In Vivo Gene Transfer by Intravenous Administration of Stable Cationic Lipid/DNA Complex

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Purpose. A stable cationic lipid/DNA complex has been developed for *in vivo* gene transfer. The formulation capitalizes on a previously described procedure to obtain stable lipid/DNA complexes for *in vitro* gene transfer (1).

Methods. Conditions for DNA/lipid complex formation were modified to yield a DNA concentration of 1 mg/ml. Heat stable alkaline phosphatase (AP) under a CMV promoter was used as a reporter gene.

Results. The resulting complex was completely insensitive to serum inactivation. Tail vein injection of a 80 μg DNA into Balb C mice yielded significant levels of reporter enzyme activity in the lung, heart, spleen, muscle, and liver. Less AP activity was observed in the kidney. No AP activity was observed in blood, bone marrow or brain. A titration of the lipid (DOSPA) to DNA-nucleotide ratio showed the optimal molar ratio for *in vivo* gene transfer to be 1/1. Using this ratio in a dose response study showed approximately 80μg of DNA/mouse yielded the highest level of gene expression. Using this dose at a 1/1 lipid to DNA nucleotide ratio, the time course for alkaline phosphatase activity was determined. Maximal AP activity was observed 24 hours after injection for all tissues. By day 5, the activity dropped approximately 10 fold for all tissues. By day 7, residual activity was detected in the lung, heart, and muscle. Histology of the lung showed both interstitial and endothelial cells to be transfected. In all other tissues, however, endothelial cells were the only transfected cell type.

Conclusions. These results demonstrate that reformulation of an existing cationic lipid can result in the formation of a stable lipid/DNA complex, which is able to reproducibly transfect lung, heart, spleen, and liver upon intravenous administration.

KEY WORDS: cationic lipid; DNA; *in vivo*; gene transfer; alkaline phosphatase.

INTRODUCTION

Several non-viral based vehicles are being developed for human gene therapy. These are lipid based (2–5) polymer based (6,7) and protein based (8–11) gene transfer vehicles. The major limitation that needs to be overcome for this technology to become a viable alternative to viral based vehicles is comparable levels and duration of transgene expression. However, before this can even begin to be addressed, formation of a stable complex that can be reproducibly manufactured and that remains active upon storage is required. Previously, we described the assembly of a stable lipid/DNA complex and characterized the storage conditions with respect to *in vitro* transfection activity (1). The complexation step of the detergent solubilized cationic lipid to the DNA has been modified to yield a cationic lipid/DNA complex that can be formed at 1mg DNA/ml. The resulting complex has a minimal shelf life stability of 3 months when stored as a suspension at 4°C, and can be administered i.v. into the tail vein of a mouse yielding significant levels of gene transfer activity in the lung, spleen, muscle, heart and liver. The following report describes the preparation procedure for these stable lipid/DNA complexes in detail, and characterizes the *in vivo* gene transfer activity.

MATERIALS AND METHODS

Materials

Lipofectamine™ was purchased from Gibco BRL/LTI (Gaithersburg, MD). Lipofectamine™ is supplied as a liposome suspension consisting of 2 mg/ml DOSPA/DOPE (3:1 w/w; or 1.53/1 mol/mol). Lipofectin™ (DOTMA/DOPE = 1/1 mol/mol) was also purchased from Gibco BRL/LTI. DOSPER and DOTAP were obtained from Boehringer Mannheim (Indianapolis, IN). Ethylated-DOPC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Octylglucoside (OG) or n-Octyl-β-D-glucopyranoside was purchased from Calbiochem (San Diego, CA). To assay *in vivo* gene expression, a plasmid was constructed encoding human placental alkaline phosphatase expressed as an extracellular integral membrane protein. Use of this as a marker gene was first described by Fields-Berry et. al. (12). Plasmid SSV9-MD-2 was derived from plasmids SSV9 (13) and pMD-G (14). pSSV9-MD-2 contains the Cytomegalovirus (CMV) immediate early gene promoter/enhancer obtained from pBC12/CMV/IL-2 exons 2 and 3 (15), a β-globin intervening sequence 2 (IVS2) and a human β-globin polyadenylation signal. This fragment was modified by PCR to incorporate restriction sites for PmlI, EcoRI and BglII for subcloning the membrane bound form of human placental alkaline phosphatase (mAP) as a reporter gene (16). A schematic representation of the plasmid is given in Figure 1.

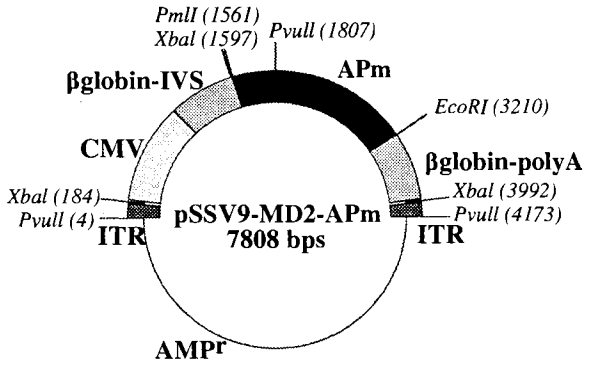

Fig. 1. A schematic representation of the plasmid used in these studies. Two AAV ITR sequences flanking a CMV promoter driving a gene encoding human placental alkaline phosphatase expressed as an extracellular integral membrane protein.

