Enhanced Efficiency of Lactosylated Poly-L-Lysine–Mediated Gene Transfer into Cystic Fibrosis Airway Epithelial Cells

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Lactosylated poly-L-lysine is a nonviral vector that transfers genes into airway epithelial cells, including those from individuals with cystic fibrosis (CF). Substitution of 40% of the ε-amino groups of poly-L-lysine with lactosyl residues not only provided a ligand for receptor-mediated endocytosis, but also reduced the toxicity when compared with nonsubstituted poly-L-lysine. Lactosylated poly-L-lysine/pCMV-*Luc* complex is not toxic to cells in amounts that gave the maximum gene expression. The level of gene expression was regulated by using different combinations of chloroquine, glycerol, and E5CA peptide. Using cultured CF cells, chloroquine, combined with E5CA peptide, increased the transfer of the pCMV*Luc*/lactosylated poly-L-lysine complex 10,000-fold compared with transfer without additives. In many systems, a high efficiency is of paramount importance and the enhancing agents can be used to modulate the expression of the gene. For example, transfer of pCMV*LacZ*/lactosylated poly-L-lysine complexes with chloroquine added to the transfection medium gave only 20% transfection efficiency of the reporter gene. However, when chloroquine was combined with glycerol, the efficiency was increased to 90%, thus approaching that reported with viral vectors. This highly efficient vector may be of great value for the future development of gene transfer systems. **Kollen, W. J. W., F. M. Schembri, G. J. Gerwig, J. F. G. Vliegenthart, M. C. Glick, and T. F. Scanlin. 1999. Enhanced efficiency of lactosylated poly-L-lysine–mediated gene transfer into cystic fibrosis airway epithelial cells. Am. J. Respir. Cell Mol. Biol. 20:1081–1086.**

Advances in molecular biology and in the identification of disease-related genes have created the potential of gene transfer as a therapeutic modality, particularly for lung diseases (1). A number of clinical trials were initiated using either recombinant viruses (2) or cationic lipids (3) as vehicles for the delivery of genes. The obstacles to achieving more than modest transgene expression *in vivo* have been reviewed (4). In an attempt to overcome these obstacles there have been renewed efforts to design more effective vectors and to employ *in vitro* systems to further elucidate the mechanisms of gene transfer and transgene expression (5).

Lactosylated poly-L-lysine, a specifically formulated molecular conjugate, has been shown to be effective in the transfer of reporter genes into human airway epithelial cells *in vitro* (6). Substitution of 40% of the ε-amino groups of the poly-L-lysine with the disaccharide lactose has provided a vector with unique features. Lactosylated poly-L-lysine is less immunogenic and less toxic than other molecular conjugates that employ polylysine with a single lysine residue substituted by either a simple or complex ligand (7, 8). It was previously shown (6) that the primary mechanism for lactosylated poly-L-lysine to enter into the cell is by receptor-mediated endocytosis. In addition, the use of the lysosomal disrupting agent chloroquine was shown to improve the level of transgene expression, although the transfection efficiency was not examined. In this report, specific combinations of agents that have different mechanisms of action are shown to enhance significantly the efficiency of transfection. These results are used to identify targets for further vector development.

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Abbreviations: cystic fibrosis, CF; cystic fibrosis transmembrane conductance regulator, CFTR; nuclear magnetic resonance, NMR; relative light unit, RLU.

Dedicated to Roger W. Jeanloz on the occasion of his 80th birthday.

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Materials and Methods

Preparation of Lactosylated Poly-L-Lysine and Characterization by ^{1}H-NMR Spectroscopy

Lactosylated poly-L-lysine was prepared as described (6). For analysis by ^{1}H-NMR spectroscopy, lactosylated poly-L-lysine (1 mg) was repeatedly treated with D_2O (99.9 atom % D, CIL) with intermediate lyophilization and finally dissolved in 0.4 ml D_2O (99.96 atom % D; Isotec, Inc.). High-resolution 500 MHz ^{1}H-NMR spectra were recorded on a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University, Utrecht, The Netherlands) at a probe temperature of 300 K. The spectral width was 5 kHz. Suppression of the HO^2H (HOD) signal was achieved by applying an advanced water eliminated Fourier transform (WEFT) pulse sequence (9). Resolution enhancement of the spectra was achieved by a Lorentzian-to-Gaussian transformation. Chemical shifts (δ) are expressed in parts per million (ppm) by reference (10) to internal acetone (δ 2.225). For comparison, ^{1}H-NMR spectra were recorded of lactose and lysine HCl and *p*-toluenesulfonate poly-L-lysine under the same conditions.

