

Lactosylated polyethylenimine for gene transfer into airway epithelial cells: role of the sugar moiety in cell delivery and intracellular trafficking of the complexes

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Abstract

Background As we have previously shown that lactosylated polyethylenimine (PEI) is the most efficient glycosylated PEI for gene transfer into human airway epithelial cells in primary culture, we have studied here the role of the lactose residue in the enhancement of gene transfer efficiency observed with lactosylated PEI as compared with unsubstituted PEI in immortalized (Σ CFTE29o- cells) and primary human airway epithelial cells.

Methods and results After three transfections of 1 h performed daily, 60% of Σ CFTE29o- cells were transfected with lactosylated PEI, whereas 25% of cells were transfected with unsubstituted PEI ($p < 0.05$). Cell viability was 1.8-fold greater with lactosylated PEI as compared with unsubstituted PEI ($p < 0.05$). As assessed by flow cytometry, the cellular uptake of lactosylated complexes was greater than that of complexes made with unsubstituted PEI ($p < 0.05$) and involved mostly a receptor-mediated endocytosis. The study of the intracellular trafficking in airway epithelial cells of complexes showed an endosomal and lysosomal accumulation of lactosylated complexes. In the presence of a proton pump inhibitor, the level of lactosylated and unsubstituted PEI-mediated gene expression was reduced more than 20-fold, whereas the cell viability increased to almost 100%. For both complexes, a nuclear localization was observed for less than 5% of intracellular complexes.

Conclusions Our results show that the greater gene transfer efficiency observed for lactosylated complexes may be attributed to a higher amount of lactosylated complexes incorporated by airway epithelial cells and a lower cytotoxicity that might be related to reduced endosomolytic properties. However, the lactose residues substituting the PEI did not promote the entry of the plasmid into the nucleus. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords polyethylenimine; lactosylated polyethylenimine; gene therapy; cystic fibrosis; intracellular trafficking; cytotoxicity

Introduction

Polyethylenimine (PEI) is a polycationic polymer that provides relatively high levels of *in vivo* gene transfer in a number of target organs (for review, see [1]). However, although PEI has been shown to be efficient for *in vivo* gene

transfer into airway cells in mice [2,3], the success was moderate. Indeed, PEI encountered barriers similar to those previously described for viral vectors, that is, a host defence mechanism of mucociliary clearance quite difficult to evade and a low binding and entry into the cells. In order to enhance this last step, we and others have developed PEI substituted with sugar residues to target cell-surface sugar-specific receptors [4–6]. Hopefully, the use of glycosylated vectors might also promote an efficient intracellular trafficking and increase the expression of transferred genes by making use of various membrane and soluble sugar receptors that act as shuttles between different cell compartments, including the nucleus (for review, see [7]). As we were interested in cystic fibrosis (CF) gene therapy, we have studied the efficiency of various glycosylated PEIs to transfer genes into airway epithelial cells and compared them with the unsubstituted polymer and to other known nonviral vectors such as polylysine and cationic lipids [6]. Among all those vectors, we have identified lactosylated PEI as the most efficient vector in both undifferentiated and differentiated airway cells. In order to identify the role of the lactose residue in the enhancement of gene transfer efficiency observed with lactosylated PEI as compared with unsubstituted PEI, we have performed a study of the intracellular trafficking of plasmids complexed to both polymers in immortalized and primary airway epithelial cells. As expected, the lactose residue appeared to favor the entry of the complexes into the cells, but also allowed a lower cytotoxicity that might be related to reduced endosomolytic properties. However, the lactose residues substituting the PEI did not promote the entry of the plasmid into the nucleus.

Materials and methods

Cell culture

The immortalized, human tracheal epithelial Σ CFTE29o-cells, kindly given by D.C. Gruenert (University of Vermont, Colchester, VT, USA), are from a CF patient homozygous for the Δ F508 *cftr* mutation and show no cAMP-dependent chloride transport [8]. They were cultured as previously described [8].

For primary culture, non-CF bronchial tissue was collected from 10 patients at the time of open thoracotomy for localized lung tumors and immediately immersed in DMEM/F12 medium supplemented with 20 mM HEPES and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). Bronchial tissue had a normal architecture at the light microscopic level. The cell isolation technique was based on that described by Wu *et al.* for epithelial cell cultures [9]. After repeated washings in phosphate-buffered saline (PBS), bronchial tissue was incubated overnight at 4 °C with 0.15% protease (type XIV; Sigma, St Louis, MO, USA) in DMEM/F12 medium supplemented with antibiotics. The dissociated surface epithelium was removed from the tissue by gentle agitation. After centrifugation at 150 g for 10 min, the cellular pellet

was suspended and seeded on glass slides coated with type I collagen associated with carbodiimide (Sigma). They were cultured in DMEM/F12 medium supplemented with 1% Ultrosor G (Biosepra, Cergy, France), glucose (55 mM), sodium pyruvate (3 mM), epinephrine (3 μ M), and antibiotics.

Lactosylated polyethylenimine

Five percent of the amino groups of polyethylenimine (PEI, 25 kDa, branched polymer) (Sigma, St Louis, MO, USA) were substituted by a lactosylthiocarbamoyl residue as previously described [6]. Briefly, to a solution of PEI dissolved in water (10 mg/ml), lactosyl phenylisothiocyanate (12 μ mol) [10,11] 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 lactosyl 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. Fluorescein-conjugated lactosylated PEI or unsubstituted PEI was obtained by adding to a 0.4 μ mol solution of the polymer in 1 ml water, 1 μ mol of fluoresceinisothiocyanate (Molecular Probes, Eugene, OR, USA) dissolved in a 1:1 ethanol/water mixture. The solution was stirred for 30 min at room temperature and the fluorescein-conjugated polymer solution was made free of ethanol and finally freeze-dried. In control experiments, the fluorescein-conjugated polymer was dissolved in a 150 mM sodium acetate solution and passed through a Biogel P10 fine column (Biorad, Ivry sur Seine, France). The fluorescent material eluted from the column together with PEI showing that fluorescein was firmly conjugated to the polymer.

Gene transfer procedure

Two days before transfection, Σ CFTE29o-cells were seeded (2×10^5 cells per well) in a 12-well plate or on coverslips (5×10^4 cells) in a 24-well plate to study either gene transfer efficiency or the intracellular trafficking of plasmid DNA, respectively. On the day of transfection, the plasmid DNA (2.5 μ g) and the desired amount of lactosylated or unsubstituted PEI for a charge ratio PEI nitrogen/DNA phosphorus (N/P) of 10 (from a stock solution of PEI, 30 mM in nitrogen) were separately diluted into 25 μ l of 150 mM NaCl and mixed together. This charge ratio was chosen because it has been shown to lead to an optimal transfection efficiency [12]. Plasmid DNA/lactosylated or/unsubstituted PEI complexes were diluted into 450 μ l of serum-free minimal essential medium (MEM) and, after removal of the growth medium, complexes were added to each culture well containing either Σ CFTE29o-cells or bronchial epithelial cells in primary culture grown at confluency. After a 1-h

incubation at 37 °C, the transfection medium was replaced with fresh medium containing 10% serum.