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DNA Purification

The plasmids were amplified in E. coli (DH10β from Gibco BRL) and grown in circle grow (Bio101, Vista, CA). Bacteria were lysed by the alkaline lysis method and plasmid DNA was purified using multiple phenol/chloroform/isoamyl alcohol extractions followed by double CsCl banding (17). The DNA was dissolved in 10mM TRIS, pH 7.4 + 1mM EDTA (TE) and dialyzed for 24 hours at 4°C against TE. Each plasmid preparation was assayed for endotoxin (LPS) activity. All plasmid preparations had LPS levels of less than 1 mU/mg DNA. A BCA protein assay (Pierce, Rockford, IL) was performed using 200 μg of plasmid showing no detectable protein. The detection limit of the assay is 2.5 μg of albumin per ml.

Preparation of Stable Lipid-DNA Complex

The protocol for formation of a stable lipid/DNA complex (1) was modified to accommodate a 50 fold increase in the DNA concentration. The water from the Lipofectamine™ was completely removed using a Speed Vac Centrifuge (Savant Instruments, Farmingdale, NY). The dry lipids were solubilized in OG buffer: 2% octylglucoside (OG), 3M NaCl, 10mM TRIS (pH 7.4). Sonication in a bath type sonicator (Laboratory Supplies, Hicksville, NY) for one minute was used to assure complete solubilization. The DNA is dissolved in OG buffer and added to the detergent solubilized lipids. Typically, 500μg DNA (1.5μmoles nucleotides) and 1.5μmoles DOSPA (1.362ml Lipofectamine™, which was dried) were mixed in a final volume of 1ml OG buffer. The clear mixture was dialyzed against 4 × 1000 volumes of 3M NaCl, 10 mM TRIS, pH 7.4, over 48 hr. at 4°C using Slid-A-Lyzer dialysis cassettes (Pierce, Rockford, IL). The clear dialysate was dialyzed against 2 × 1000 volumes of 0.15M NaCl, TRIS, pH 7.4 for 16 hours (with continuous mixing for the first 10 minutes). Changing the dialysis buffer from 3M NaCl to 0.15M NaCl causes a slight increase in the final volume of the lipid/DNA suspension. The final product was a homogeneous, turbid suspension with no visible aggregates.

In Vitro Cell Transfection

Twenty four well plates were seeded with 6×10^4 NIH 3T3 cells (ATCC, Rockville, MD) and transfected 24 hours later. The medium was removed and the appropriate amount of stable lipid/DNA complex was diluted in 0.5 ml DMEM (either with or without 10% calf serum) and added to the well. The cells were incubated at 37°C for 5 hours. The lipid/DNA complex containing medium was replaced by DMEM containing 10% calf serum (Bio-Whittaker, Walkersville, MD), and cells were cultured for another 24 hours. Alkaline phosphatase expression was determined with the colorimetric pNPP assay.

In Vivo Administration of Stable Lipid/DNA Complex

Female Balb/C mice of 18–22 grams were obtained from Taconic (Germantown, NY). Prior to injection the mice were anesthetized with Sodium Pentobarbital (Anthony Products; Arcadia, CA), which was diluted to a 6.5mg/ml solution in saline and administered intraperitoneally at a dose of 75–80mg/kg. Typically, each mouse received 80μg DNA intravenously via the tailvein injection in a maximum volume of 300μl. The mice were sacrificed 24 hours after injection. The tissues were harvested, immediately frozen in liquid nitrogen, and stored at −80°C until assayed for AP activity using the immunocapture assay.

Alkaline Phosphatase pNPP Assay

The cells were washed twice with 1 ml Dulbeco's PBS. The cells were lysed with 200μl lysis buffer containing 250mM TRIS (pH 7.4) + 1.0% Triton X-100. The cell lysates were heat inactivated at 65°C for 30 minutes, to eliminate endogenous AP activity. The samples were cooled on ice and 100μl of the sample was placed in the wells of a 96 well plate. An equal volume of substrate buffer containing 4.5mg/ml p-nitrophenyl phosphate (pNPP) (Sigma, Milwaukee, WI), 1M diethanolamine, 0.28M NaCl, and 0.5mM $MgCl_2$ was added. The change in OD405 was measured over time using a Molecular Devices plate reader. The AP activity is expressed in total mUnits per 10^5 cells transfected. 1 mU is defined as the amount of AP that hydrolyzes 1nmol of pNPP per minute at 37°C. A standard curve of purified AP (Sigma) was measured with each set of samples. V_{max} was calculated for each sample.