Cell Culture

The immortalized cell line CF/T43, nasal airway epithelial cells from a cystic fibrosis (CF) patient homozygous for the ΔF508 mutation (11), and an immortalized CF tracheal epithelial cell line, CF/T1(wt) transfected with wild-type CFTR (12), were kindly provided by Dr. J. R. Yankaskas (University of North Carolina). An immortalized normal tracheal cell line, BEAS-2B, was obtained from Dr. J. F. Lechner, National Institutes of Health (Bethesda, MD) (13, 14). All cells were seeded 24 h prior to transfection at 1.2 to 2 × 10^5 cells per 25 mm well in a 12-well plate or at 0.5 to 1 × 10^5 cells per 15 mm well in a 4-well plate. CF/T43 cells were cultured in KGM medium from Clonetics (San Diego, CA); BEAS-2B and CF/T1(wt) cells were cultured in LHC-9 medium from Biofluids (Rockville, MD).

Conditions of Transfection

Lactosylated poly-L-lysine was added dropwise to the reporter gene pCMV*Luc* (Cayla, Toulouse, France) or pCMV*LacZ* (kindly supplied by Dr. P. Ballard, The Children's Hospital of Philadelphia, PA) in a 3:1 (wt:wt) ratio unless otherwise noted, and held for 30 min at ambient temperature (6). E5CA peptide (GLFEAIAEFIEGGWEG-

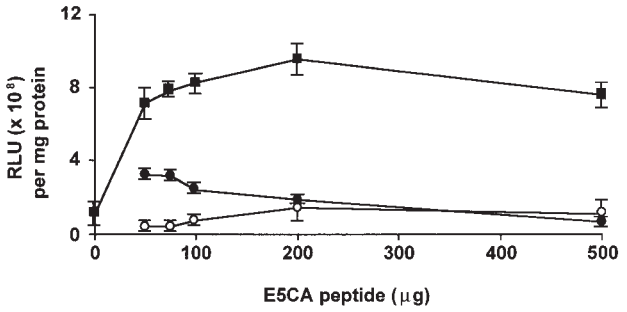

Figure 1. Enhancement of luciferase gene expression in CF/T43 cells with E5CA peptide alone and in combination with glycerol or chloroquine. Transfection of CF/T43 cells was performed with pCMV*Luc* (1 μg) complexed to lactosylated poly-L-lysine (3 μg) in the presence of the noted concentrations of E5CA peptide and 100 μM chloroquine (*closed squares*), 5% glycerol (*closed circles*), or E5CA peptide without other additives (*open circles*). After subsequent culture for 48 h, luciferase gene expression was detected. Data represent mean values of RLU per mg protein ± standard deviation (SD) of duplicate samples in two different experiments (n = 4). In the case of 100 μM chloroquine and 500 μg E5CA peptide, data were duplicate samples of one experiment. With the addition of 5% glycerol alone 1.8 × 10^7 (± 3.0 × 10^6) RLU/mg protein were detected.