To investigate the uptake of complexes by the cells, cells were preincubated in MEM containing either wortmannin (Sigma, 1 μ M), filipin (Sigma, 5 μ g/ml) or nystatin (Sigma, 50 μ g/ml) or in hypertonic medium (0.45 M sucrose) for 45 min at 37 °C. Then, cells were incubated in the presence of complexes made with fluorescein-conjugated lactosylated PEI and diluted in the same medium for 1 h. In some control experiments, cells were incubated in the presence of fluorescein-labeled transferrin (Molecular Probes) or fluorescein-labeled dextran-70 000 (Sigma). After transfection, cells were rinsed twice with sodium citrate buffer, pH 4.6, to remove cell-surface-bound complexes and trypsinized. The fluorescence of 5000 single cells was measured with an EPICS Elite flow cytometer (Beckman Coulter, Fullerton, CA, USA) used with 488-nm excitation and 520-nm emission wavelength filters.

To investigate the endosomolytic properties of the vectors, transfection experiments were conducted in the presence of bafilomycin A1 (Sigma; final concentration 200 nM in 2% ethanol) which was added to the transfection medium just after dilution of the complexes in MEM.

Gene expression measurements

Two expression plasmids were used: pCMV*Luc* (pUT 650, 5.15 kb; Cayla, Toulouse, France) and pCMV*GFP* (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.

Luciferase gene expression

Luciferase gene expression was measured by luminescence according to de Wet *et al.* [13]. The luminescence generated upon automatic addition of 150 μ l of 167 μ M luciferin dissolved in water was recorded in duplicate samples for 4 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 [14] modified according to Hill and Straka [15]. Results are expressed as RLU per mg of protein.

Green fluorescent protein expression

For green fluorescent protein expression, the number of fluorescent Σ CFTE290- cells was determined using a FACSort. Cells were also observed with a Leitz epifluorescence microscope (Leica, Rueil-Malmaison, France) after fixation *in situ* in 3% paraformaldehyde at room temperature and mounting in Vectashield-diamidinophenylindole (DAPI) solution (Vector Laboratories, Burlingame, CA, USA).

Assessment of cellular toxicity

Twenty-four hours after the gene transfer procedure, cytotoxicity was evaluated by using the colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [16]. MTT (5 mg/ml in PBS) was added to each cell culture and incubated for 4 h. MTT converted to an insoluble dye in living cells was then solubilized with acidic isopropanol. Absorbance was measured at a wavelength of 570 nm and expressed as a percentage of the absorbance observed for nontransfected cells cultured in the wells under the same conditions as transfected cells. Membrane integrity was also assessed using a lactate dehydrogenase (LDH) assay. The amount of LDH released into the medium was measured using a commercial test kit (DG 1340-K; Sigma).

Intracellular localization of plasmid DNA/lactosylated or/unsubstituted PEI complexes

The plasmid was biotinylated using the FastTag nucleic acid labeling system (Vector Laboratories) and complexed to lactosylated or unsubstituted PEI. In some experiments, fluorescein-conjugated PEIs were used. Σ CFTE290- cells or bronchial epithelial cells in primary culture grown at confluency were incubated with complexes for 1 h at 4 °C to allow complex binding to the cell membrane, but not their uptake. Then, the complexes remaining free in the supernatant were withdrawn and cells were incubated at 37 °C in growth medium from 10 min up to 48 h. At the indicated times, cells were processed as previously described [17]. Briefly, cells were fixed in 3% paraformaldehyde for 15 min, incubated for 10 min with 0.1 M glycine in PBS, and then for 15 min with 0.2% bovine serum albumin and 0.05% saponin in PBS. Finally, the biotinylated plasmid DNA was labeled with rhodamine-labeled streptavidin (Molecular Probes; dilution: 1 : 200) and some intracellular organites were labeled by immunocytochemistry. Then, coverslips were mounted in Vectashield-DAPI solution and examined with an MRC-1024 Bio-Rad confocal system (Hercules, CA, USA) mounted on a Diaphot 300 inverted microscope. The krypton/argon laser was tuned to produce excitation wavelengths of 488, 568 or 647 nm. Serial sections collected at increments of 0.5- μ m thick for endosomal and lysosomal localizations, and of 0.1- μ m thick for nuclear localization, were used to define the intracellular localization of plasmid DNA/lactosylated or/unsubstituted PEI complexes. For quantification experiments, 25 cells from adjacent fields were analyzed: all complexes within a given cell were counted and their localization inside or outside a given organelle was determined. Percentages represent the number of colocalized complexes among all the complexes present inside a cell. Images were obtained with a Kalman acquisition device and processed with Adobe Photoshop 6.0 software.

Antibodies for organelle immunolabeling

The following primary antibodies were used: the mouse monoclonal antibody (mAb) directed against the human transferrin receptor, clone H68.4 (Zymed Laboratories, San Francisco, CA, USA; diluted 1:200) as a marker of early endosomes; the mouse mAb directed against the lysosomal-associated membrane protein 1 (LAMP-1), clone H4A3 (PharMingen, San Diego, CA, USA; diluted 1:500) as a marker of lysosomes; and the goat polyclonal Ab directed against a peptide mapping at the amino terminus of lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA; fixation: methanol-acetone and diluted 1:100) as a marker of the internal nuclear membrane. The following secondary antibodies were used: fluorescein-conjugated goat anti-mouse Ab (Molecular Probes; diluted 1:200); fluorescein-labeled donkey anti-goat Ab (Jackson ImmunoResearch, West Grove, PA, USA; diluted 1:100); and cyanine Cy5-labeled donkey anti-goat Ab (Jackson ImmunoResearch; diluted 1:100).

Intracellular plasmid microinjections

Σ CFTE29o- cells were grown on CELLocate microgrid coverslips (Eppendorf, Hamburg, Germany). Free plasmid DNA pCMVGFP (50 ng/ μ l in H₂O) or plasmid DNA/lactosylated PEI or/unsubstituted PEI complexes were diluted in a 0.5% tetramethylrhodamine isothiocyanate-dextran (155 kDa) solution. Nuclear or intracytoplasmic microinjections were carried out under visual control on a Nikon Diaphot inverted phase-contrast microscope, using a Micromanipulator 5170 and a Microinjector 5242 (Eppendorf). Injections were performed with the Z (depth) limit option, using a 0.2-s injection time and 150-hectopascal injection pressure via glass micropipettes having tip diameters ranging from 0.3–0.7 μ m (Femtotips; Eppendorf). The presence of tetramethylrhodamine isothiocyanate-dextran in the injected solution allowed the determination of the exact number of injected cells and of the localization of microinjection, nuclear or cytoplasmic: usually, 50–100 cells were injected over a 15–30 min period. Twenty-four hours post-injection, cells were fixed, mounted in Vectashield-DAPI solution, and analyzed using a Leitz epifluorescence microscope. The overnight survival rate of injected cells (usually about 50%) was calculated after determination of the number of cells stained by tetramethylrhodamine isothiocyanate-dextran. Then, the number of GFP-expressing cells was determined.

