inefficient.

In order to evaluate whether the plasmid DNA remained as a complex or as a vector-free molecule within the cell, complexes were made with a biotinylated plasmid and fluoresceinylated Lac-PEI. Three hours upon nasal instillation, the plasmid DNA was always observed as a complex with Lac-PEI (Fig. 4a). Eight and 24 h upon nasal instillation, dissociation between the plasmid DNA and Lac-PEI was observed for most complexes (Fig. 4b and c).

The nuclear localization of the biotinylated plasmid DNA and the fluoresceinylated Lac-PEI was studied after labeling the internal nuclear membrane. Three hours upon nasal instillation, a nuclear localization was seldom observed and mostly occurred for Lac-PEI (Fig. 5a). Eight hours upon nasal instillation, Lac-PEI was clearly localized inside the nucleus while the plasmid was not (Fig. 5b). Twenty-four hours upon nasal instillation, both plasmid DNA and Lac-PEI could be observed in the nucleus, but to a low extent (Fig. 5c). The nuclear localization of plasmid DNA and Lac-PEI was similar when the mice that have received complexes made with unlabeled pCMV Luc and fluoresceinylated Lac-PEI or complexes made with biotinylated pCMV Luc and unlabeled Lac-PEI were studied.

The lysosomal localization of the biotinylated plasmid DNA and the fluoresceinylated Lac-PEI was then studied, lysosomes being labeled with an antibody recognizing LAMP-1, a lysosomal glycoprotein. Three hours upon nasal instillation, complexes were very seldom observed in lysosomes. Eight hours upon nasal instillation, lysosomal localization was frequently observed and mostly occurred for the plasmid DNA (Fig. 6a). Twenty-four hours upon nasal instillation, the localization in lysosomes was low (Fig. 6b). The lysosomal localization of plasmid DNA and Lac-PEI was similar when the mice that have received complexes made with unlabeled pCMV Luc and fluoresceinylated Lac-PEI or complexes made with biotinylated pCMV Luc and unlabeled Lac-PEI were studied.

4. Discussion

Several authors have demonstrated that unsubstituted linear 22 kDa PEI or branched 25 kDa PEI are suitable vehicles for gene transfer into respiratory epithelial cells *in vivo* in mice with a higher efficiency than cationic lipids [5,6]. *In vitro*, the presence of sugar residues on glycosylated PEI allowed the targeting of cell-surface sugar-specific receptors leading the complexes to bind the cell surface with a higher efficiency and to be more taken up inside the cells [16]. Moreover, *in vitro* in airway epithelial cells, Lac-PEI was less toxic and more efficient than unsubstituted PEI [17]. We here show for the first time that glycosylated PEIs are as efficient as

unsubstituted PEI for *in vivo* gene transfer into the mouse lungs upon nasal instillation and that they display low toxicity. Moreover, we have studied the intracellular trafficking of complexes upon nasal