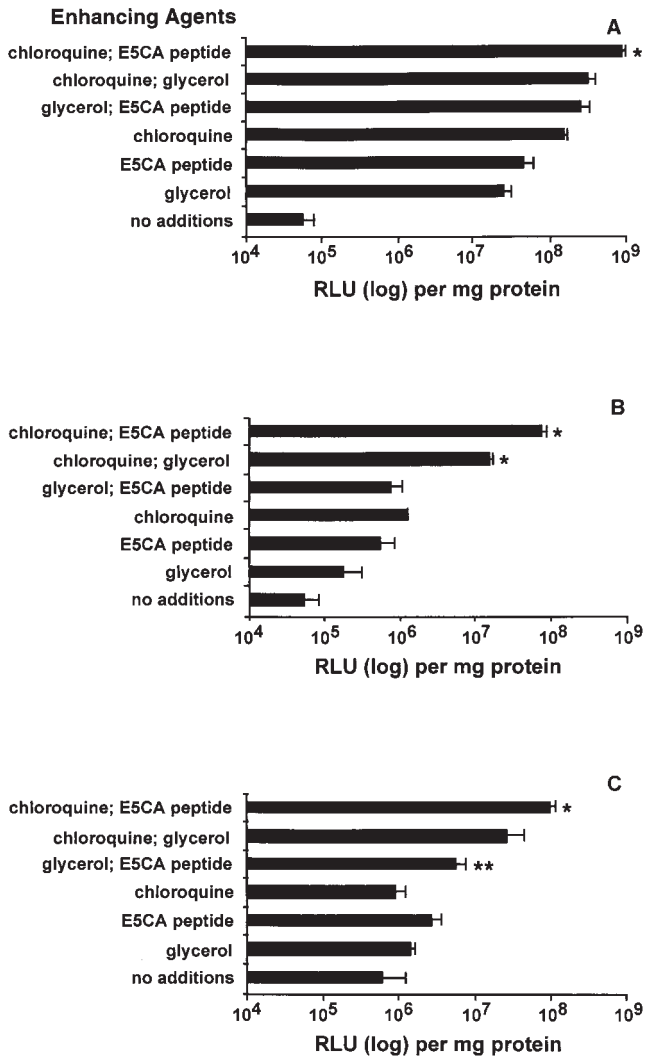

Figure 2. Luciferase gene expression in immortalized cells with optimized enhancing agents. Transfection of the cells was performed with pCMV*Luc* (1 μg) complexed to lactosylated poly-L-lysine (3 μg), in the presence of 100 μM chloroquine, 5% glycerol, 75 μg E5CA peptide, or combinations of these agents as noted. Luciferase gene expression was detected after subsequent culture for 48 h. Data are expressed as mean values of RLU per mg protein ± standard deviation from two separate experiments with CF/T43 (n = 4) (*A*); BEAS-2B (n = 6) (*B*); and CF/T1(wt), (n = 6) (*C*). *$P < 0.001$ when compared with chloroquine; ** $P <$ 0.01 when compared with chloroquine.

LIEGCA) was synthesized by Core Laboratories of the Louisiana State University Medical Center (New Orleans, LA). Chloroquine (Sigma, St. Louis, MO), glycerol (Fisher, Pittsburgh, PA), or combinations of these agents were added in the noted concentrations. The final mixture was added to each culture after removal of the growth medium and the cells were incubated at 37°C. After 4 h, the transfection medium was removed and the cells were further incubated at 37°C in growth media. The cells were examined morphologically in a Zeiss Opton microscope (Oberkochen, Germany), after both transfection and expression time. Except when specified, all cells appeared normal and after 48 h the cells were processed to detect reporter gene expression.

Reporter Gene Expression

Luciferase gene expression was detected as described (6, 15) with a luciferase assay system from Promega (Madison, WI). Luminescence was recorded on a Lumat LB 9501 luminometer (Berthold Systems Inc., Pittsburgh, PA) for 5 s and reported as relative light units (RLU). One picogram of luciferase (Boehringer Mannheim, Indianapolis, IN) was equivalent to 11,000 RLU under these assay conditions. Proteins were determined (16) on cells lysed with 0.1 M NaOH. Luciferase gene expression was reported as RLU/mg of cell protein. Statistical analysis was performed using the double-tailed paired Student's *t* test. *LacZ* gene expression was detected with X-Gal stain. The cells were fixed with 2.2% paraformaldehyde/0.2% glutaraldehyde in phosphate-buffered saline for 15 min and stained overnight with X-Gal stain (1 ml; Sigma, St. Louis, MO). Transfection efficiency was determined by counting the percentage of blue-stained cells in the well, using a Nikon Diaphot 300 microscope (Garden City, NY). A minimum of 600 cells was counted.

