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Formation of stable cationic lipid/DNA complexes for gene transfer

(transfection/liposome/DOSPA/non-viral/detergent)

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ABSTRACT **Stable cationic lipid/DNA complexes were formed by solubilizing cationic liposomes with 1% octylglucoside and complexing a DNA plasmid with the lipid in the presence of detergent. Removal of the detergent by dialysis yielded a lipid/DNA suspension that was able to transfect tissue culture cells up to 90 days after formation with no loss in activity. Similar levels of gene transfer were obtained by mixing the cationic lipid in a liposome form with DNA just prior to cell addition. However, expression was completely lost 24 hr after mixing. The transfection efficiency of the stable complex in 15% fetal calf serum was 30% of that obtained in the absence of serum, whereas the transient complex was completely inactivated with 2% fetal calf serum. A 90-day stability study comparing various storage conditions showed that the stable complex could be stored frozen or as a suspension at 4°C with no loss in transfection efficiency. Centrifugation of the stable complex produced a pellet that contained approximately 90% of the DNA and 10% of the lipid. Transfection of cells with the resuspended pellet and the supernatant showed that the majority of the transfection activity was in the pellet and all the toxicity was in the supernatant. Formation of a stable cationic lipid/DNA complex has produced a transfection vehicle that can be stored indefinitely, can be concentrated with no loss in transfection efficiency, and the toxicity levels can be greatly reduced when the active complex is isolated from the uncomplexed lipid.**

The field of gene therapy has produced a diverse spectrum of gene delivery vehicles ranging from replication incompetent viruses to DNA formulated with various delivery vehicles. Administration of DNA alone has yielded successful gene transfer for isolated applications (1). However, the majority of applications requires the assistance of a delivery vehicle to facilitate gene transfer. Gene delivery vehicles have included: cationic lipids (2–6), poly-L-lysine conjugates (7–11), liposomes (12), and polymers (13, 14). Each system has advantages and disadvantages and all vehicles have achieved some level of gene transfer. Of these vehicles, cationic lipids are the most widely used. This class of lipids has been under development for the past several years yielding several different lipids capable of achieving gene transfer. The lipids are synthetic in nature and vary from monocation head groups, such as DOTMA (15), DMRIE (2, 5), DOTAP (16), and DC-Chol (17), to polycation head groups, such as DOSPA and DOGS (18). They are usually formulated as an aqueous liposome suspension with a helper lipid, DOPE, except for DOGS, which is active by itself (18). *In vitro*, several parameters are examined to optimize transfection efficiency. These parameters are the ratio of cationic lipid to helper lipid, the molar ratio of cationic lipid to DNA nucleotide, and the amount of DNA offered to the cells.

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Cationic liposome-mediated transfection is very useful as a research tool. However, as a therapeutic gene delivery system, the technology is limiting. The limitations are the finite lifetime of the transfection complex, the inactivation of the complex by serum proteins, and cell toxicity. This later point is particularly problematic in obtaining optimal gene expression because toxicity limits the amount of cationic lipid that can be offered to the cells.

The following report describes a new methodology for the formation of cationic lipid/DNA complexes that maintain the same transfection efficiency for up to 3 months. The level of gene transfer is equivalent or greater than that obtained with the more traditional cationic liposome/DNA complexes. These complexes are able to transfect cells in the presence of serum. The methodology allows the active lipid/DNA complex to be separated from the uncomplexed lipid yielding a dramatic reduction in toxicity.

MATERIALS AND METHODS

Materials. Lipofectamine was purchased from GIBCO/BRL/Life Technologies. Lipofectamine is supplied as a liposome suspension consisting of 2 mg/ml of DOSPA/DOPE (3:1 wt/wt; or 1.53:1 mol/mol). DOTAP liposomes were obtained from Boehringer Mannheim. Plasmid DNA containing the β-galactosidase (β-gal) reporter gene sequence under the CMV promoter (pCMVβ) was purchased from Clontech. β-gal was purchased from Boehringer Mannheim.

DNA Purification. The plasmids were amplified in *E. coli* (DH10β from GIBCO/BRL) and grown in circle grow (Bio 101). Bacteria were lysed and plasmid was purified according to Qiagen (Chatsworth, CA) giga-kit preparation protocol. Care was taken to remove endotoxin (lipopolysaccharide) from the plasmids using polymixin-B columns (Bio-Rad). The DNA was mixed with the polymixin-B resin and agitated at 4°C on a rocker overnight. The DNA was separated from the column support and concentrated by ethanol precipitation with 0.3 M sodium acetate. The DNA was resuspended in water and dialyzed for 24 hr at 4°C against water. This last step was found to remove components that affected gene transfer activity. Each plasmid preparation was assayed for endotoxin activity. All plasmid preparations after polymixin-B adsorption had lipopolysaccharide levels of less than 20 endotoxin units/mg DNA. A BCA protein assay (Pierce) 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 Transient Liposome–DNA Complex. The DNA was complexed with DOSPA/DOPE liposomes according to the protocol of the manufacturer. Briefly, the DNA was diluted to a concentration of 0.4–2 μg per ml of DMEM tissue culture medium. DOSPA/DOPE was added to the DNA in a volume of 0.5 ml.