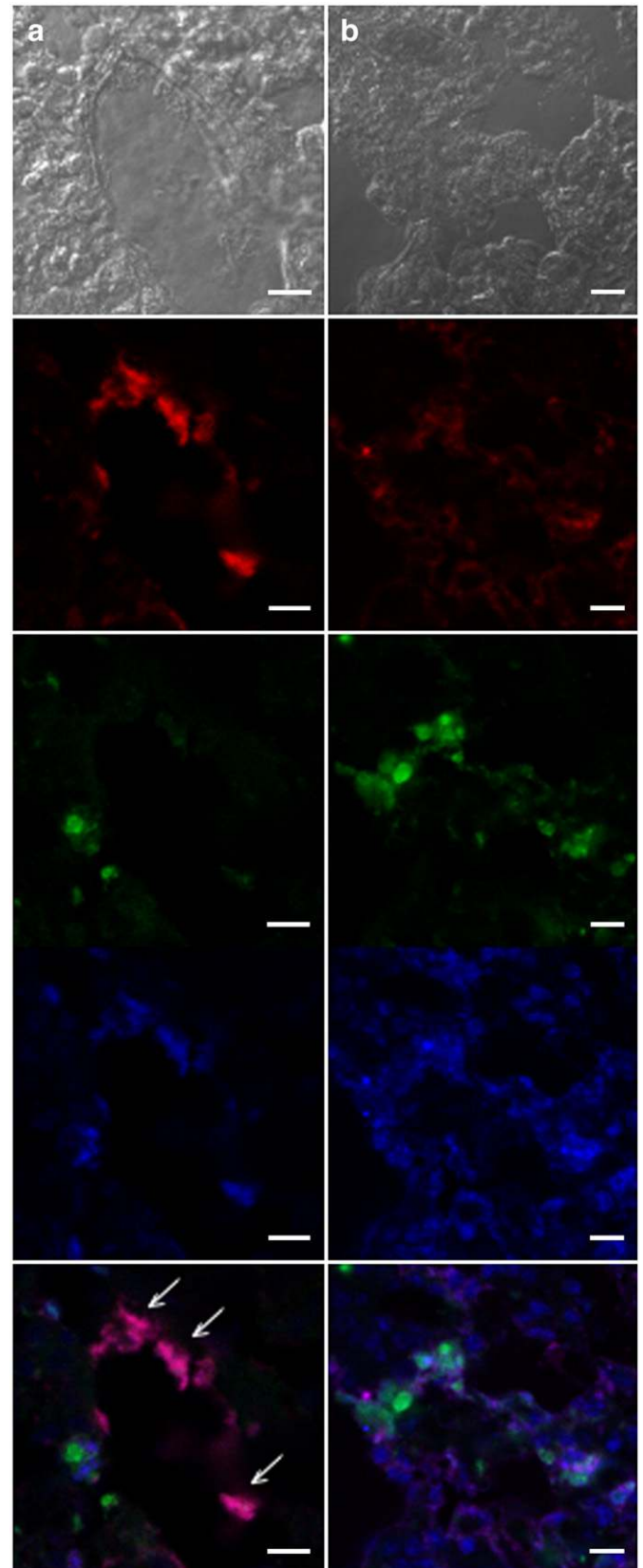


Fig. 6. Lysosomal localization of plasmid DNA/Lac-PEI complexes. Complexes were made with fluoresceinylated Lac-PEI and a biotinylated plasmid and instilled intranasally into mice. Eight (a) and 24 (b) hours later, animals were killed, and pulmonary tissues were analyzed by confocal microscopy after labeling of the biotinylated plasmid DNA with Qdot 655®-coupled streptavidin and lysosomes, with anti-LAMP1 antibodies. From top to bottom: phase-contrast image of cells; the plasmid DNA which appears red; the fluoresceinylated Lac-PEI which appears green; the labeling of lysosomes which appear blue and merged images of the plasmid DNA, the Lac-PEI and the labeled lysosomes. The arrows show the lysosomal localization of the plasmid DNA (bar = 10 μ m).

instillation and showed that it is quite different than the one described *in vitro*.

The level of luciferase activity we obtained was similar to that described by Bragonzi et al. [5] upon intratracheal instillation in mice of complexes made with unsubstituted PEI. The optimal formulations we found (complexes generated in a 5% glucose solution, with 100 µg of plasmid and an N/P ratio between 10 and 15) are those described by others [2,20]. Glycosylated PEIs made with branched 25 kDa PEI were as efficient as the unsubstituted polymer and, in contrast with *in vitro* experiments, there was no difference in the efficiency of the various glycosylated polymers. This might be due to differences in the physico-chemical properties of the complexes made for *in vivo* administration as compared with those made for *in vitro* experiments: for *in vivo* experiments, the amounts of plasmid (and of PEI derivative) were higher, the N/P ratios were sometimes a little higher too and this might lead to high nonspecific uptake. However, despite a luciferase activity between 10⁴ and 10⁵ RLU/mg proteins, the number of GFP-expressing cells was quite low for all PEI derivatives. This discrepancy between relatively high levels of luciferase expression and low numbers of GFP-expressing cells has been previously reported [21]. It was attributed to either a GFP expression below the detection limit in any given cell, to a differential expression level of the luciferase and GFP genes present in the plasmid, or to a high level of luciferase expression in a few lung cells. In any case, although direct visualisation of GFP expression is simple and allows the localization of gene expression, more sensitive methodologies for the detection of nonviral *in vivo* gene transfer is needed.