Results and Discussion

Enhancement of Luciferase Gene Expression with Specific Agents

Lactosylated poly-L-lysine was used as a vector to transfer pCMV*Luc* into three airway epithelial cell lines. To en-

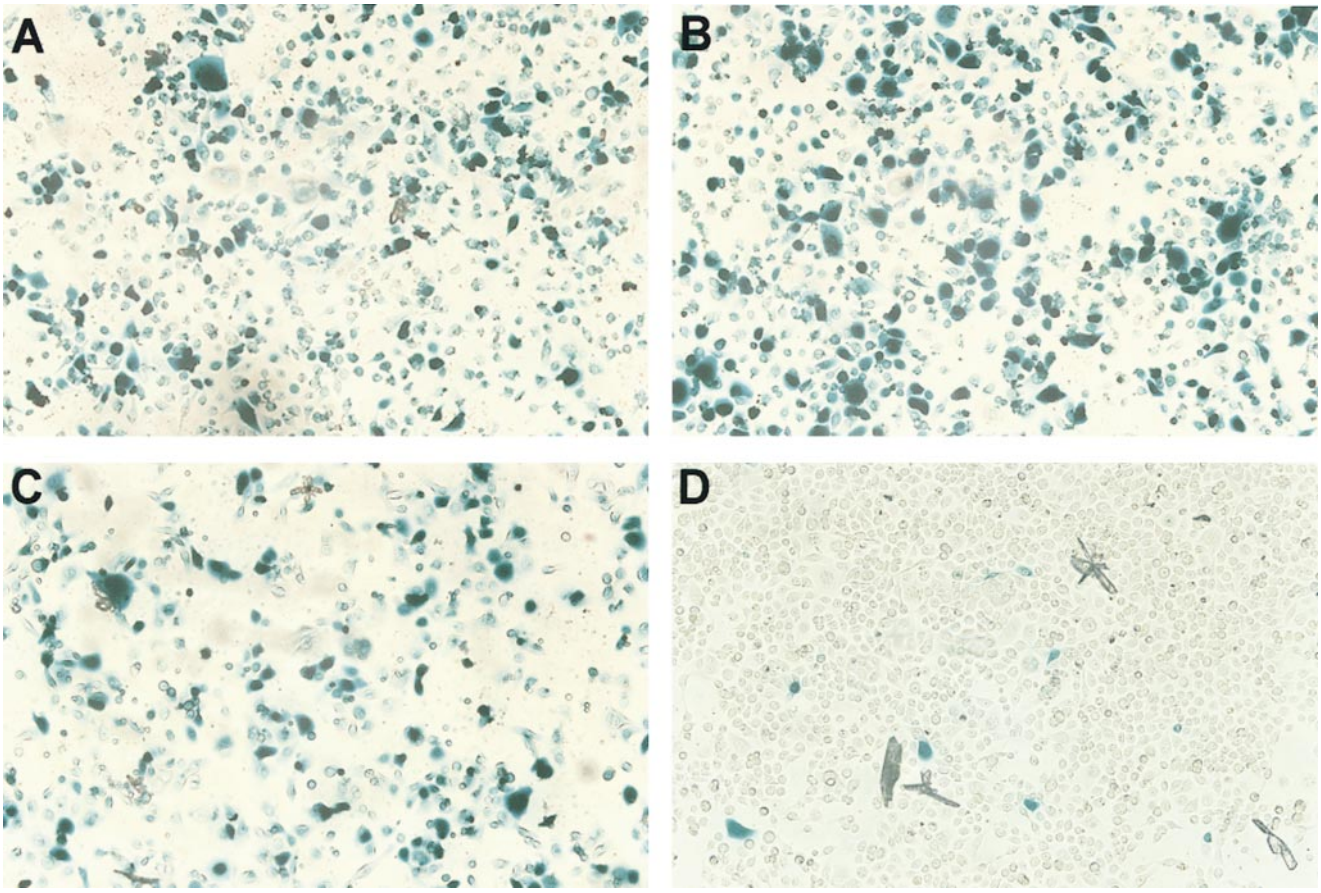

Figure 3. Photomicrographs of the expression of β-galactosidase after transfection with pCMV*LacZ*. The efficiency of gene transfer into CF/T43 cells was examined using lactosylated poly-L-lysine complexed to pCMV*LacZ* in the presence of 100 μM chloroquine and 5% glycerol. The cells were transfected for 4 h at 37°C on either one or three sequential days and incubated in growth medium. Gene expression was detected after fixation in 2% paraformaldehyde/0.2% glutaraldehyde and incubation for 18 h in X-Gal stain. 7.5 μg of pCMV*LacZ* complexed to 22.5 μg of lactosylated poly-L-lysine added once (*A*); 2.5 μg of pCMV*LacZ* complexed to 7.5 μg lactosylated poly-L-lysine added each day for three sequential days (*B*); 2.5 μg of pCMV*LacZ* complexed to 7.5 μg lactosylated poly-L-lysine added once (*C*); 2.5 μg pCMV*LacZ* without lactosylated poly-L-lysine added on three sequential days (*D*). The cells were examined with a Nikon Diaphot 300 microscope (original magnification: ×50).

hance luciferase gene expression, agents known to effect intracellular trafficking were explored as additives to the transfection medium. Glycerol, chloroquine, and E5CA peptide were examined individually or in specific combinations. The combination of 100 μM chloroquine and 100 μg E5CA peptide gave the highest enhancement of luciferase gene expression in all the airway epithelial cell lines. A 10,000-fold increase was observed in CF/T43 cells, compared with transfer without additions (Figures 1 and 2). Titration of E5CA peptide alone, or in combination with glycerol or chloroquine, showed that concentrations higher than 200 μg E5CA peptide did not give additional luciferase gene expression. This indicates that the maximum optimization with E5CA peptide was achieved (Figure 1).