Abbreviation: β-gal, β-galactosidase.

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Preparation of Stable Lipid/DNA Complex. DOSPA/DOPE was first solubilized in 1% octylglucoside/10 mM Tris (pH 7.4). Five micrograms of DNA (15.15 nmol DNA nucleotide) was added to the detergent solubilized lipid in 0.5 ml of 1% octylglucoside buffer. The mol/mol ratio of DOSPA to DNA nucleotide ranged from 0.33:1 to 16.5:1. Each formulation was dialyzed 3 times against 2000 volumes of 10 mM Tris/5% dextrose (pH 7.4) over a 48-hr period at 4°C. The dialysis tubing used had a molecular weight cut off of 12–14 k (Spectra/Por no. 2).

***In Vitro* Cell Transfection.** Twenty-four well plates were seeded with 6×10^4 NIH 3T3 cells (American Type Culture Collection) and transfected 24 hr later. For transfections, complexes containing 0.2 μg of DNA were diluted into 0.5 ml of DMEM tissue culture medium (GIBCO/BRL) without serum, and added to each well. The cells were incubated at 37°C for 4–5 hr. The complex containing medium was replaced by DMEM containing 10% calf serum (BioWhittaker), and cells were cultured for 24 hr before harvesting the cell lysates.

β-Gal Assay. The cells were washed twice with 1 ml of Dulbecco's PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. Subsequently, the cells were lysed with 200 μl of lysis buffer containing 250 mM Tris (pH 7.4) and 0.1% Triton X-100. The cell lysates were analyzed using the colorimetric *o*-nitrophenyl β-D-galactopyranoside (ONPG) (Sigma) assay (19) in a 96-well plate format. β-gal activity is expressed in total milliunits per well with 10^5 cells per well. One milliunit is defined as the amount of β-gal that hydrolyzes 1 nmol of ONPG per min at 28°C. A standard curve of β-gal from 0.05 milliunit to 100 milliunits was measured with each set of samples. V_{max} was calculated for each sample using a Molecular Devices plate reader.

Shelf Life. The effect of storage conditions on the transfection efficiency of stable lipid/DNA complexes was studied by preparing a 6-ml batch of stable complex, according to the above protocol, at a DOSPA/DNA nucleotide ratio of 6.6:1. The 1.2-ml aliquots of the complex were stored at −20°C (with and without 5% dextrose), 4°C, ambient temperature, and 37°C. The stable complex stored at −20°C was thawed slowly at 4°C prior to testing. DNA (0.2 μg) was used to transfect NIH 3T3 cells.

RESULTS

Characterization of *in Vitro* Gene Transfer. Lipid/DNA complexes were prepared by detergent dialysis according to conditions described in *Materials and Methods*. The following lipid compositions: DOSPA/DOPE, DOTAP, and DC-Chol/DOPE were tested for transfection of NIH 3T3 cells. All three lipid compositions formed lipid/DNA suspensions that were able to transfect cells 48 hr after formation. Of the three lipid formulations, DOSPA/DOPE gave the highest level of β-gal expression (results not shown). Further characterization of the stable DNA/lipid complexes was performed with DOSPA/DOPE. The detergent dialyzed complex will be referred to as the stable complex and addition of cationic lipid containing liposomes to DNA will be referred to as the transient complex. Transfection efficiency of the stable complex versus a transient DNA complex was compared by titrating the DOSPA to DNA nucleotide ratio over a DNA concentration range of 0.1–0.8 μg per well. The transfection efficiencies were determined by quantitating β-gal expression and measuring cell viability as a function of total protein in the cell lysates. The results are shown in Fig. 1. Transfections with the transient complex are shown in *A–D* and transfections with the stable complex are shown in *E–H*. The transient complex showed that β-gal expression at the optimal DOSPA/DNA nucleotide ratio increased from 10 milliunits to 120 milliunits as the DNA concentration increased. The stable complex yielded β-gal levels ranging from 70 milliunits to 130 milliunits with increasing DNA concentrations. The optimal DOSPA/DNA nucleotide ratio decreased as the DNA dose was increased for both the transient and stable complexes. At the lower DNA doses, 0.1–0.4 μg, the stable complex gave higher levels of β-gal. Comparison of cell viability, based on total cellular protein, showed that the stable complex also yielded higher levels of viable cells compared with the transient complex at all DNA doses.