Alkaline Phosphatase Immunocapture Assay

A 96 well plate was coated with goat anti-mouse Ab (Sigma) in 0.1M $NaHCO_3$, pH 9.8 overnight at 4°C. After washing with TBS (154 mM NaCl, 50mM TRIS pH 7.4, 1mM $MgCl_2$), the plate was blocked with 1% BSA in 250mM NaCl, 20mM TRIS (pH 7.4) for 1 hour at room temperature. Mouse anti-human Alkaline Phosphatase Ab (Sigma) in blocking buffer was added to the plate and incubated overnight at 4°C. The plate was washed with TBS before applying the tissue homogenates. The tissues were homogenized in AP-lysis buffer containing 250mM TRIS (pH7.4), 1% Triton X100, and 0.1mM PMSF with a Vitrishear tissue homogenizer (Virtis, Gardiner, NY) at a concentration of 100mg tissue per ml AP-lysis buffer. The homogenates were heat inactivated at 65°C for 30 minutes. Samples were centrifuged at 3000× g at 4°C, for 15 min. 300μl of supernatant was applied per well and incubated at 4°C, overnight. After washing with TBS, AP activity was determined by pNPP assay as described above. The sensitivity of the immunocapture assay was approximately 0.01mU of AP per 100mg tissue.

Histology

Histochemistry was used to determine the cell types that were transfected. After the animals were deeply anesthetized the trachea was intubated and the lungs were inflated with 5 ml air to avoid collapse. The whole animal was then perfused through the aorta with ice cold PBS, followed by perfusion with 4% paraformaldehyde (PFA). The spleen, skeletal muscle, heart, liver, and kidney were removed, and postfixed in 4% PFA by immersion for 30 min. Tissues were washed with PBS and transferred to 30% sucrose solution until saturated. The organs were then cut into pieces of 4 × 4mm, submerged in OCT compound (VWR), frozen in dry ice/isopentane bath, and sectioned in a cryostat to a thickness of 10μm.

Alkaline Phosphatase (AP) Histochemistry

The alkaline phosphatase histochemistry was a modified procedure from S.C. Fields-Berry et al. (12). Briefly, the sec-

tions were washed with PBS, and heated at 65°C for 1 hour, to inactivate endogenous AP activity. After cooling to room temperature the sections were incubated in AP-buffer (100mM TRIS,100mM NaCl, 5 mM $MgCl_2$,pH 9.5) for 20 minutes, followed by incubation with AP substrate buffer (0.1mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 1.0mg/ml nitroblue tetrazolium, 0.24mg/ml levamisol (Sigma) in AP-buffer) for 2 hr in the dark at room temperature. The reaction was stopped by adding EDTA to a final concentration of 20 mM. Sections were then washed in PBS, counter stained with nuclear fast red, and coverslipped with aqueous mounting medium.

Lung Processing

After whole animal perfusion, the lungs were removed from the animals and infused through the trachea with 4% PFA. The degree of fixation was increased by immersing the lungs in the same fixative for 30 minutes. The lungs were washed with PBS, and heated at 65°C for 1 hour. The lungs were then perfused and immersed with AP buffer for 20 minutes at room temperature. The lungs were perfused and immersed with substrate buffer , for 1 hour at room temperature. The reaction was stopped by 20mM EDTA. The lungs were rinsed with PBS, and were equilibrated in 30% sucrose. The tissue was embedded in paraffin and cut into 5 μm sections. The sections were counter stained with nuclear fast red.

RESULTS

The formation and *in vitro* characterization of stable lipid/DNA complexes has been previously described (1). Concentrating these complexes more than 10 fold by various dehydration methods led to formation of large aggregates, and thus to inactivation of the complex (results not shown). Hence, the other alternative was to increase the DNA concentration during manufacture rather than after particle formation. A series of conditions were tested to increase the DNA concentration during the initial lipid/DNA complex formation step and turbidity measurements were used to monitor the effect of these different conditions at relatively high DNA concentrations.

The effect of different conditions on complex solubility at 0.5mg of DNA/ml was tested by monitoring % transmittance at 380 nm as an indication of aggregation. Figure 2 shows the result of an increasing salt concentration on the turbidity of a DNA/lipid mixture in the presence of octylglucoside. A 0.5mg/ml DNA (i.e. 1.515μmol DNA nucleotides/ml) solution in the presence of 2.724mg/ml detergent solubilized Lipofectamine™ (i.e. 1.515μmol DOSPA/ml) had a low % transmittance at 380nm indicative of aggregation. The % transmittance increased abruptly when the $MnCl_2$ or NaCl concentration was increased from 0.3 to 1M. At 1M, the % transmittance returned to the original value of 0.5mg/ml DNA in the absence of detergent solubilized lipid. The stabilizing effect of high salt concentration allows for complexation of high concentrations of DNA to lipid.

Dialysis of the lipid/DNA complex against the same salt concentration used for solubilization resulted in a clear dialysate. Subsequent dialysis against either 5% dextrose or 0.15 M NaCl produced a turbid suspension with no visible aggregates with high transfection efficiency. Lack of aggregates was dependent upon the lipid to DNA nucleotide ratio. At ratios of 1/1 (mol/mol) or above no aggregates were observed.

