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In vivo gene transfer using cetylated polyethylenimine✪

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This report describes gene transfer *in vitro* as well as *in vivo* using cetylated low-molecular mass (600 Da) polyethylenimine (28% of amine groups substituted with cetyl moieties), termed CT-PEI. This compound is hydrophobic and has to be incorporated into liposomes in order to be suitable for gene transfer studies. Serum-induced plasmid DNA degradation assay demonstrated that CT-PEI-containing liposomal carriers could protect complexed DNA (probably *via* condensation). *In vitro* luciferase gene expression achieved using medium supplemented with 10% serum was comparable to that achieved in serum-reduced medium and was highest for CT-PEI/cholesterol liposomes, followed by CT-PEI/dioleoylphosphatidylcholine liposomes and PEI 600 Da (uncetylated) carrier. *In vivo* systemic transfer into mice was most efficient when liposome formulations contained CT-PEI and cholesterol. Higher luciferase expression was then observed in lungs than in liver.

In conclusion: liposomes containing cetylated polyethylenimine and cholesterol are a suitable vehicle for investigating systemic plasmid DNA transfer into lungs.

A continuous need exists for constructing more efficient non-viral vectors allowing transfer of therapeutic genes. Such vectors might be useful for studies exploring inhibi-

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Abbreviations: Chol, cholesterol; CT-PEI, cetylated polyethylenimine 600 Da; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; FBS, fetal bovine serum; LAL, Limulus Amebocyte Lysate; LPS, lipopolysaccharide (endotoxin); PEG, polyethylene glycol; PEI, polyethylenimine; RLU, relative luminescence units.

tion of angiogenesis, promotion of apoptosis, destruction of target cells by activated pro-drugs, restoration of cellular sensitivity to growth inhibitors or destruction of cancer cells' metastatic abilities (Luo & Saltzman, 1999; Pedroso de Lima *et al.*, 2000). More efficient and target-specific vectors should also be non-immunogenic and pose negligible risk of genetic recombination (see for example: Erbacher *et al.*, 1999; Kichler *et al.*, 2000; Oupicky *et al.*, 2001; Hood *et al.*, 2002; Kircheis *et al.*, 2002). They would be an alternative to viral carriers (retroviruses, adenoviruses, adeno-associated viruses, herpesviruses, lentiviruses and hybrid /retro-adeno/ viruses, see for example: Benihoud *et al.*, 1999; Monahan & Samulski, 2000; Trono, 2000) as well as to the clinically cumbersome physical methods.

A more widespread use of synthetic carriers has been hampered so far by the fragility of DNA-carrier complexes in robust biological environments (e.g. blood) (Dash *et al.*, 1999; Li *et al.*, 1999; Eliyahu *et al.*, 2002) resulting in relative inefficiency of DNA transfer.

Non-viral carriers take advantage of the chemical properties exhibited by certain compounds capable of binding to DNA and forming complexes that can readily adsorb onto cell surface and become endocytosed. Most importantly, such compounds are cationic in nature (Godbey *et al.*, 1999; Barron *et al.*, 1999).

Among the well known non-viral carriers of this kind are polyethylenimines. They are linear or branched polymers with a considerable density of positive surface charge (Godbey *et al.*, 1999). They are particularly effective in terms of DNA transfer to the cells. This probably results from the PEIs exhibiting the so-called "proton sponge effect". Upon endosomal pH decrease, increased PEI protonation leads to osmotic swelling and then bursting of the endosomes. As a result, the PEI-DNA complexes are released into the cytoplasm.

There have been numerous attempts to modify polyethyleneimines in order to increase their targeting specificity. Suitable examples are provided by polyethyleneimine with an Arg-Gly-Asp (RGD) motif-containing peptide ligand, recognized by cellular integrin receptors (Erbacher *et al.*, 1999), polyether–polyethylenimine graft copolymers (Nguyen *et al.*, 2000) or PEG–transferrin–PEI complexes (Ogris *et al.*, 1999). Other derivatives, such as alanine-substituted and dodecylated PEI were also synthesized (Thomas & Klibanov, 2002).