Transcription assay

To investigate the putative inhibition of transcription efficiency attributed to the condensation of DNA with lactosylated or unsubstituted PEI, a nuclease S1 transcription assay adapted from Sambrook *et al.* [18] was

used as previously described [17]. Briefly, free plasmid DNA pCMVLuc (75 ng) or plasmid DNA/lactosylated or/unsubstituted PEI complexes were incubated with a HeLa cell nuclear extract to allow the formation of the preinitiation complex. Transcription was then initiated by adding NTPs and allowed to proceed for 30 min at 30 °C. After phenol/chloroform extraction, the transcripts were hybridized overnight at 42 °C with [³²P]-labeled (10 000 cpm/assay) 60-mer oligonucleotide corresponding to the CMV sequence (from –20 to +40). The single-stranded DNA was then digested with 100 U of nuclease S1. The reaction was stopped by adding 10 μ g of tRNA and loaded onto an 8% polyacrylamide gel.

Statistical analysis

Data are expressed as means \pm s.e.m. of at least three independent experiments or of the analysis of 25 cells for confocal studies. Comparisons were made using the nonparametric Mann–Whitney U-test. Values of $p \leq 0.05$ were considered to be statistically significant.

Results

Lactosylated PEI-mediated gene transfer into Σ CFTE29o- cells

Gene transfer efficiency was studied in Σ CFTE29o- cells using a plasmid encoding the *gfp* gene complexed to either lactosylated or unsubstituted PEI. One, two, or three transfections for 1 h were applied daily on three consecutive days and the number of transfected cells was evaluated on the fourth day (Figure 1A). After a single 1-h transfection, one-third of the cells incubated in the presence of plasmid/lactosylated PEI complexes were transfected, as assessed by epifluorescence microscopy (Figure 1A). By flow cytometry, lactosylated PEI allowed an efficient gene transfer into $34 \pm 4\%$ of cells, while only $24 \pm 3\%$ of cells expressed GFP after incubation in the presence of plasmid/unsubstituted PEI complexes ($p < 0.05$) (Figure 1B). After two transfections applied on the first and the second day, lactosylated and unsubstituted PEI allowed an efficient gene transfer into $54 \pm 2\%$ and $30 \pm 3\%$ of cells, respectively ($p < 0.05$). After three transfections performed daily, lactosylated PEI allowed $61 \pm 2\%$ of cells to be transfected, while unsubstituted PEI mediated gene transfer into only $24 \pm 6\%$ of cells ($p < 0.05$).

Using an MTT assay, we analyzed the toxicity of lactosylated and unsubstituted PEI (Figure 1B). When lactosylated PEI was used, cellular viability remained high after one, two or three transfections performed daily (between 82 ± 1 and $88 \pm 1\%$ of cells). In contrast, when unsubstituted PEI was used, cellular viability decreased with the number of transfections applied. After one, two or three transfections applied daily, viable cells decreased from 61 ± 1 to 53 ± 4 and

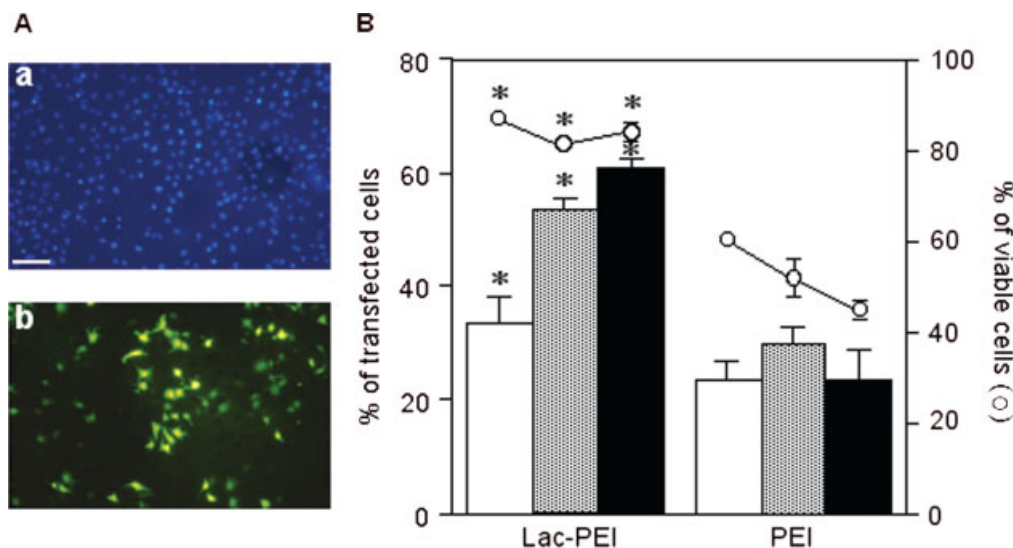


Figure 1. Lactosylated and unsubstituted PEI-mediated gene transfer and cytotoxicity in Σ CFTE290- cells. (A) Twenty-four hours after a single transfection of 1 h with a plasmid encoding the *gfp* gene complexed with lactosylated PEI, Σ CFTE290- cells were fixed and examined by epifluorescence microscopy. DAPI-stained nuclei appear blue (a) and transfected cells appear green (b) (bar = 30 μ m). (B) A plasmid encoding the *gfp* gene was complexed with lactosylated PEI (Lac-PEI) or unsubstituted PEI (PEI) and incubated for 1 h with Σ CFTE290- cells either once on the first day (□), or twice on the first and the second day (▨) or thrice on the first, the second and the third day (■). Cells were subsequently cultured in MEM and the percentage of fluorescent cells was determined by flow cytometry on the fourth day (* $p < 0.05$ when the percentages of transfected cells were compared between Lac-PEI and PEI). Cytotoxicity (○) was evaluated by an MTT assay (* $p < 0.05$ when the percentages of viable cells were compared between Lac-PEI and PEI)

46 \pm 2% of cells, respectively ($p < 0.05$ when cell viability was compared between vectors, for each number of transfections applied). Similar results were obtained when cytotoxicity was assessed using LDH release (data not shown).

Taken together, these data show that lactosylated PEI is more efficient and less toxic in Σ CFTE290- cells than unsubstituted PEI. These results are in agreement with previous data obtained on normal airway epithelial cells in primary culture [6]. To understand how the lactose residues substituting the PEI modify the intracellular trafficking of the complexes and thus improve the gene transfer efficiency of PEI, we studied the cellular uptake and intracellular trafficking of complexes made with lactosylated or unsubstituted PEI.

Uptake of complexes made with lactosylated or unsubstituted PEI by Σ CFTE290- cells

Σ CFTE290- cells were incubated for 1 h at 37°C in the presence of complexes made with fluorescein-conjugated lactosylated or unsubstituted PEI and the cell fluorescence intensity was measured by flow cytometry. As expected, the fluorescence intensity of cells incubated in the presence of complexes made with lactosylated PEI was significantly higher than that observed with complexes made with unsubstituted PEI ($p < 0.05$; Figure 2A).