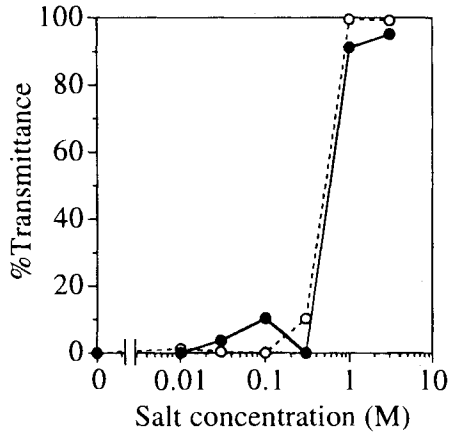

Fig. 2. The effect of both $MnCl_2$ (—●—) and NaCl (---○---) on lipid/DNA complex formation in the presence of 2% detergent (OG). Percent transmittance at 380nm is a measurement of sample turbidity, indicative of aggregate formation.

Serum stability of the complex was tested by incubating the lipid/DNA complex in DMEM containing various percentages of calf serum at 37°C for 1 hour, prior to *in vitro* transfection (Figure 3). The formulations were then diluted to either 0.5, 1.0, and 1.5μg DNA per 0.5 ml DMEM with a final serum concentration of 10%. The control cells were transfected in the absence of serum with stable lipid/DNA complex pre-incubated at 37°C for 1 hour in DMEM without calf serum. The results show that at all DNA and serum concentrations tested, no decrease in AP activity was observed. Even an overnight incubation of the complex in 100% serum at 37°C did not decrease transfection efficiency (results not shown). Moreover, transfection in the presence of serum was more effective than in the absence of serum. This is most probably due to the effect of serum on the endocytotic index. Transfection in the presence of 20μM chloroquine or 2mM ammonium chloride reduced AP expression 7 fold showing the primary route of entry to be

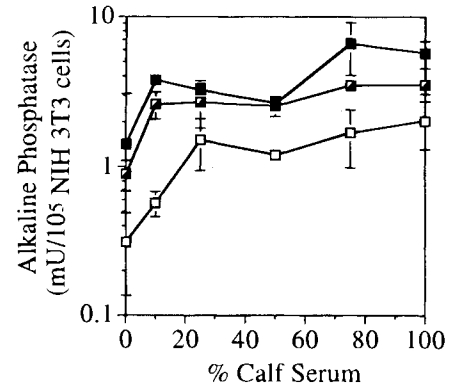

Fig. 3. Serum stability of the stable lipid/DNA complex at a 1/1 DOSPA/DNA-nucleotide molar ratio. The complex was incubated in DMEM containing various amounts of calf serum at 37°C for one hour prior to transfection. The cells were transfected with 3 different doses of DNA in a final volume of 0.5ml DMEM containing 10% calf serum, i.e. 0.5μg (—□—), 1.0μg (—◪—), and 1.5μg (—■—). The negative controls (0% calf serum) that pre-incubated and transfected in DMEM without serum. The data points indicate average ± standard deviation (n=3).

endocytosis (results not shown). Increasing the transfection time from 5 hours to an overnight incubation did not lead to an increase in transfection efficiency, suggesting that the uptake process was saturated after 5 hours. The fact that these complexes can be incubated in 100% serum at 37°C, overnight, indicated that serum components should not inactivate the complexes upon intravenous administration.

The DOSPA to DNA-nucleotide ratio was titrated from 0.5/1 to 1.5/1 (mol/mol) at a dose of 80 μg DNA per tail vein injection volume of 0.3 ml. Tissue was harvested 24 hours after administration and assayed for AP activity. The results are shown in Figure 4. The 1.0 and 1.5 DOSPA/DNA nucleotide ratios yielded similar results whereas the AP activity from the 0.5 was approximately 10 fold lower. Toxicity was observed with the 1.5 ratio with only 1 mouse out of 5 surviving after 24 hours. All 5 mice survived the 1.0 ratio. The 1/1 molar ratio was selected for further optimization.

The effect of escalating the DNA dose on AP activity per tissue was tested. Doses of 30μg, 45μg, 60μg, 80μg and 100μg DNA were administered in 0.3ml saline intravenously. Figure 5 shows the biodistribution of AP activity as a function of DNA dose. There was a distinct correlation with respect to gene transfer vs. dose offered. The 80 μg dose yielded the highest AP activity in all tissues. This was the maximimal tolerated dose, since a further increase to 100μg DNA per injection was lethal. The lung and spleen had the highest level of AP activity followed by the heart, muscle and liver.

The possibility to use other lipids than DOSPA/DOPE to form stable lipid/DNA complexes was investigated. The optimal cationic lipid/DNA molar ratio was determined by studies similar to those presented in Figure 4. The expression levels obtained by these optimized lipid/DNA complexes is summarized in Table 1. The *in vivo* biodistribution of all lipid/DNA complexes tested was similar, however, levels of transgene expression varied significantly. DOSPA/DOPE mediated gene transfer resulted in at least 10 fold higher transgene expression than could be obtained by any of the other lipids. A small change in the chemical structure of the lipid could have a strong impact on the transfection efficiency of the complex. For example,