A PEI derivative obtained by partially substituting amine groups with cetyl moieties (CT-PEI) was used to prepare liposomes with increased resistance against the degrading activity of serum proteins (Yamazaki *et al.*, 2000).

The purpose of this study was to investigate suitability of CT-PEI for *in vivo* DNA transfer. By substituting amine groups of a low-molecular-mass polyetylenimine with cetyl residues a derivative was obtained which, unlike the hydrophilic PEI, was used to prepare liposomal formulations together with such neutral lipids as phosphatidylethanolamine (PE) or phosphatidylcholine (PC), or with cholesterol.

Preliminary toxicity screening *in vitro* of such liposomes showed no adverse effects upon the cell lines studied. A series of *in vitro* experiments followed in order to optimize transfer conditions (DNA amount, carrier-to-DNA ratio). Finally, systemic *in vivo* studies of luciferase reporter gene transfer into mouse lungs and liver were performed.

MATERIALS AND METHODS

CT-PEI synthesis. Synthesis of cetylated polyethylenimine (CT-PEI) was performed according to Yamazaki *et al.* (2000). In brief, the synthesis was initiated by reacting Epomin (low-molecular-mass polyethylenimine 600,

Nippon Shokubai Ltd., Japan) with cetyl bromide (Sigma-Aldrich, Milwaukee, WI, U.S.A.) in the presence of triethylamine for 12 h at 63°C. The product was dialyzed, first against 40% ethanol and then water. Finally, it was lyophilized. The end product was ^{1}H NMR-analyzed using a Unity Inova-300 spectrometer operating at 300 MHz (Institute of Organic Chemistry and Technology, Silesian Technical University in Gliwice, Poland) with deuterated chloroform as solvent and tetramethylsilane as reference.

CT-PEI liposomes. The liposomes were prepared by mixing aliquots of chloroform solutions (10 mg/ml) of CT-PEI and other lipids (DOPE, DPPC or cholesterol) at appropriate molar ratios and spin-evaporating the resulting preparations. Dry lipid film was hydrated at 4°C for 16 h and sonicated with a tip probe until the emulsion began to clear (two or three 1-min cycles, medium power, Branson sonifier). The sonicated liposomes were then extruded using gas-tight glass Hamilton syringes and a hand-held extrusion device fitted with two polycarbonate membranes of 100-nm pore size (Sigma).

DNA preparations. pVR1255 plasmid DNA containing luciferase reporter gene from *Photinus pyralis,* under the control of CMV promoter was kindly provided by Dr. R. H. Zaugg (Vical Inc., San Diego, CA, U.S.A.). The plasmids were isolated from DH5α *E. coli* (Gibco-BRL) cultures using a procedure developed by Horn *et al.* (1995). Crude plasmid DNA was endotoxin-purified on a Sephacryl S1000 column (h = 100 cm, ID = 1.6 cm, 150 cm^3 packed bed) and ethanol-precipitated. The endotoxin content in purified DNA preparations was assessed spectrophotometrically using the Limulus Amebocyte Lysate (LAL) reagent (BioWhittaker). One microgram of DNA preparation typically contained 0.03–0.04 LPS units.

Serum-induced degradation tests. Migration retardation of CT-PEI–liposome-complexed plasmid DNA was characterized in 1% agarose gel (not shown). Optimized (i.e. capable of protecting plasmid DNA) complex formulations were tested for stability in the presence of 10% serum. Aliquots of 2 μg pVR1255 plasmid DNA were incubated for 15 min at room temperature with increasing amounts of liposomes. Fetal bovine serum (FBS, final conc. = 10%) was added to the complexes formed. The samples were incubated for 4 h at 37°C. The complexes were subsequently treated with EDTA (final conc. = 10 mM), SDS (final conc. = 0.5%) and incubated for 20 min at 55°C. Plasmid DNA was phenol/chloroform- extracted and precipitated before performing electrophoresis.

Cell culture. Studies of transfection efficiency were performed using three cell lines: B16(F10) (murine melanoma) and Renca (murine renal cell carcinoma), both obtained from Wistar Institute (Philadelphia, PA, U.S.A.), and HeLa (human cervical epithelioid carcinoma) obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw, Poland). Cells were grown in T-25 (Corning) flasks in RPMI 1640 medium supplemented with 10% FBS and antibiotics (streptomycin at 100 μg/ml and ampicillin at 100 U/ml), at 37°C and 5% CO_2.