Complexes made with unsubstituted PEI have been shown to enter the cells through nonspecific endocytosis involving electrostatic interactions between the positively charged complexes and the negatively charged components of the cell membranes, such as heparan sulfate proteoglycans (for review, see Zuber *et al.* [19]). To study whether the sugar residues substituting the PEI modified the mechanism of uptake of complexes made with lactosylated PEI, the effect of hypertonic medium was investigated on the basis that an hypertonic medium should inhibit any receptor-mediated endocytosis by rendering clathrin unavailable for assembly into coated pits [20]. To ascertain that the incubation in hypertonic medium of Σ CFTE290- cells inhibits receptor-mediated (clathrin-dependent) endocytosis, fluorescein-labeled transferrin was first used. The incubation of cells in hypertonic medium containing 0.45 M sucrose resulted in a 90% decrease in fluorescein-labeled transferrin uptake, as compared with the uptake observed in control medium ($p < 0.01$; data not shown). In hypertonic medium, the uptake of lactosylated complexes was decreased by 85% as compared with that observed in control medium ($p < 0.05$; Figure 2B). The possibility that macropinocytosis or caveolae-mediated endocytosis could also be involved in the uptake of lactosylated complexes was investigated by using wortmannin, an inhibitor of the formation of macropinosomes which does not affect receptor-mediated endocytosis [21,22], and filipin and nystatin which disrupt internalization via caveolae [23]. To ascertain that incubation of Σ CFTE290- cells in the presence of wortmannin modifies macropinocytosis, fluorescein-labeled

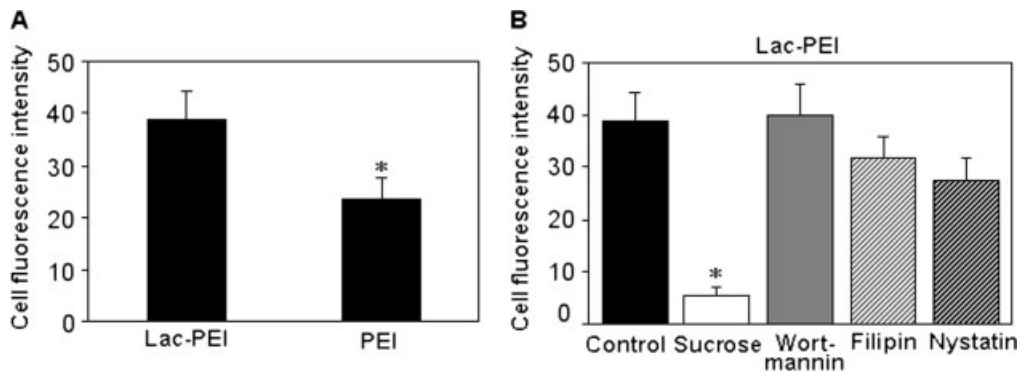


Figure 2. Flow cytometric analysis of the uptake of complexes made with fluorescein-labeled lactosylated PEI by Σ CFTE290- cells. (A) Σ CFTE290- cells were incubated for 1 h at 37°C in the presence of complexes made with fluorescein-labeled lactosylated PEI (Lac-PEI) or unsubstituted PEI (PEI). Cell fluorescence intensity was measured by flow cytometry (expressed as relative light units). (B) After a 45-min preincubation either in MEM (■) or in MEM containing sucrose (0.45 M, □), wortmannin (1 μ M, ▨), filipin (5 μ g/ml, ▩) or nystatin (50 μ g/ml, ▤), cells were incubated for 1 h in the same medium in the presence of complexes made with fluorescein-labeled lactosylated PEI. Cell fluorescence intensity was measured by flow cytometry (expressed as relative light units) (* $p < 0.05$)

dextran-70 000 (Sigma) was used. Incubation of cells in MEM containing 1 μ M wortmannin resulted in a 60% decrease in FITC-dextran uptake, as compared with the uptake observed in control medium ($p < 0.05$; data not shown). In the presence of wortmannin (1 μ M), incorporation of lactosylated complexes was unchanged (Figure 2B). In the presence of filipin (5 μ g/ml) or nystatin (50 μ g/ml), a slight decrease in uptake of complexes made with lactosylated PEI was observed (20 and 30%, respectively, $p > 0.05$; Figure 2B). These results suggest that the uptake of complexes made with lactosylated PEI is mostly related to a receptor-mediated endocytosis.

Endosomal localization of complexes made with lactosylated or unsubstituted PEI in Σ CFTE290- cells

To determine the subcellular localization of complexes made with lactosylated or unsubstituted PEI, early endosomes were visualized by using an anti-human transferrin receptor antibody. Both types of complexes were clearly localized in early endosomes after 30 min (Figures 3A and 3B). However, the amounts of complexes present in early endosomes and the subsequent time course of endosomal localization were different for complexes made with lactosylated PEI as compared with those made with unsubstituted PEI. Localization in early endosomes of lactosylated complexes was maximal after 1 h with 35% of complexes present in early endosomes, and then the amount of endosomal lactosylated complexes slowly decreased (Figures 3A and 3B). In contrast, endosomal localization of complexes made with unsubstituted PEI was maximal after 30 min with 27% of complexes localized in early endosomes and then rapidly decreased to 16% of complexes present in early endosomes after 1 h (Figures 3A and 3B).

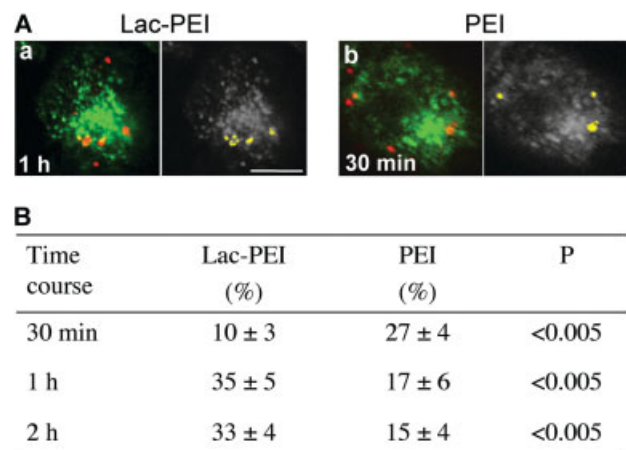


Figure 3. Endosomal localization of complexes made with lactosylated or unsubstituted PEI. (A) Σ CFTE290- cells were incubated with biotinylated DNA/lactosylated PEI (a) or unsubstituted PEI (b) complexes for 1 h at 4°C, then washed, incubated at 37°C for the indicated times, fixed and examined by confocal microscopy. Biotinylated DNA labeled with rhodamine-labeled streptavidin appears red and endosomes immunolabeled with anti-transferrin receptor antibodies and fluorescein-labeled anti-mouse antibodies appear green in the left-hand panels. In the right-hand panels, pixel analysis of colocalization of plasmid DNA and endosomes appears in yellow (bar = 10 μ m). (B) Experiments were quantified by confocal microscopic analysis of 25 Σ CFTE290- cells at the indicated times: all complexes within a given cell were counted and their localization inside or outside endosomes was determined. The mean percentages \pm s.e.m. of endosomal complexes among all the complexes present inside a cell are indicated