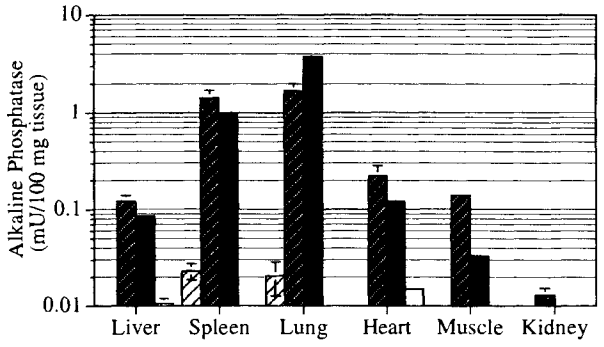

Fig. 4. Optimization of DOSPA/DNA nucleotide ratio for *in vivo* gene transfer. The DOSPA to DNA-nucleotide ratio was titrated from 0.5 (▨), 1.0 (▩), to 1.5 (■) at a dose of 80 μg DNA per tailvein injection volume of 0.3 ml. Five mice were injected for each ratio. The negative control group (□) consists of 5 mice injected with stable lipid/DNA complex of irrelevant (non-AP) plasmid DNA. Tissues were harvested 24 hours after injection. The amount of transgene (alkaline phosphatase) expression was determined by immunocapture assay. The error bars indicate the standard deviation.

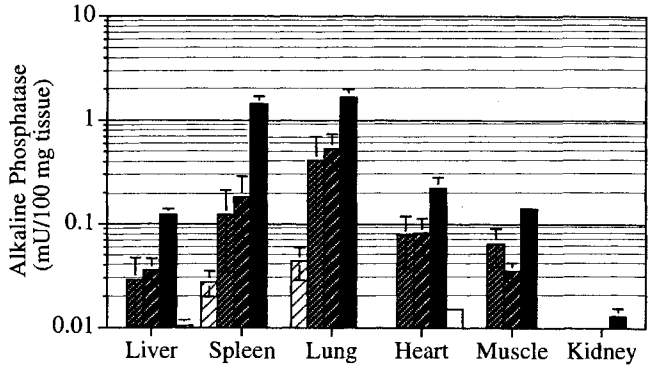

Fig. 5. Effect of DNA dose on *in vivo* gene transfer. Stable lipid/DNA complex suspensions containing 30 μg (▨), 45 μg (▩), 60μg (▩), and 80 μg (■) DNA at a 1/1 molar ratio of DOSPA/DNA nucleotide, were administered intravenously in 0.3ml saline (n=5). The negative control group (□) consists of 5 mice injected with stable lipid/DNA complex of irrelevant (non-AP) plasmid DNA. Tissues were harvested 24 hours after injection. The amount of transgene (alkaline phosphatase) expression was determined by immunocapture assay. The error bars indicate the standard deviation.

changing the link by which the acyl chain is attached to the headgroup from an ether (DOSPA) to an ester (DOSPER) bond resulted in a 25 fold decrease in transfection efficiency. This may be due to the instability of the ester bond. Comparing the transfection efficiency of these formulations *in vitro* vs. *in vivo* showed similar relative transfection efficiencies. *In vitro* the (DOSPA/DOPE)/DNA complex gave a 10 fold higher transgene expression than the DOTAP/DNA complex, a 100 higher expression as compared to Ethylated-DOPC/DNA complex, and a 1000 fold higher expression than could be achieved by (DOTMA/DOPE)/DNA complexes.

The final characterization of *in vivo* gene transfer involved the duration of AP expression. The maximal tolerated dose of approximately 80μg DNA at a 1/1 (mol/mol) DOSPA to DNA-nucleotide ratio was used. The results are shown in Figure 6. The highest level of activity was in the lung and spleen followed by the heart, muscle and liver. The AP activity in lung was almost maximal as early as 6 hours after injection. For all tissues maximal AP activity was observed 24 hours after injection. By day 5 the activity drops approximately 10 fold. By day 7, residual activity can only be detected in the lung, heart and muscle. The time course experiments also revealed that the symptoms of toxicity, i.e. scruffy appearance of the mice, were reversible and dissappeared by the third day after injection.

To determine the transfected cell types in each tissue, 80μg of DNA at a DNA nucleotide/DOSPA ratio of 1/1 was administered. Tissue was harvested 24 hours later, fixed and sectioned. The sections were stained for AP activity. High and low magnification micrographs of stained lung sections are shown in Figure 7. Panel B shows many positively stained foci at low magnification whereas lungs from control mice, Panel A show no staining. Higher magnification of the section, Panel C , reveals flattened endothelial cells with attenuated cytoplasm located along the lining of alveolar capillaries and small vessels that were positive for AP. In addition, rounded and spindleloid

Table 1. *In Vivo* Expression of Alkaline Phosphatase Gene by Cationic Lipid Mediated Gene Transfer Via i.v. Administration at a Dose of 80μg Per Injection

Cationic lipid	Cationic lipid/ DNAnucleotide[a]	Lung	Spleen	Liver
DOSPA/DOPE	1/1	1.68 (±0.32)	1.43 (±0.27)	0.12 (±0.02)
DOSPER	1/1	<	<	<
DOSPER/DOPE	1/1	0.065 (±0.06)	0.016 (±0.002)	<
DOTAP[b]	9/1	0.179 (±0.001)	0.071 (±0.005)	0.025 (±0.0004)
DOTMA/DOPE[c]	3/1	<	<	<
Ethyl-DOPC*[4]	3/1	<	<	<
Ethyl-DOPC/DOPE[d]	3/1	<	<	<

Note: AP expression is givem in mU enzyme per 100mg tissue weight. < indicates that the AP levels were below the detection limit of 0.01mU/100mg tissue.