In vitro transfection studies. One day prior to transfection cells were seeded in 24-well plates, at 5×10^4 cells/well. When the cells reached about 80% confluence, appropriate dilutions of CT-PEI/DOPE liposomes or PEI were prepared in 100 μl final volume using polystyrene tubes (Sarstedt). Separately, appropriate dilutions of 2 μg plasmid DNA in 150 mM NaCl were prepared, using identical volumes and tubes. The carrier emulsions were added dropwise to DNA solutions and the tube was gently mixed. Following 30-min incubation at room temperature, 850 μl of suitable medium (RPMI+Fbs, RPMI-FBS or Opti-MEM) was added to each sample. The transfection mixtures were then transferred to the wells, following removal of growth medium. Cells were next incubated for 4 h at 37°C and 5% CO_2. At the end of in-

cubation, the transfection medium was replaced with regular growth medium. After 24 h cells in each well were lysed using a Promega kit-provided lysis buffer.

In vivo transfection studies. *In vivo* transfer of CT-PEI-containing complexes into mice was performed as follows: aliquots of pVR1255 plasmid DNA were complexed to aliquots of polycarbonate membrane-extruded (100 nm nominal pore size) liposomes in 5% dextrose, by adding plasmid DNA dropwise to liposomal emulsion. Six- to eight-week-old C57 BL/6 mice were injected 600 μl of the above transfection mix containing varying amounts of plasmid DNA and liposomes. This volume was required to accommodate the complex components without causing precipitation. The animals were sacrificed at various time-points after injection of complexes in order to recover lung and liver tissues. The tissues were rinsed with ice-cold PBS^- and homogenized in Promega lysis buffer. Samples were centrifuged at 15 000 r.p.m. at 4°C and supernatant aliquots were assessed for luciferase activity.

Determining luciferase enzymatic activity in cell lysates. After 24 h the cultures in each well were lysed using the Luciferase Assay System buffer (Promega). The lysates were centrifuged for 30 min at 15 000 r.p.m. and 4°C, supernatants (100 μl) transferred into fresh test tubes and luciferase activity determined, using a Berthold luminometer, after adding 100 μl aliquots of enzyme substrate to the lysates (20 μl or less). Protein content in the lysates was measured by the Bradford method using a kit from BioRad (and bovine serum albumin as a standard).

RESULTS

Synthesis of cetylated polyethylenimine

The cetyl derivative of polyethylenimine (CT-PEI) was synthesized as described in Materials and Methods. The synthesized yellowish compound was insoluble in water; following dialysis it became white (yields usually varied between 60 and 70%).

^{1}H NMR analysis of CT-PEI

The structure of the synthesized compound and the number of substituted amine groups were confirmed by ^{1}H NMR. The degree of substitution in different syntheses was found to be similar (between 22 and 28 mole %). The spectrum shown in Fig. 1 refers to one of the syntheses. The triplet at 0.881 p.p.m. corresponds to $-CH_3$ from the cetyl substituents. The multiplet between 1.15–1.56 p.p.m. corresponds to the remaining protons from the cetyl substituents, except for the protons at the carbon atom linked to the amine group; the latter signals overlap the multiplet (2.3–3.3 p.p.m.) from protons of PEI ethyl groups ($-CH_2-CH_2-$). Signal intensities represented as integration curves proportional to the number of hydrogen atoms in corresponding moieties demonstrate that the synthesized CT-PEI contains, on average, 14 ethylene units and 4 cetyl groups. This corresponds to 28 mole% substitution yielding stoichiometric formula where m = 14 $(C_2H_4N)_m(C_{16}H_{33})_{0.22-0.28m}$.

Stability of CT-PEI-containing complexes in the presence of serum

CT-PEI liposomes were prepared as described in Materials and Methods. The stability of the complexes in the presence of 10% serum was investigated for CT-PEI/ DOPE (0.65:1, mol/mol) (not shown); CT-PEI/ DPPC/Chol (0.65:1:1, by mol) (not shown) and for CT-PEI/Chol (1:1, mol/mol) (Fig. 2).