Chloroquine, a weak base, is the most established agent used to enhance the level of gene expression with molecular conjugates. It neutralizes the acidic endosomes and inhibits the hydrolases in lysosomes, therefore decreasing degradation of the lactosylated poly-L-lysine complex (6).

The use of E5CA peptide alone to enhance luciferase gene expression with lactosylated poly-L-lysine was described previously in HepG2 cells in culture (17). E5CA, a modified E5 peptide derived from the *N*-terminal segment of the HA2 subunit of hemagglutinin, is thought to mimic the fusogenic activity of the influenza virus HA2 hemagglutinin (18). E5CA peptide has a pH- and concentration-dependent membrane permeabilization activity, allowing endosome disruption between pH 5 and 6 (19). By morphologic examination, E5CA peptide did not show evidence of toxicity in amounts up to 500 μg peptide, the highest amount examined (Figure 1).

Glycerol was shown to enhance luciferase gene expression using lactosylated poly-L-lysine as a vector (Figures 1 and 2). Zauner and colleagues (20) described enhanced reporter gene expression with a transferrin-polylysine construct using glycerol, without influencing the uptake of DNA complexes or increasing promoter activity. Furthermore, they showed that an osmotic effect on intracellular vesicles was unlikely.

TABLE 1

Assignments of ^{1}H-chemical shifts (ppm) of characteristic protons

Residue/Proton	Lysine*	Lysine.HCl	Lactose	*p*-TSA-Lys†	Lac-Lys‡
NH	8.41	—	—	—	—
αH	4.36	3.750	—	4.286	4.286
βCH_2	1.85, 1.76	1.901	—	1.740	1.769
γCH_2	1.45, 1.45	1.470	—	1.390	1.378
δCH_2	1.70, 1.70	1.723	—	1.614	1.645
εCH_2	3.02, 3.02	3.024	—	2.892	2.936
εNH_3^+	7.52	—	—	—	—
Glc H-1α	—	—	5.223	—	—
Glc H-1β	—	—	4.769	—	5.112
Gal H-1β	—	—	4.451	—	4.475
p-TSA $2,6H_2$	—	—	—	7.686	7.685
p-TSA $3,5H_2$	—	—	—	7.351	7.356
p-TSA CH_3	—	—	—	2.381	2.367
Phenyl $2,6/3,5H_4$	—	—	—	—	7.139

*Values from Wüthrich (24).
†*p*-Toluenesulfonate polylysine.
‡Lactosylated polylysine.