[a] The cationic lipid to DNA nucleotide molar ratios were varied from 0.5/1 through 12/1. The ratios given in this table were optimal for the respective formulations.

[b] DOTAP formulated with equal mol% of helper lipid DOPE resulted in an unstable formulation.

[c] DOTMA/DOPE did not give detectable levels of AP expression *in vivo. In vitro* the levels of DOTMA/DOPE expression were 500 fold lower than those obtained with DOSPA/DOPE.

[d] Ethylated DOPC either with or without DOPE did not give detectable levels of AP expression *in vivo. In vitro* the levels of Ethylated-DOPC expression were 100 fold lower than those obtained with DOSPA/DOPE.

cells representing phagocytic cells and/or fibroblastic elements were also AP positive. High magnification micrographs of stained sections from heart, muscle, liver and spleen are shown in Figure 8. No muscle fibers were stained positive for AP in the skeletal muscle, nor in the heart muscle. All positive cells that were stained in these tissues were clearly endothelial cells lining the capillaries. In the liver, cells along the sinusoidal lining and small capillaries were AP positive. In spleen, the AP positive cells were also endothelial in nature (Figure 8). No positive staining was found in the kidney.

The predominant transfected cell type in all tissues was endothelial, although other transfected cell types were observed in the lung. Two possibilities for the limitiation in transfected cells types are restricted access of transfection complexes to other cell types or resistance of other cell types to transfection. The lung, however, was the exception. Endothelial cells and interstitial cells (primary and secondary pneumocytes, and alveolar macrophage) were stained positive (Figure 7). Further characterization of these transfected cell types is in progress.

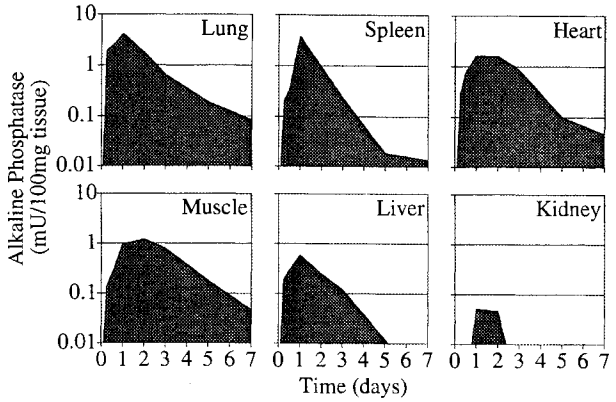

Fig. 6. Duration of AP activity per tissue upon single administration of stable lipid/DNA complex. The complex was prepared at 0.5mg DNA/ml and at a mol ratio DOSPA/DNA-nucleotide of 1/1.80μg DNA was injected in a volume of 300μl per mouse (n=5). Tissues were harvested at various times after injection. The amount of transgene (alkaline phosphatase) expression was determined by immunocapture assay.

DISCUSSION

This report describes a method to complex high concentrations of DNA to cationic lipids for *in vivo* use. This complexation method is very reproducible and leads to the formation of highly stable lipid/DNA complexes. The key to the formation of stable lipid/DNA complexes appears to be the maintenance of the distinguishable intermediate mixtures during complexation in a thermodynamically stable state. Unstable intermediates will result in irreversible precipitation, i.e. formation of large, inactive aggregates. Three different stages can be recognized during the complex formation. The first stage is the lipid/DNA mixture in the presence of detergent. At DNA concentrations exceeding 50μg/ml large aggregates will form resulting in turbid suspensions (cf. Figure 2) with a dramatic decrease in transfection efficiency. Salts at high enough concentrations may serve as chaotropes, keeping the lipid/DNA/detergent mixture stable in solution without precipitation. When precipitation occurred an increase in either the detergent and/or the salt concentration cleared the suspension. The second stage is the lipid/DNA complex in high salt after detergent removal. Higher salt concentrations may be required to keep the lipid/DNA complexes in solution once the detergent is removed. For example, when 1 or 2 M NaCl was used (Figure 2), a slightly hazy suspension was obtained after detergent removal. However, after dialysis against 3M NaCl the lipid/DNA mixture remained clear and was 3–5 times more active than that obtained after 1M NaCl dialysis. The third stage is the final product where the high salt is reduced to physiological concentrations by dialysis against e.g. saline or 5% dextrose containing 10mM TRIS (pH 7.4). The final product is a slightly hazy (milky) suspension, without any visible aggregates. The particle size of the stable (DOSPA/DOPE)/DNA complex, estimated from electron micrographs, is approximately 250 nm (results not shown).