Complexes formed at the liposome/DNA (w/w) ratios 4:1 and higher (lanes 7–10) are able to protect plasmid DNA against degradation by 10% serum during a 4-h incubation at

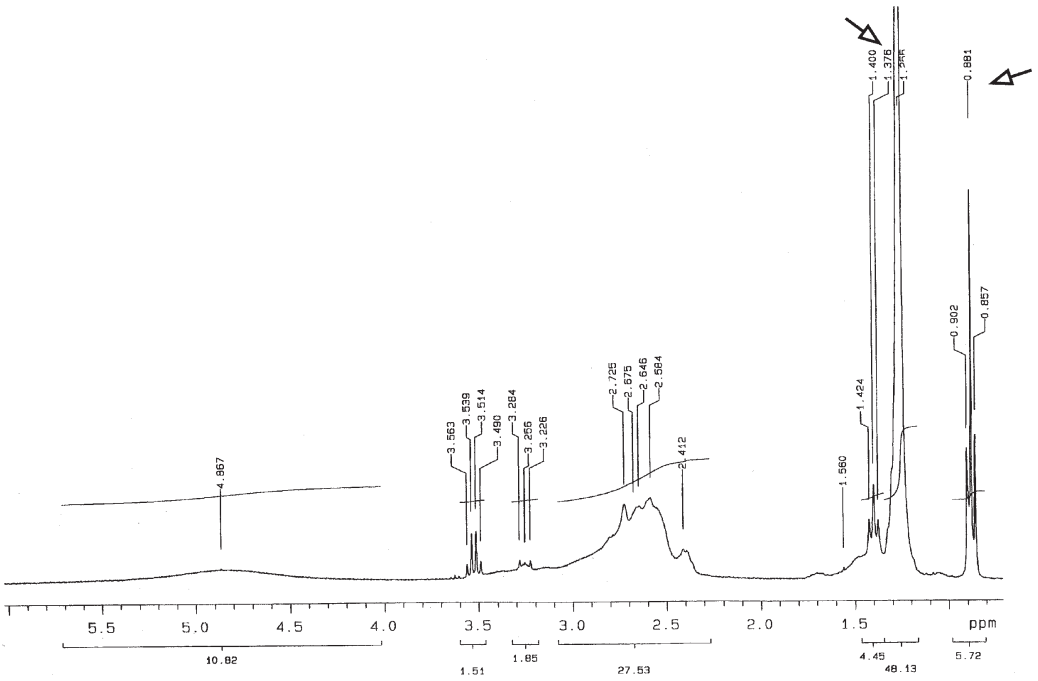

Figure 1. ^{1}H NMR spectrum of CT-PEI.

Note peaks (triplet at 0.881 p.p.m. and multiplet at 1.15–1.56 p.p.m.) indicative of the presence of cetyl moieties in CT-PEI (arrows). See Results for details.

37°C. Lower ratio formulations, i.e. 1:1 through 3:1 (lanes 1–6) do not protect DNA completely (Fig. 2).

In vitro transfection of neoplastic cell lines

In vitro transfections of the luciferase reporter gene (Fig. 3) were performed in the presence of 10% serum using either uncetylated poliethylenimine (control) or CT-PEI liposomes formulated with either DOPE or cholesterol. Three different cell lines were used: (B16(F10), HeLa and Renca (only the first is shown).

Luciferase expression, which indirectly illustrates the efficiency of gene transfer, was lowest when plasmid DNA was complexed to uncetylated PEI, compared to both liposomal formulations. CT-PEI liposomes containing DOPE yielded the reporter gene expression higher by one order of magnitude. When DOPE was substituted with cholesterol, luciferase expression in cells transfected using