Synergistic effects were noted when E5CA peptide was combined with either glycerol or chloroquine and when glycerol was combined with chloroquine (Figure 2). One interpretation of these results is that these enhancing agents increased the luciferase gene expression by different mechanisms. It was observed that glycerol and polylysine seemed to synergize in their ability to enhance transfection (20). Others confirmed additional luciferase gene expression when chloroquine was combined with a fusogenic peptide (21). The enhanced luciferase gene expression, which resulted from combining chloroquine and E5CA peptide was greater than expected (Figures 1 and 2). Fusogenic peptides are reported to be activated by a low pH in the endosomes (18), whereas chloroquine raises the pH (6), therefore having contradictory effects. Nevertheless, a synergistic effect was noted. The observation that chloroquine facilitates the dissociation of the plasmid-vector complex may actually be more relevant than chloroquine raising the pH in the endosomes (22).

Efficiency of Transfection with Lactosylated Poly-L-Lysine as a Vector in the Presence of Glycerol and Chloroquine

To determine the efficiency of lactosylated poly-L-lysine as a vector, the expression of the *LacZ* gene was examined following transfection with different combinations and concentrations of agents. Using 5% glycerol and 100 μM chloroquine as enhancing agents, approximately 89% transfection efficiency was obtained with 7.5 μg plasmid complexed to 22.5 μg of lactosylated poly-L-lysine (Figure 3A). Three repeated administrations with one-third of the amount of complex on three sequential days resulted in a similar (85%) efficiency (Figure 3B). In contrast, using one-third the amount of complex for one administration resulted in 68% efficiency (Figure 3C). Repeated administration will be of value, as in some cases small volumes of the plasmid/vector complex may be required. In six separate experiments, chloroquine as the only enhancing agent gave only 20% transfection efficiency (data not shown). pCMV*LacZ* without the lactosylated poly-L-lysine vector gave $< 1\%$ transfection efficiency (Figure 3D).

The seemingly modest enhancement of luciferase gene expression when 5% glycerol was combined with chloro-

TABLE 2

Calculation of the number of lactose residues in lactosylated polylysine by integration values

Galβ H-1 (1 proton)	Galβ H-1 (1 proton)	Lys αH (1 proton)	Lys εCH_2 (2 protons)	Lac/100 Lys
629	—	1,567	—	40.1
629	—	—	3,014	41.7
132	—	343	—	38.5
132	—	—	639	41.3
—	618	1,567	—	39.4
—	618	—	3,014	41.0
—	135	343	—	39.4
—	135	—	639	42.3
Average number of lactose residues per 100 lysine residues:				40.5

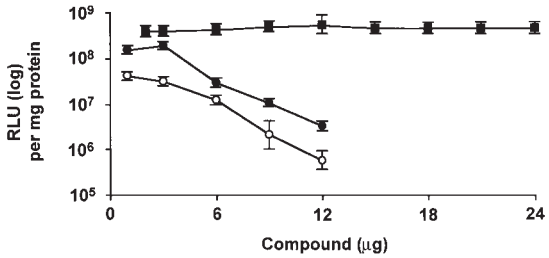

Figure 4. Toxicity of poly-L-lysine HBr and *p*-toluenesulfonate poly-L-lysine compared with lactosylated poly-L-lysine. CF/T43 cells were transfected with pCMV*Luc* (1 μg) complexed to either poly-L-lysine HBr (*closed circles*), *p*-toluenesulfonate poly-L-lysine (*open circles*), or lactosylated poly-L-lysine (*closed squares*) in the concentrations as noted. Transfection was performed in the presence of 100 μM chloroquine. After subsequent culture of 48 h in KGM medium the cells were lysed and luciferase gene expression was detected. Data represent mean values of RLU per mg protein ± SD of duplicate samples.

quine provided gene delivery into all cells. In fact, the conditions that increased the level of gene expression 10-fold as observed with pCMV*Luc* (Figure 2) improved the efficiency to an extent, comparable with that reported with viral vectors.

Glycerol in concentrations higher than 5% caused morphologic changes of the cells. However, combining 5% glycerol with chloroquine reduced toxicity and gave high expression of the reporter gene. Glycerol is of special interest in the transfer of the CFTR gene because it has been reported to reverse the misfolding phenotype of the ΔF508 CF mutation by stabilizing the protein (23). Thus, the use of glycerol has a dual effect on CF cells: enhancement of wtCFTR gene transfer and stabilization of newly synthesized wtCFTR protein as well as the ΔF508 CFTR expressed in the mutant cells.