Despite numerous reports concerning the use of PEI for nonviral gene transfer into the mouse lung [1,2], few have identified the type of the transfected cell upon nasal instillation [5,10,21,22] and, to our knowledge, none has investigated the intracellular trafficking of the plasmid DNA. We chose to further study the Lac-PEI vector because it was the most efficient glycosylated PEI for *in vitro* gene transfer into airway epithelial cells [16]. By labeling the complexes and some respiratory cell types, we have determined that macrophages which are the first line of defence in the lungs do not take up most of the complexes. In contrast, complexes were observed in epithelial cells. However, these were mostly epithelial alveolar cells which are of poor interest for cystic fibrosis gene therapy. The uptake of complexes by the airway cells was very uneven, from very few to numerous cells having taken up the complexes in the same mouse. This uneven distribution has been reported after nasal instillation of complexes [21]. Aerosol delivery would probably have yielded more relevant results for cystic fibrosis gene therapy since gene expression is higher after nebulization than after nasal instillation and gene expression has been reported to be mainly localized in the bronchial epithelium of large airways [10,12]. Although the complex localization was not the one that could be expected to be the most appropriate for cystic fibrosis gene therapy, the number of complexes that have been taken up by the alveolar or the bronchial epithelial cells was high. This shows that although these complexes were positively charged (N/P= 12.5) and likely to interact with the negatively charged macromolecules of the mucus lining the airways and of the surfactant lining the alveolar space, it did not prevent a large quantity of complexes to access the epithelial cells. Besides the targeting of sugar receptors, it may be hypothesized that the sugar ligand has an additional effect of shielding positive charges.

A large number of Lac-PEI complexes were taken up by the cells. However, a low gene transfer efficiency was observed. It suggested that an inefficient intracellular trafficking occurred *in vivo* as it is the case *in vitro* [17,23]. We therefore studied the intracellular trafficking of Lac-PEI complexes *in vivo* after nasal instillation. *In vitro*, in various epithelial cell types including airway epithelial cells, we [17] and others [24–27] have reported that when complexed with a PEI derivative, a plasmid enters the nucleus mostly as a complex. We here show that it is not the case *in vivo* and that an early dissociation of the

complex occurs, leading to a possible degradation of the plasmid and to different intracellular trafficking for the plasmid and the vector. While the small Lac-PEI enters the nucleus, the plasmid is mostly localized in lysosomes where it will finally be degraded. These results partly explain the low numbers of GFP-expressing cells that we found. They emphasize the need to study the different steps of gene transfer in the most *in vivo* appropriate models, since the mechanisms described *in vitro* in various cell lines are likely to be very different than the ones that occur *in vivo*. Finally, these results point out the need to engineer a compacted plasmid able by itself to target the nucleus. In this event, gene transfer would be a multiple step process with a vector acting as a Trojan horse, able to complex the plasmid, to target the cells of interest and to favor an endosomal exit. After dissociation between the plasmid and its vector, the engineered plasmid would then enter the nucleus to be transcribed.

In conclusion, we have shown that glycosylated PEIs are as efficient as unsubstituted 22 kDa linear or 25 kDa branched PEI for *in vivo* gene transfer into the mouse lungs and that transfected cells are mostly epithelial cells. However, the number of transfected cells was lower than that of cells having taken up the complexes. This was mostly due to an inefficient intracellular trafficking of complexes. While *in vitro*, complexes mostly stayed in an associated form during their intracellular trafficking, *in vivo*, complexes rapidly dissociated impeding an efficient nuclear localization of the plasmid DNA. This study emphasizes the need to engineer a plasmid able by itself to overcome the nuclear barrier. It also shows that while the first *in vitro* proof-of-concepts are necessary when studying innovative vectors or plasmids, it is essential to quickly move to the most *in vivo* appropriate models since the underlying mechanisms of gene transfer may be very different between *in vitro* and *in vivo* models.

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