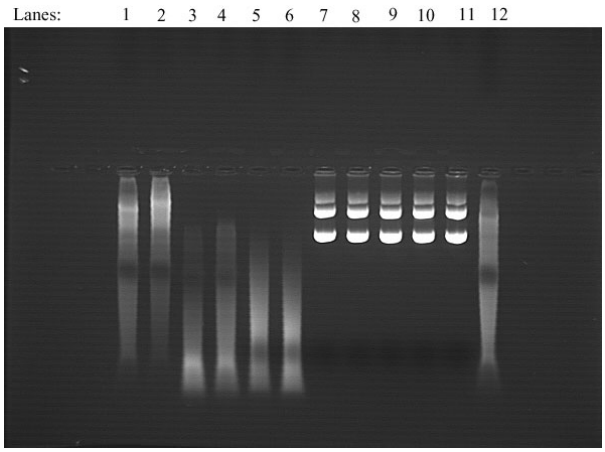

Figure 2. Protection assay of plasmid DNA by complexes containing CT-PEI/Chol liposomes following exposure to 10% serum.

Aliquots (2 μg) of pVR1255 DNA were mixed with increasing amounts of liposomes; the resulting complexes were then exposed to 10% serum (4 h) following which DNA was extracted from samples and electrophoresed, as described in Materials and Methods. Legend: 2 μg CT-PEI/Chol liposomes 1:1 (mol/mol) (lanes 1–2); 4 μg (lanes 3–4); 6 μg (lanes 5–6); 8 μg (lanes 7–8); 10 μg (lanes 9 and 10); uncomplexed plasmid DNA (lane 11); uncomplexed and serum-exposed plasmid DNA (lane 12).

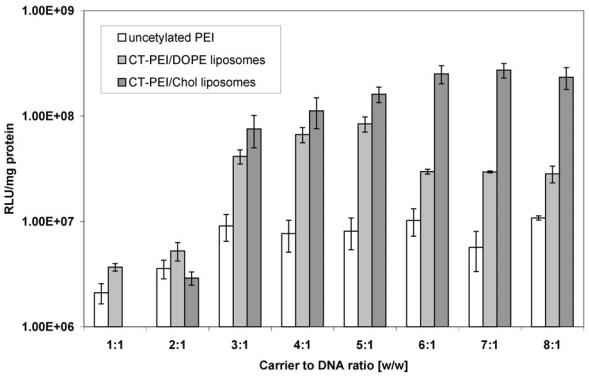

Figure 3. Luciferase gene expression in B16(F10) murine melanoma cells following *in vitro* transfer (in the presence of serum) of plasmid DNA complexed to uncetylated PEI or CT-PEI-containing liposomes.

Complexes were formed containing 1 μg of pVR1255 (DNA amount optimization data not shown) and different amounts of PEI or CT-PEI liposomes. Complexes were formed either in Opti-MEM (not shown) or in RPMI medium supplemented with 10% FBS. The complexes were used to transfect cells seeded on 24-well plates, as described in Materials and Methods. Each bar represents mean (±S.D.) of triplicate determinations of luminescence. Open bars: uncetylated PEI-600; light grey bars, optimized lipoplexes containing CT-PEI/DOPE 0.65:1 (mol/mol); dark grey bars, optimized lipoplexes containing CT-PEI/Chol 1:1 (mol/mol); composition of both lipoplexes was previously optimized in separate experiments (not shown).

serum-containing medium was the highest (above 5×10^8 RLU/mg protein) and comparable to that seen in the absence of serum (not shown). CT-PEI/cholesterol liposomes were chosen for *in vivo* studies.

In vivo gene transfer into mice

Transfer of plasmid DNA containing the luciferase reporter gene was also attempted *in vivo*, using C57Bl/6 mice. Mice were given a single intravenous injection (tail) of liposome carrier-plasmid DNA complexes formed at a chosen weight ratio of liposomes to plasmid DNA (see Materials and Methods for details).

As a plasmid carrier *in vivo*, CT-PEI liposomes formulated with cholesterol (1:1, mol/mol) were chosen. It had been initially found that addition of phosphatidylcholine (DPPC) to CT-PEI/cholesterol liposomes did not augment luciferase expression neither when a comparable amount of the cationic component was used in the formulation nor at the ratio previously proposed by Yamazaki *et al.* (2000) (not shown).