Determination of the endosomolytic activity of lactosylated or unsubstituted PEI

An important part of PEI transfection efficiency relies on its ability to destabilize the lipid bilayer, an event required to allow the plasmid DNA to leave endosomes in order to reach the cytosol. This endosomolytic property

is due to the ability of PEI to increase the ionic strength of the endosomal lumen by capturing protons entering the endosomes and by promoting the accumulation of chloride ions during endosomal acidification [24]. To evaluate a possible effect of glycosylation of PEI on this endosomal swelling property, lactosylated or unsubstituted PEI-mediated transfection was investigated in the presence of a proton pump inhibitor, bafilomycin A1; the inhibition of endosomal acidification is expected to significantly decrease the gene expression by lowering the availability of the plasmid. For both lactosylated and unsubstituted PEI, the level of gene expression was similar between cells treated with ethanol, the solvent used to solubilize bafilomycin A1, and untreated cells (data not shown). In the presence of bafilomycin A1, the level of lactosylated and unsubstituted PEI-mediated gene expression was reduced 20- and 35-fold, respectively, as compared with gene expression obtained in the absence of bafilomycin A1 ($p < 0.01$ for both types of complexes; Figure 4). These results show that an important part of the efficiency of lactosylated PEI relies on its buffer-based endosomolytic activity.

In order to evaluate if the cytotoxicity observed for complexes made with unsubstituted PEI could be related to endosomal acidification and swelling, an MTT assay was performed in the presence of bafilomycin A1. Twenty-four hours after a 1-h incubation of complexes made with lactosylated or unsubstituted PEI, in the presence of bafilomycin A1, cellular viability increased to 99 ± 1 and $94 \pm 1\%$, respectively, as compared with cellular viability observed in the absence of bafilomycin A1 (88 ± 1 and $61 \pm 1\%$, respectively, $p < 0.05$ for both types of

complexes, when cell viabilities in the presence or in the absence of bafilomycin A1 were compared; Figure 4).

Lysosomal localization of complexes made with lactosylated or unsubstituted PEI in Σ CFTE290- cells

Since entrapment of complexes in lysosomes is thought to be associated with their degradation, the lysosomal localization of complexes made with lactosylated or unsubstituted PEI was then investigated. Lysosomes were labeled with an antibody recognizing the lysosomal membrane glycoprotein LAMP-1. Complexes made either with lactosylated or unsubstituted PEI were observed in vesicles expressing LAMP-1 after 1 h (Figures 5A and 5B). The subsequent time course of lysosomal localization was different for complexes made with lactosylated PEI as compared with those made with unsubstituted PEI. Maximal accumulation in LAMP1⁺ vesicles of complexes made with lactosylated PEI was observed between 2 and 6 h and concerned about 30% of the intracellular complexes (Figures 5A and 5B). In contrast, the LAMP1⁺ vesicle localization of complexes made with unsubstituted PEI was maximal after 1 h with 20% of complexes localized in LAMP1⁺ organelles, and decreased afterwards (Figures 5A and 5B).

Nuclear localization of complexes made with lactosylated or unsubstituted PEI in Σ CFTE290- cells

To evaluate the nuclear localization of complexes, the internal nuclear membrane was labeled using an anti-lamin A/C antibody and cells were studied by confocal microscopy on 0.1- μ m thick serial sections. For complexes made with lactosylated or unsubstituted PEI, the plasmid was detected inside the nucleus after 4 h and up to 8 h, with a maximal localization at 6 h (Figure 6A). This time course observed for nuclear localization of the plasmid is consistent with the expression kinetics of a plasmid encoding the luciferase gene and complexed to lactosylated or unsubstituted PEI since luciferase expression began to be detected at 4 h (Figure 6B). In order to evaluate if the plasmid was present in the nucleus as a complexed or as a vector-free molecule, we labeled the internal nuclear membrane, the vector and the plasmid. For both lactosylated and unsubstituted PEI, the plasmid entered the nucleus as a complex: the plasmid/lactosylated PEI complex is shown in Figure 7. However, with both vectors, very few complexes were observed inside the nucleus (<5% of the intracellular complexes), most of the complexes being gathered in aggregates at the periphery of the nucleus (Figures 6A and 7). In addition and for both types of complexes, nuclear complexes were observed in the nucleus of only 20% of the cells.

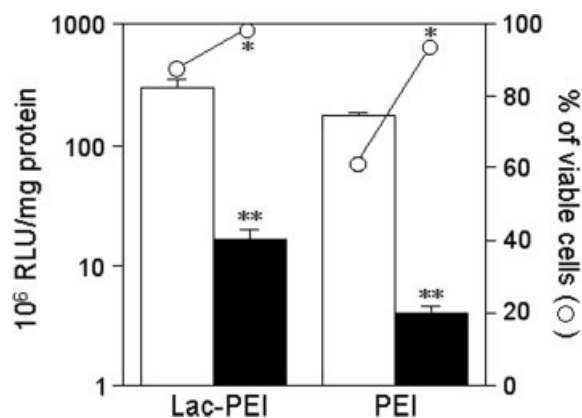


Figure 4. Influence of bafilomycin A1 on lactosylated or unsubstituted PEI-mediated gene transfer efficiency and cellular toxicity. Σ CFTE290- cells were incubated for 1 h with a plasmid pCMVLuc complexed to lactosylated or unsubstituted PEI in the absence (□) or in the presence (■) of the proton pump inhibitor bafilomycin A1 (200 nM). Twenty-four hours later, cells were lysed and luciferase activity was measured by chemiluminescence. The relative light units (RLU) measured for 4 s are expressed as means \pm s.e.m. per mg of protein. Cytotoxicity (○) was also evaluated by an MTT assay (* $p < 0.05$ when the percentages of viable cells in the presence or in the absence of bafilomycin A1 were compared and ** $p < 0.01$ when gene transfer efficiencies obtained in the presence or in the absence of bafilomycin A1 were compared)