Shelf Life. The stability of the stable DNA/lipid complex was tested at a DOSPA/DNA nucleotide ratio of 6.6:1 and aliquots were stored under various conditions over a 90-day period. The storage conditions were −20°C, −20°C with 5% dextrose, 4°C, ambient temperature, and 37°C. Stability was evaluated by assaying the transfection efficiency of the com-

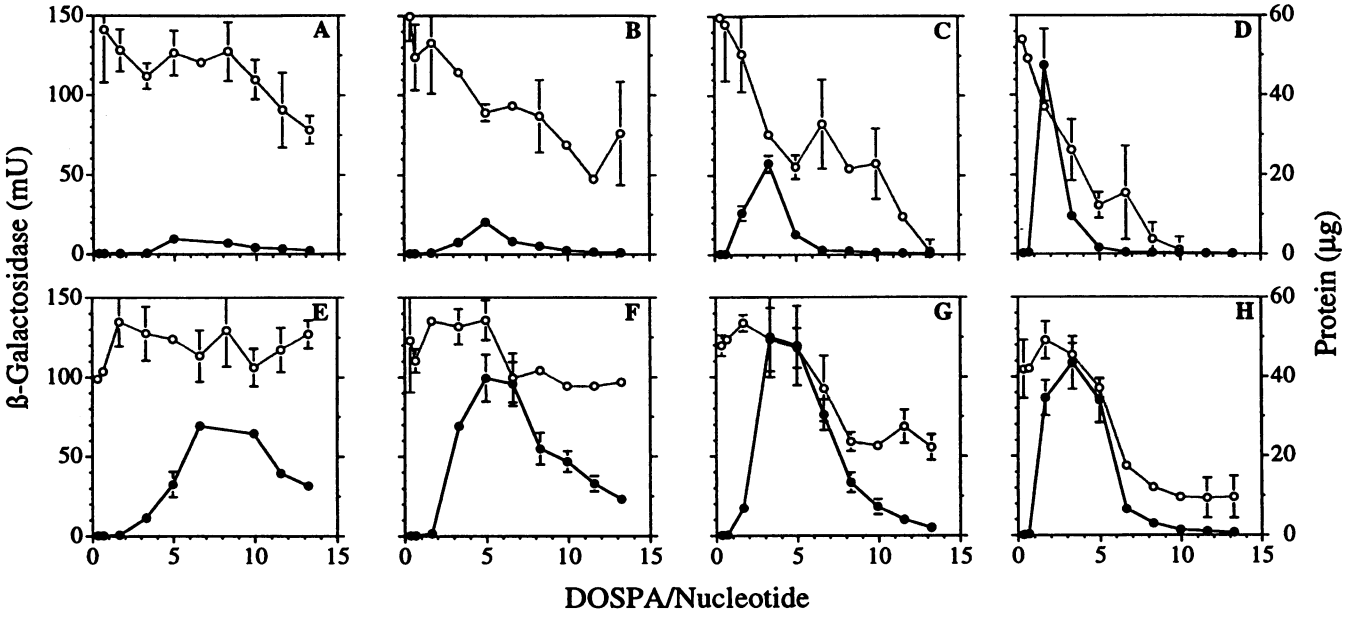

FIG. 1. β-gal reporter gene transfer by transient (*A–D*) versus stable (*E–H*) cationic lipid/DNA complex. Murine 3T3 fibroblasts were transfected with pCMVβ. The DOSPA to DNA nucleotide (mol/mol) ratio was varied over a range of 0.3:1–15:1, and the DNA concentration was varied at 0.1 μg per well (*A* and *E*), 0.2 μg per well (*B* and *F*), 0.4 μg per well (*C* and *G*), and 0.8 μg per well (*D* and *H*) of a 24-well plate. The β-gal activity (●) was plotted on the left *y*-axis, and compared with the protein recovery (○), plotted on the right *y*-axis.

plexes. The results are shown in Fig. 2. The −20°C with dextrose and the 4°C storage conditions retained 100% of transfection activity up to 90 days. Storage at −20°C, without dextrose, resulted in an initial 5-fold decrease in β-gal activity with a continued decline over time. Storage at ambient temperature and 37°C resulted in complete loss of transfection activity after 14 days.

Serum Stability. Another concern was the ability to transfect cells in the presence of serum proteins. The transfection efficiency of the stable complex was compared with the transient complex in the presence of serum. The DOSPA/DNA nucleotide was 6.6 for both stable and transient complexes, and the DNA transfection concentration was 0.4 μg/ml. β-gal activity was measured as a function of the percentage of fetal calf serum in the cell media. The results are shown in Fig. 3. The transfection efficiency of the transient complex was completely inhibited with 2% serum. The same percentage of serum decreased the transfection efficiency of the stable complex to 30% of that observed in the absence of serum. This level of β-gal expression was maintained for up to 15% serum. The 30% expression level is equivalent to 10% of the cells expressing β-gal as determined by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining (results not shown).