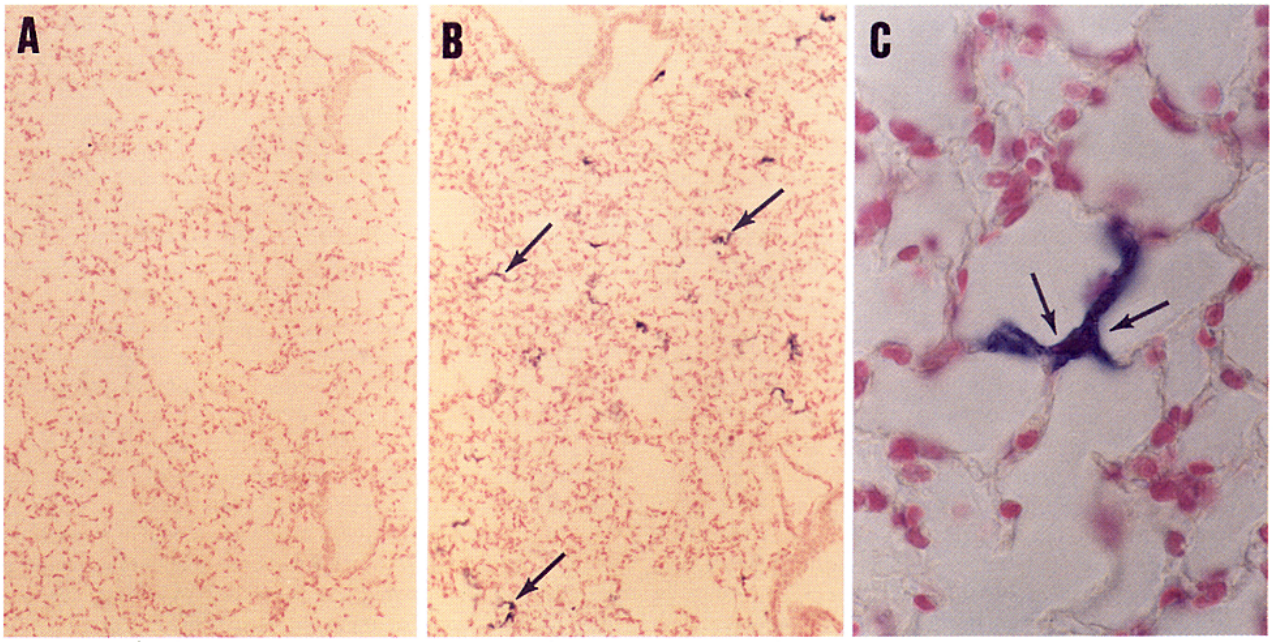

Fig. 7. Histochemical analysis of transfected cell types in the lung after i.v. administration of stable lipid/DNA complex. Panel A is a 100× magnification of control lung tissue. Panel B is the same magnification of the transfected lung. Panel C is a 630× magnification of transfected tissue. Tissues were processed 24 hours after administration. The arrows indicate positive cells.