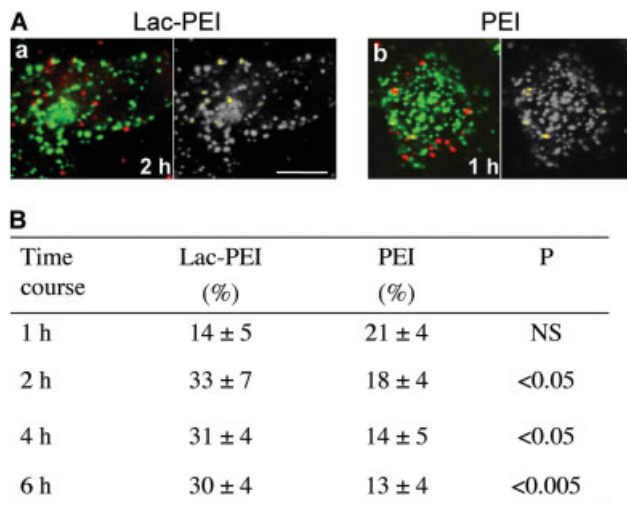


Figure 5. Lysosomal localization of complexes made with lactosylated or unsubstituted PEI. (A) Σ CFTE290⁻ cells were incubated with biotinylated DNA/lactosylated PEI (a) or/unsubstituted PEI (b) complexes for 1 h at 4°C, then washed, incubated at 37°C for the indicated times, fixed and examined by confocal microscopy. Biotinylated DNA labeled with rhodamine-labeled streptavidin appears red and lysosomes immunolabeled with anti-LAMP1 antibodies and fluorescein-labeled anti-mouse antibodies appear green in the left-hand panels. In the right-hand panel, pixel analysis of colocalization of plasmid DNA and lysosomes appears in yellow (bar = 10 μ m). (B) Experiments were quantified by confocal microscopic analysis of 25 Σ CFTE290⁻ cells at the indicated times: all complexes within a given cell were counted and their localization inside or outside lysosomes was determined. The mean percentages \pm s.e.m. of complexes present in lysosomes among all the complexes present inside a cell are indicated

As these confocal studies suggested that the nuclear entry of the complexes is a main limiting step for gene expression, a vector-free plasmid DNA encoding the *gfp* gene on the one hand, and the same plasmid DNA complexed to either lactosylated or unsubstituted PEI on the other, were microinjected into the cytoplasm of the cells (Figure 8A). Twenty-four hours after cytoplasmic microinjections, the number of cells expressing GFP was low when the free plasmid DNA was microinjected (20 \pm 9% of all injected cells expressed GFP) or when the plasmid complexed to lactosylated PEI (35 \pm 12% of cells) or to unsubstituted PEI (27 \pm 9% of cells) was microinjected. These results show that the entry into the nucleus is indeed a limiting step.

Transcription efficiency of the plasmid complexed to lactosylated or unsubstituted PEI

As there was no direct evidence for a dissociation of the plasmid from its vector, further experiments were conducted to test the hypothesis of a transcription efficiency limitation related to the condensation of the plasmid with the polycation. Using an S1 nuclease transcription assay and Hela cell nuclear extract (10 μ g/ μ l, either 5 μ l:

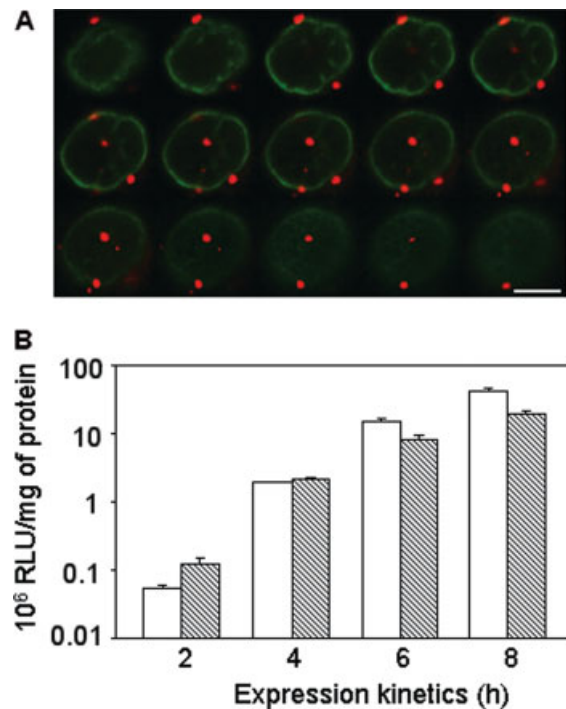


Figure 6. Nuclear localization of biotinylated DNA complexed to lactosylated PEI (A) and kinetics of gene expression by using lactosylated or unsubstituted PEI as vectors (B). Σ CFTE290⁻ cells were incubated with plasmid pCMVLuc/lactosylated PEI (A & B, \square) or unsubstituted PEI (B, \square) for 1 h at 4°C, then washed, incubated at 37°C in culture medium. (A) After 6 h, cells were fixed, biotinylated DNA was labeled with rhodamine-labeled streptavidin and appears red; the nuclear membrane was immunolabeled with anti-lamin A/C antibodies, followed by fluorescein-labeled anti-goat antibodies, and appears green. Cells were analyzed by confocal microscopy and serial sections 0.1- μ m thick were collected; every section in five is shown (bar = 10 μ m). (B) At the indicated times, cells were lysed and luciferase activity was measured by chemiluminescence in a luminometer. The relative light units (RLU) measured for 4 s are expressed as means \pm s.e.m. per mg of protein

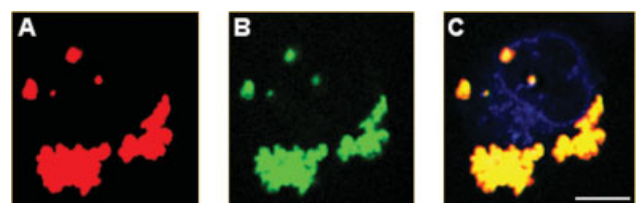


Figure 7. Lack of evidence for the dissociation of plasmid/lactosylated PEI complexes. Σ CFTE290⁻ cells were incubated with biotinylated plasmid DNA/fluorescein-conjugated lactosylated PEI complexes for 1 h at 4°C, then washed, incubated at 37°C in culture medium for 6 h and fixed. Biotinylated plasmid DNA was labeled with rhodamine-labeled streptavidin and the nuclear membrane was immunolabeled with anti-lamin A/C antibodies, followed by cyanine Cy5-conjugated anti-goat antibodies. Cells were analyzed by confocal microscopy. (A) The biotinylated DNA appears red; (B) the fluorescein-labeled lactosylated PEI appears green; and (C) images were merged and the nuclear membrane appears blue (bar = 10 μ m)

Figure 9, lanes 1, 3, 5 or 10 μ l: lanes 2, 4, 6), the transcription efficiencies of free plasmid DNA, plasmid DNA