Characterization of Lactosylated Poly-L-Lysine

High-resolution ^{1}H-NMR spectroscopy was used to characterize the lactosylated poly-L-lysine. The assignments of relevant protons in the ^{1}H-NMR spectrum of lactosylated poly-L-lysine were achieved by comparison with the values of lysine reported in the literature (24), and to the spectra of lysine, lactose, and *p*-toluenesulfonic acid–substituted poly-L-lysine, respectively, as listed in Table 1. The average number of lactose residues bound per lactosylated poly-L-lysine molecule was calculated by ratio of the integration values of the characteristic protons (Table 2) and was found to be 40.5 lactosyl residues per 100 lysine residues. The number of *p*-toluenesulfonic acid residues was calculated using the same method, showing an average of 61.2 residues per 100 lysine residues.

The toxicity of bromide-substituted poly-L-lysine and *p*-toluenesulfonic acid–substituted poly-L-lysine was compared with that of lactosylated poly-L-lysine. The vector/plasmid complexes were formed using different ratios of vector to plasmid. Gene transfer using poly-L-lysine neutralized with *p*-toluenesulfonic acid but not lactose, gave decreased luciferase expression in higher ratios (Figure 4) and caused extensive cell lysis (Figure 5B). Similar results were obtained with bromide-substituted poly-L-lysine (Figure 4). In contrast, lactosylated poly-L-lysine with 40% of the ε-amino groups substituted with lactose did not show any toxicity, even at an eightfold higher concentration than usually used (Figures 4 and 5A). Di Stefano and coworkers (7) reported that positively charged polylysine lost toxicity and immunogenicity *in vivo* when substituted with carbohydrate moieties.

The mono- or disaccharide-substituted polylysines may serve as specific vectors, which provide a significant level of transfection for a variety of cell types *in vitro*. Lactosylated poly-L-lysine, when used with enhancing agents, proved to be an efficient vector for gene transfer into airway epithelial cells in culture, with a transfection efficiency equivalent to that of viral vectors. The potential use of the enhancing agents for increased efficiency with other polylysine-ligand vectors or even liposomes remains to be explored. These agents in specific combinations may become of general use when it is necessary to improve or modulate the efficiency of transfection. In terms of efficiency the expression of the *LacZ* gene was raised to 90%. The specific characteristics of lactosylated poly-L-lysine, which include low toxicity and the targeting of airway epithelial cells, make it an ideal system for a variety of transfection experiments *in vitro*.

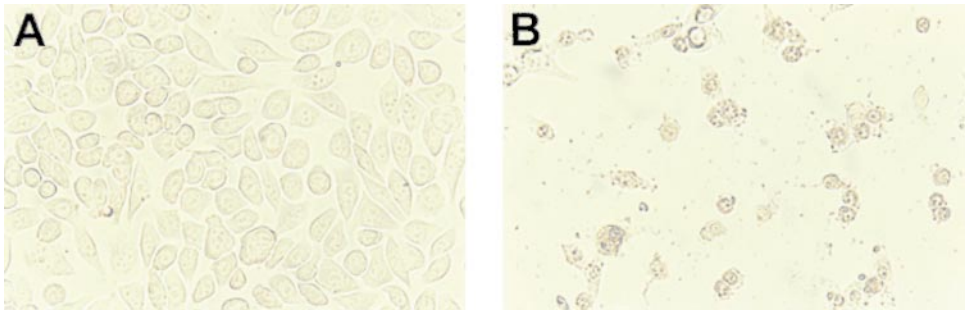

Figure 5. Photomicrographs of toxicity of *p*-toluenesulfonate poly-L-lysine compared with lactosylated poly-L-lysine. CF/T43 cells were transfected with either lactosylated poly-L-lysine/pCMV*Luc* (ratio 24:1 w/w) (*A*); or *p*-toluenesulfonate poly-L-lysine/pCMV*Luc* (ratio 12:1 w/w) (*B*). Transfection was performed in the presence of 100 μM chloroquine and the cells were incubated for 4 h at 37°C. After subsequent culture for 48 h in KGM growth medium, visualization was with a Nikon Diaphot 300 microscope (original magnification: ×200).

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