The time course of the reporter gene expression following administration of complexes formed at a constant weight ratio of liposomes to plasmid DNA was first determined in lungs and livers at various time points following injection. It was found (Fig. 4) that luciferase expression was highest 24 h after complex administration and that lungs were consistently transfected more efficiently than liver.

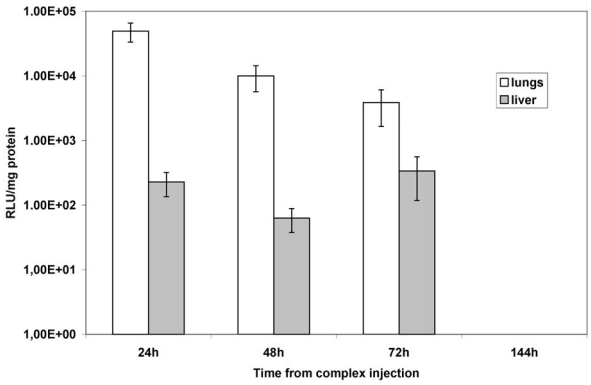

Figure 4. Kinetics of luciferase gene expression in murine lungs and liver following *in vivo* transfer of plasmid DNA complexed to extruded CT-PEI/Chol (1:1, mol/mol) liposomes.

Complexes containing 60 μg of pVR1255 (DNA amount optimization data: see Fig. 5) and 360 μg extruded liposomes were injected (in 600 μl total volume of 5% dextrose) intravenously (tail) to 6–8 week-old C57 Bl/6 mice. Animals were sacrificed at indicated time points after complex administration and luciferase activity was measured in lung and liver tissue homogenates. Each bar represents mean (±S.D.) of triplicate determinations of luminescence.

The dependence of luciferase expression in the lung and liver upon the DNA dose administered was also investigated (Fig. 5). Eighty micrograms of plasmid DNA was found to be

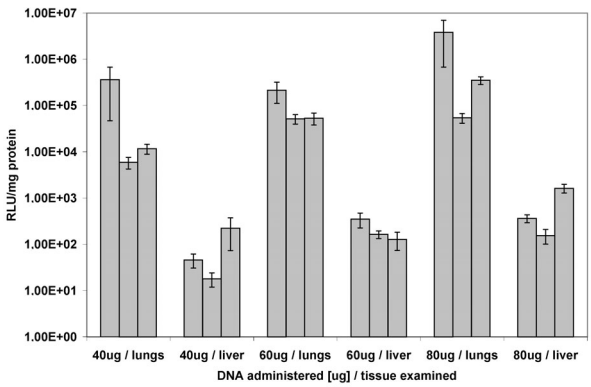

Figure 5. Distribution of luciferase gene expression as a function of plasmid DNA dose as assessed in murine lungs and liver following *in vivo* transfer of plasmid DNA complexed to extruded CT-PEI/Chol liposomes.

Complexes containing the indicated amounts of pVR1255 and varying amounts of extruded CT-PEI/Chol (1:1, mol/mol) liposomes (keeping the DNA/lipids weight ratio at 1:6; complex optimization not shown) were injected as described in Materials and Methods and Fig. 4. Animals were sacrificed at 24 h post complex administration and luciferase activity was measured in lung and liver tissue homogenates. Each bar represents mean (± S.D.) of triplicate determinations of luminescence.

the highest administrable dose. Higher amounts of DNA caused complex precipitation when the indicated volume was used, while larger volumes were detrimental to mice. Therefore, the amount of 60 μg of plasmid DNA was adopted for further *in vivo* studies.

DISCUSSION

The present report describes *in vitro* and *in vivo* studies of plasmid DNA transfer achieved with a liposomal carrier containing cetylated polyethylenimine (CT-PEI), a novel low-molecular-mass cationic polymer. The goal of this study was to assess the suitability of this compound for systemic gene transfer as well as to establish its *in vivo* targets.

Cetylated PEI was synthesized according to a protocol published by Yamazaki *et al.* (2000). ^{1}H NMR spectra showed that 22–28 mole % of PEI amine groups were successfully substituted with cetyl moieties. The latter value obtained in one of the syntheses is somewhat higher than the one originally reported (see Fig. 1).