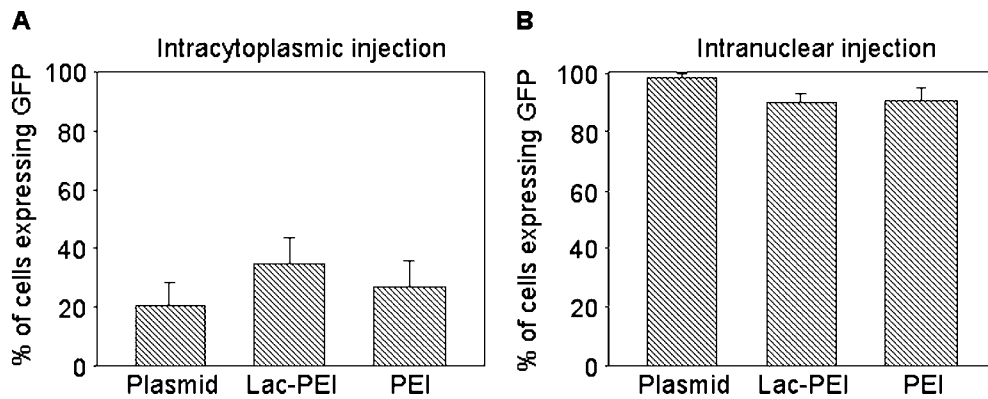


Figure 8. Expression of GFP by Σ CFTE290- cells upon intracytoplasmic or intranuclear microinjections of vector-free pCMVGFP plasmid or of plasmid complexed with either lactosylated or unsubstituted PEI. Free plasmid (pCMVGFP) or plasmid complexed to either lactosylated (Lac-PEI) or unsubstituted PEI (PEI) was diluted in a tetramethylrhodamine isothiocyanate-dextran solution and microinjected into the cytoplasm (A) or the nucleus (B) of Σ CFTE290- cells. Twenty-four hours later, the number of cells expressing GFP and the total number of microinjected cells were determined by using an epifluorescence microscope and results are expressed as percentages of microinjected cells expressing GFP

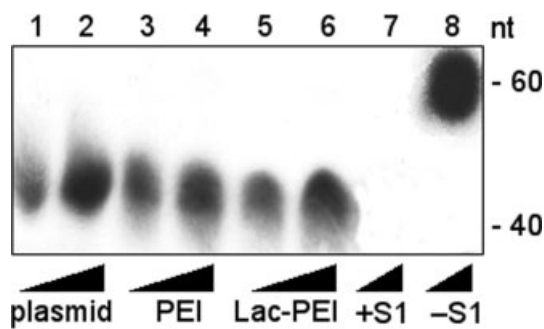


Figure 9. Lactosylated and unsubstituted PEI-dependent transcription. Free plasmid (pCMVLuc) (lanes 1, 2) or plasmid complexed with either unsubstituted (lanes 3, 4) or lactosylated PEI (lanes 5, 6) were tested for transcription activity in a nuclease S1 assay: transcription was initiated in the presence of either 5 μ l (lanes 1, 3 and 5) or 10 μ l (lanes 2, 4 and 6) of a 10 μ g/ μ l solution of an HeLa cell nuclear extract and NTPs. Transcripts were then hybridized with [32 P]-labeled 60-mer oligonucleotide corresponding to the CMV sequence and the single-stranded DNA was digested with 100 U of nuclease S1. Control lanes 7 and 8 represent the nuclease S1 (100 U)-digested and the undigested radiolabeled probe, respectively (nt: nucleotides)

complexed to lactosylated PEI or to unsubstituted PEI were assessed. Initiation of free plasmid DNA transcription was slightly more efficient (lanes 1, 2) than the initiation of transcription of plasmid DNA complexed to unsubstituted PEI (lanes 3, 4) or to lactosylated PEI (lanes 5, 6). This analysis was completed by determining the number of cells expressing GFP 24 h upon nuclear microinjection of the vector-free plasmid DNA, the plasmid DNA complexed with lactosylated PEI and with unsubstituted PEI (Figure 8B): under such conditions, 98 ± 2 , 90 ± 4 and $90 \pm 3\%$ of cells expressed GFP, respectively. Therefore, the presence in the nucleus of the lactosylated or of the unsubstituted PEI complexed to the plasmid slightly lowers the transfection efficiency, both to the same extent.

Gene transfer efficiency and intracellular trafficking of complexes made with lactosylated PEI in bronchial epithelial cells in primary culture

After a single 1-h transfection of bronchial epithelial cells in primary culture grown at confluency, 1 or 2% of the cells incubated in the presence of plasmid/unsubstituted PEI complexes or plasmid/lactosylated PEI complexes expressed GFP, as assessed by flow cytometry. The intracellular trafficking of lactosylated complexes was studied in a similar manner in bronchial epithelial cells in primary culture. Complexes were observed in about 60% of the cells (Figure 10A). Fewer complexes per cell were observed in primary bronchial epithelial cells, as compared with Σ CFTE290- cells. Lactosylated complexes were seen in early endosomes between 15 and 60 min, with a maximal localization after 30 min ($35 \pm 6\%$ of complexes; Figure 10B). Similarly, lactosylated complexes were observed in lysosomes between 30 min and 3 h, with a maximal localization after 30 min ($38 \pm 4\%$ of complexes; Figure 10C). The plasmid was detected inside the cell nucleus between 3 and 4 h and was complexed with lactosylated PEI. Nuclear complexes were observed in less than 5% of the cells and, each time, very few complexes were present in the nucleus (Figure 10D). Taken together, these data show that the intracellular trafficking of complexes made with lactosylated PEI is similar in human airway cells in primary culture to that observed in immortalized airway epithelial cells: the maximal percentages of complexes present in endosomes, lysosomes and nucleus were similar. However, the intracellular trafficking of lactosylated complexes appeared to be quicker in human airway cells in primary culture than in immortalized airway cells.

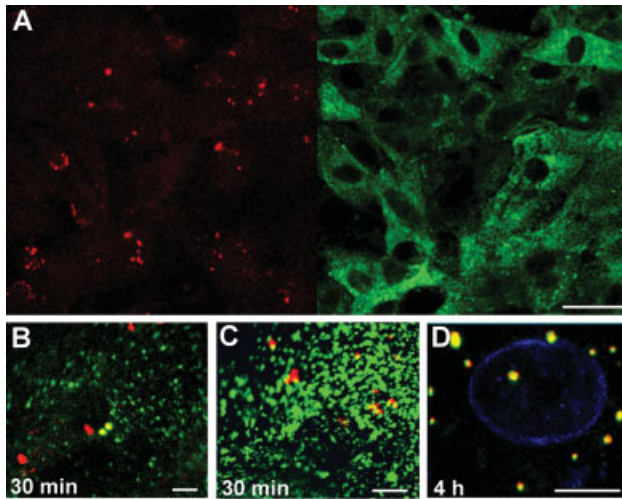


Figure 10. Intracellular trafficking of complexes made with lactosylated PEI in bronchial epithelial cells in primary culture. Bronchial epithelial cells grown at confluency were incubated with biotinylated DNA/lactosylated PEI complexes for 1 h at 4 °C, then washed, incubated at 37 °C for the indicated times, fixed and examined by confocal microscopy. Biotinylated DNA labeled with rhodamine-labeled streptavidin appears red. Endosomes were immunolabeled with anti-transferrin receptor antibodies and fluorescein-labeled anti-mouse antibodies and appear green (A, bar = 10 μm ; B, bar = 2.5 μm). Lysosomes were immunolabeled with anti-LAMP1 antibodies and fluorescein-labeled anti-mouse antibodies and appear green (C, bar = 2.5 μm). The biotinylated DNA was complexed to fluorescein-conjugated lactosylated PEI which appears green. The nuclear membrane was immunolabeled with anti-lamin A/C antibodies, followed by cyanine Cy5-conjugated anti-goat antibodies and appears blue (D, bar = 2.5 μm)