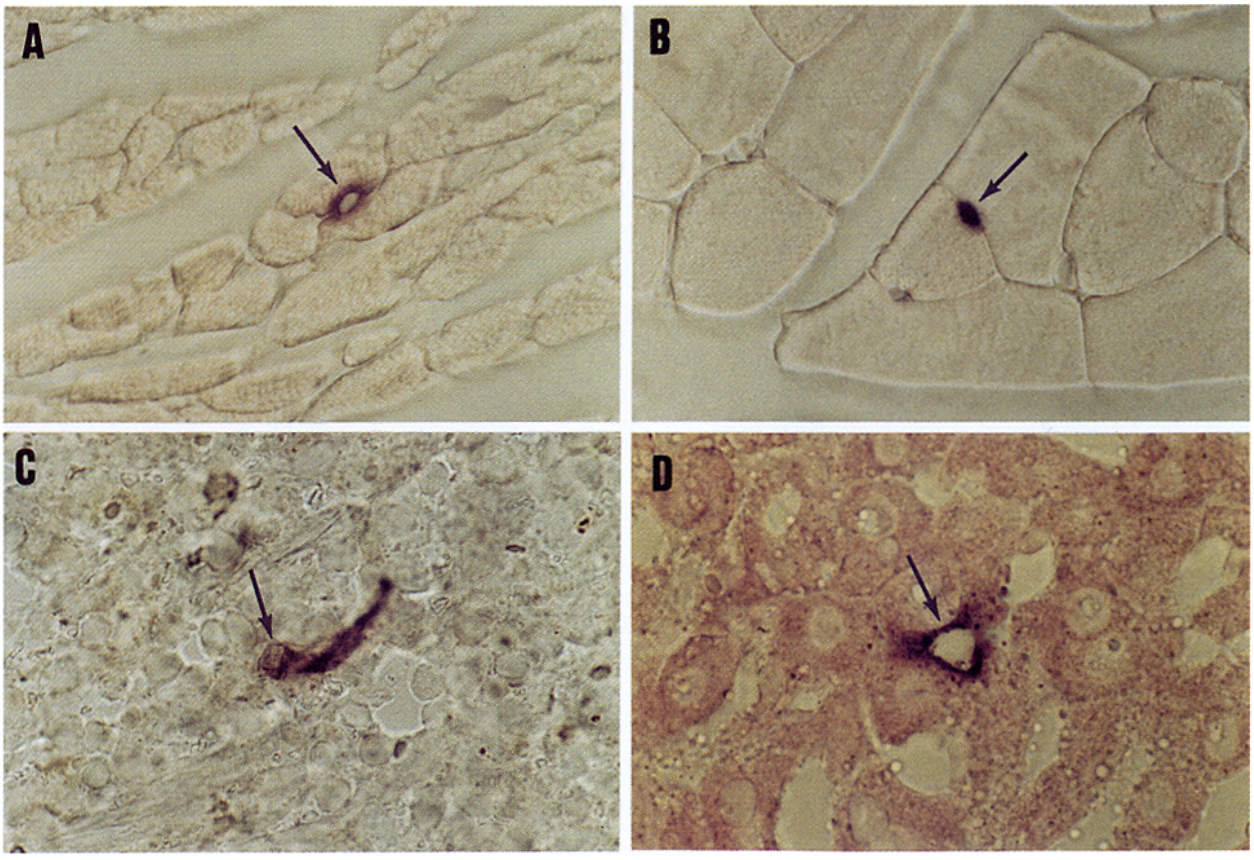

Fig. 8. Histochemical analysis of transfected cell types in the heart (Panel A), muscle (Panel B), spleen (Panel C), and liver (Panel D). Tissue sections were processed 24 hours after administration. Arrows indicate transfected cells.

The transfection activity of DNA/lipid complexes prepared by the modified protocol was identical to activity obtained from complexes formed using the original protocol(1), even though the concentration had been increased 50–100 fold (results not shown). This concentration of DNA was sufficient to allow intravenous administration such that the administered dose was not restricted by the injected volume. Furthermore, the modified protocol yielded complexes that were insensitive to serum inactivation of *in vitro* transfection activity. Hence, the modified protocol yielded a product with attributes highly suited for *in vivo* gene transfer.

Intravenous administration of DNA/cationic liposome complexes has been previously reported using both monocationic and polycationic lipids (18,19). The expressed reporter genes were luciferase and chloramphenicol acetyltransferase (CAT), and enzyme activity was observed in the lung, heart, spleen and liver. The transfection complexes for each study were formed by either hydrating a lipid film with the DNA solution or complexing the DNA with preformed liposomes. In both cases, however, the suspensions were immediately administered suggesting that the transfection complexes had short storage half lives. Both CAT and luciferase enzymatic activity assays are very sensitive for quantitating expression but require indirect immunohistochemistry to determine the transfected cell types. The ability to use the same reporter gene for quantitation of activity and for histochemical determination of transfected cell types removes any ambiguity imposed by indirect detection methods. Hence, this is the rationale for the selection of placental alkaline phosphatase to characterize the *in vivo* gene transfer activity of the stable lipid/DNA complex. Unlike β-galactosidase, endogenous AP activity can be eliminated by heating at 65°C for 0.5 hr. The immunocapture assay was developed to expedite tissue processing and eliminate components from the tissue homogenate that could either interfere with the enzymatic assay or inhibit enzyme activity.

In the first set of *in vivo* gene transfer experiments, Figures 5 and 6, conditions were optimized to maximize gene transfer activity. The conditions were the lipid/DNA nucleotide ratio and the injected DNA dose. The optimized conditions were then tested for duration of expression showing very significant activity in the lung within 6 hours of administration with maximal activity observed 24 hours after administration. The stability of the lipid/DNA complex was exemplified by testing complexes stored for 3 months at 4°C for gene transfer activity yielding the same biodistribution and tissue activity as that observed on day 1 (results not shown).

Gene expression was not restricted to the plasmid construct described in this paper. A commercial available plasmid, pCMVβ, containing the LacZ reporter gene under the CMV promoter was used to prepare stable lipid/DNA complexes at a DOSPA/DNA-nucleotide ratio of 1/1, and injected intravenously at a dose of 80μg DNA. The lung yielded 2.5mU(+/−0.25) β-galactosidase activity per 100mg tissue. The detection limit of the immunocapture assay for β-galactosidase was 0.5mU/100mg tissue. Assuming a similar biodistribution and expression levels as obtained with the AP expression plasmid, the expression of the other tissues were below the detection limits of this assay.

Stable lipid/DNA complexes can be prepared with other polyamine lipids or monovalent cationic lipids (table 1). The rationale behind testing several cationic lipids in this study was to show that the method to prepare stable lipid/DNA complexes is generally applicable, and is not restricted to the use of DOSPA/DOPE alone. Screening of all commercially available lipids was beyond the scope of this paper. The stable lipid/DNA complex prepared by detergent dialysis method was 5 to 10 fold more efficient than the liposome/DNA complex prepared according to the manufacturers directions *in vitro* (1). However, no *in vivo* transgene expression could be detected using these conventional liposome/DNA complexes. Moreover, the stable lipid/DNA complex was at least 8 fold less toxic than the conventional liposome/DNA complexes, which were lethal at a dose of 10μg DNA using a lipid/DNA molar ratio of 1/1. Although the levels of transgene expression varied significantly, the biodistribution of transgene expression was similar for all lipid/DNA complexes tested.

The stable lipid/DNA complex appears to be able to achieve consistent expression levels per tissue from multiple batches and from different DNA preparations. It has a minimal shelf life of approximately 30 days when stored as a suspension at 4°C. The advantage of the prolonged shelf life is realized in the ability to perform multiple experiments with the same batch of material. This also removes variability in doing multiple administrations. Future goals for these complexes are to incorporate components into the formulation that can increase the levels and duration of expression of the transgene product.

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