The cetyl derivative of PEI, due to its hydrophobic nature, has to be formulated into liposomes in order to be useful for DNA transfer. In our study the liposomes, besides CT-PEI, contained either DOPE or cholesterol (and in some cases also DPPC).

All three investigated liposomal formulations were able to complex plasmid DNA. Complex formation, presumably accompanied by DNA condensation, has long been regarded as imparting to DNA resistance against degradation by serum. The ability of these liposomes to protect DNA was checked by studying electrophoretic migration patterns of plasmid DNA aliquots extracted from complexes that had been previously exposed to 10% serum (Fig. 2). The CT-PEI-containing complexes effectively protected transferred DNA at certain liposome/DNA ratios. A stepwise increase of retardation ability, similar to that seen in Fig. 2, was repeatedly noticed in several experiments. Its nature requires explanation; this was not, however, the goal of our study.

The applicability of the studied liposomes for DNA transfer was further examined in a series of *in vitro* transfection experiments, in the presence of serum (Fig. 3). They showed that CT-PEI polycationic liposomes were suitable for *in vitro* DNA transfer, matching the efficiencies obtainable either with high molecular mass PEIs or with other well established liposomal formulations (e.g. Bragonzi *et al.*, 1999; Sochanik *et al.,* 1999). In our hands, the best were CT-PEI liposomes formulated with cholesterol at 1:1 (mol/mol), as opposed to 0.65:1 (mol/mol) CT-PEI/DOPE previously proposed by Yamazaki *et al.* (2000). In that study DNA transfer was higher in the presence of serum. Here we confirm similar relationships for HeLa and Renca cell lines (re-

sults not shown). Transfection of B16(F10) cells with CT-PEI liposomes in the presence of serum yielded luciferase gene expression that was decreased by half to one order of magnitude.

Several investigators have pointed out the composition and stoichiometric formulae of nonviral carriers, carrier to DNA weight ratios, volumes, media used, etc., as crucial factors for successful DNA transfer *in vivo* (see for example: Liu *et al.*, 1997; Loisel *et al.*, 2001). After having established the kinetics of luciferase gene expression (Fig. 4) we determined an optimum safe dose of administered plasmid DNA (Fig. 5). Our results *in vivo* fully confirmed the essential significance of the mentioned parameters. In our hands, the three-component liposomes (CT-PEI, cholesterol and DPPC) described by Yamazaki *et al.* (2000) were not as effective in transfecting lungs as the liposomes containing just CT-PEI and cholesterol.

The efficiency of gene transfer with non-targeted complexes depends on the amount of the complexes reaching cells in target tissues. Destructive interactions between the elecrostatically unshielded cationic complexes and blood components may require increased quantities of carrier to be administered in order to achieve efficient gene transfer. Yet, biosafety considerations limit the tolerable doses of both plasmid DNA and carrier (Senior *et al.*, 1991; Eliyahu *et al.*, 2002). We thus explored another possibility of increasing transfer efficiency *in vivo* by preinjecting cationic liposomes (results not shown). This approach had been used previously by other investigators (Liu *et al.*, 1995; 1997; Song *et al.*, 1998). In fact, it did increase expression of the reporter gene but higher expression was offset by marked toxicity in mice, most likely resulting from the total dose of cationic lipid administered. For our *in vivo* studies we used liposomes extruded through 100-nm pore polycarbonate membranes. This markedly improved luciferase expression, especially in the lungs that are, it has to be remembered, the first capillary bed encountered by complexes upon intravenous tail injection. Volumes required to achieve successful injection of such tolerable liposome/DNA complex quantities (without causing precipitation) were in our experiments close to the borderline values reported (Thierry *et al.*, 1995; Goula *et al.*, 1998; 2000; Loisel *et al.*, 2001).

In summary, low-molecular mass PEI substituted with hydrophobic cetyl moieties was formulated into cationic liposomes either with DOPE or with cholesterol, or with both phosphatidylcholine and cholesterol. Such liposomes can be used as efficient DNA carriers for transfecting various cell lines *in vitro*. Extruded CT-PEI-containing liposomes formulated with cholesterol proved effective in systemic gene transfer *in vivo* allowing marked reporter gene expression in murine lungs.

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