Discussion

The cationic polymer PEI is among the most efficient synthetic vectors for gene transfer *in vitro* into various cell types, including airway epithelial cells [12,25,26]. However, *in vivo* in mice, the success of gene transfer by the use of PEI was moderate [2,3]. In order to obtain an enhanced cell-specific transfection, we have substituted PEI with sugar residues and among various glycosylated PEIs, PEI bearing lactose residues was shown to be the most efficient for *in vitro* gene transfer into airway epithelial cells [6]. We have confirmed here that lactosylated PEI is an efficient vector for gene transfer into airway epithelial cells and studied the role of the sugar moiety in the enhanced gene transfer efficiency. We first showed that, even upon three daily transfections, the cytotoxicity due to lactosylated PEI remained low (around 15%). In contrast, unsubstituted PEI appeared to be highly cytotoxic, even after one transfection (40%), and this cytotoxicity increased with the number of transfections applied, up to 60% after three transfections. Knowing that PEI permeabilizes Gram-negative bacterial outer membranes [27], the cytotoxic effects of PEI have been attributed to a putative capacity to permeabilize plasma membranes of transfected cells [28]. However, the cytotoxic effects of PEI could also

be due to its ability to disrupt endosomes. Indeed, this “proton sponge effect” of PEI allows a high gene transfer capacity [24]. As demonstrated by the reduced gene transfer efficiency of lactosylated PEI in the presence of bafilomycin A1, a proton pump inhibitor, we show here that lactosylated PEI retains the “proton sponge effect” of the unsubstituted polymer and that this effect is quite necessary for an efficient gene transfer. However, this ability to increase the ionic strength of the endosomal lumen which results in endosomal disruption may also be detrimental to cells in case of disruption of a too large number of vesicles. This mechanism for PEI cytotoxicity is supported by an almost complete absence of cytotoxicity observed when the ability of PEI to increase the ionic strength of the endosomal lumen has been inhibited by bafilomycin A1. While lactosylated PEI retains the ability of the unsubstituted polymer to disrupt endosomes and displays a high gene transfer efficiency, the low cytotoxicity observed with complexes made with lactosylated PEI suggests that the endosomal swelling properties of lactosylated PEI are reduced, as compared with unsubstituted PEI.

The role of the sugar moiety in the uptake of complexes was studied and, as expected, we observed that a greater amount of lactosylated complexes were taken up by $\Sigma\text{CFTE290}$ -cells and that these complexes mainly entered the cells via a receptor-mediated endocytosis. This is quite different from the mechanism of uptake of complexes made with unsubstituted PEI which have been shown to enter the cells through nonspecific endocytosis such as fluid-phase endocytosis [19]. The subsequent intracellular trafficking was quite different for both complexes. Endosomal localization of complexes made with unsubstituted PEI was observed at early time points (30 min) and was transient. Similarly, lysosomal localization of complexes made with unsubstituted PEI was observed at 1 h; it concerned only 20% of complexes and this localization decreased afterwards. These results confirm and expand previous reports from other groups showing that complexes made with PEI are localized in cytoplasmic vesicles from which they rapidly escape and that a lysosomal localization also occurs [24,29,30]. In contrast, for complexes made with lactosylated PEI and in both immortalized and primary human airway epithelial cells, a massive endosomal and lysosomal localization was observed. This low endosomal escape of complexes made with lactosylated PEI further suggests reduced endosomal swelling properties for lactosylated PEI as compared with unsubstituted PEI. However, this apparent unfavorable intracellular trafficking of lactosylated complexes did not result in a lower gene transfer efficiency. On the contrary, lactosylated PEI was more efficient for gene transfer and less toxic than unsubstituted PEI and, for both types of complexes, the kinetics of gene transfer, the time required to reach the nucleus and the amount of plasmid in the nucleus were similar. These results suggest that the pre-nuclear steps of intracellular trafficking may be efficient without too many demands. Indeed, with the vectors presently at our disposal, the majority of the

delivered DNA does not participate in the expression of the transgene. Most of the delivered DNA is either trapped in vesicles as it occurs for lactosylated complexes, or present in aggregates at the periphery of the nucleus for both types of complexes. In either case, a very small and quite comparable amount of the delivered DNA is able in some cells to reach the nucleus.

Nuclear lectins, i.e. nuclear sugar-binding proteins, have been described and the presence of glycosylated proteins in the cytosol and the nucleus supports the idea that lectins and glycoproteins may interact and play a role in the trafficking of macromolecules between the cytosol and the nucleus [31,32]. In order to identify whether the lactose residue substituting the PEI could promote the nuclear entry of plasmid DNA, we studied the nuclear trafficking of the complexes. No differences were observed between complexes made with lactosylated or unsubstituted PEI: complexes were localized in the nucleus mainly between 4 and 8 h and very low amounts of intracellular complexes were observed in the nuclei of 20% of the cells. Our results obtained on airway cells in primary culture or by using intracytoplasmic microinjections confirmed other reports showing that the nuclear entry is a major limiting step for an efficient gene transfer by using PEI as vectors [30,33–35]. It has been suggested that the lactose residues substituting polylysine, another cationic polymer, were involved in the nuclear localization of complexes by means of targeting a putative galactose-specific lectin [36]. Our results do not support this hypothesis and are in agreement with the fact that neoglycoproteins bearing lactose residues are not imported in the nucleus [37]. For both lactosylated and unsubstituted PEI, our results show an absence of dissociation of the plasmid from its vector and this has been described previously for unsubstituted PEI [29]. We show here, both in a cellular and an acellular model, that this absence of dissociation between the plasmid and its vector did not cause a major impairment of transcription. This may be due to the fact that at the N/P ratio of 10 that we used (because it is known to lead to an optimal transfection efficiency [12,35]), the compaction of DNA does not prevent its access to the transcription machinery.

In conclusion, our study suggests that for gene transfer with the presently available synthetic vectors for which most of the complexes remain outside the nucleus, the pre-nuclear steps of intracellular trafficking may not be as demanding as previously thought. Indeed, a level of endosomolytic activity is necessary. However, in our study, complexes made with lactosylated PEI displayed an accumulation in endosomes and lysosomes in both immortalized and primary airway epithelial cells. Nevertheless, lactosylated PEI allowed a more efficient gene transfer than complexes made with unsubstituted PEI. This greater gene transfer efficiency could be attributed to a greater amount of lactosylated complexes incorporated by airway epithelial cells and to a lower cytotoxicity that we hypothesize may be due to reduced endosomolytic properties. Finally, the lactose residues substituting the PEI did not promote the entry of the

plasmid into the nucleus and further work is needed to use a sugar-dependent import of complexes into the nucleus, in order to increase the expression of transferred genes.

Acknowledgements

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