Separation of Toxic Component from Active Complex. During the course of the stability study of the stable complex, a precipitate was observed upon storage at 4°C after 14 days. The precipitate was pelleted by centrifugation. Transfection with the resuspended pellet versus the supernatant showed that most of the transfection activity was in the pellet. This phenomenon was investigated in more detail by preparing stable complexes at DOSPA/DNA nucleotide ratios of 3.3:1, 6.6:1, and 16.5:1. These complexes were centrifuged at 3000 × *g*, at 4°C for 15 min, and the supernatants were separated from the pellets. The pellets were resuspended in 5% dextrose/10 mM Tris. The original mixtures, the resuspended pellets, and the supernatants were tested for transfection efficiency. The amounts of pellet and supernatant used were based on equivalent volumes of the starting mixture. The results are shown in Fig. 4. The 3.3:1 DOSPA/DNA nucleotide ratio showed equivalent β-gal activity in the supernatant and the mixture with no activity in the pellet. The 6.6:1 DOSPA/DNA nucleotide ratio showed equivalent β-gal activity in the pellet and

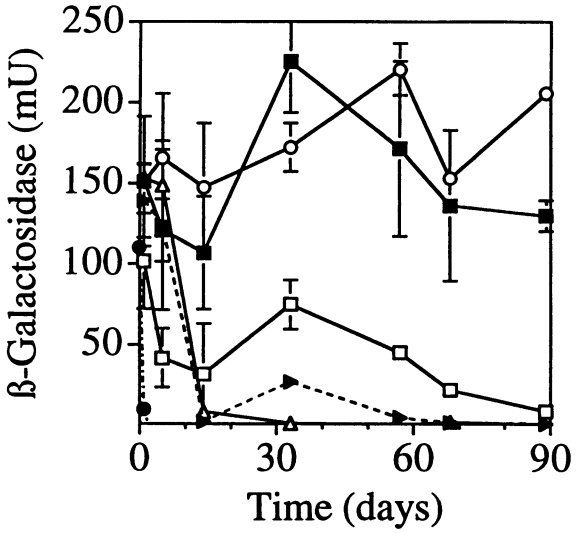

FIG. 2. Stability of stable versus transient complex under different storage conditions. The stable cationic lipid/DNA complexes were stored at −20°C + 5% dextrose (■), −20°C (□), 4°C (○), room temperature (▲), and 37°C (Δ). The transient complex was stored at 4°C (●). The storage time begins at the time of harvest, i.e., after 48 hr of dialysis for the stable complex, and directly upon mixing of the liposomes and the DNA for the transient complex.

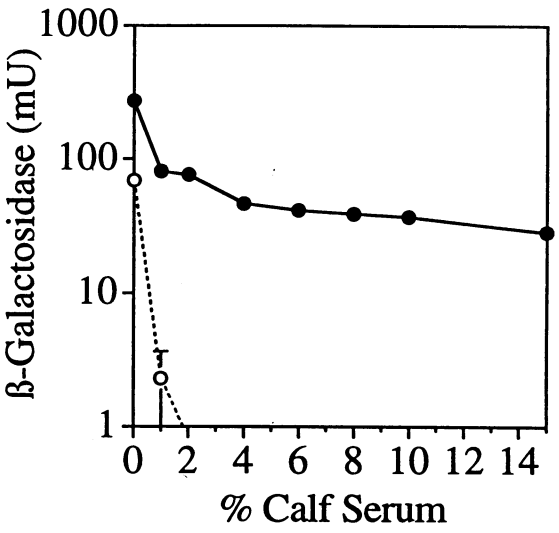

FIG. 3. Effect of calf serum on gene transfer by stable (●) versus transient (○) cationic lipid complex. Each complex was prepared at a DOSPA/DNA nucleotide ratio of 10:1. The complexes were added to 0.5 ml of media containing increasing amounts of fetal calf serum. DNA (0.2 μg) was added to 10^5 NIH 3T3 cells per well.

mixture with marginal activity in the supernatant. The 16.5:1 DOSPA/DNA nucleotide ratio showed greater β-gal activity to be in the pellet compared with the mixture with marginal activity in the supernatant. DNA and lipid analysis of the pellet and supernatant showed that all the DNA was in the supernatant for the 3.3:1 ratio, whereas 90% of the DNA was in the pellet for the 6.6:1 and 16.5:1 ratios. Lipid analysis of the 6.6:1 and 16.5:1 ratios showed no marked difference in the DOSPA/DOPE ratio and approximately 10% of the total lipid was in the pellet, whereas 90% of the lipid was in the supernatant (results not shown). The implication is that for higher DOSPA/DNA nucleotide ratios low speed centrifugation is sufficient to separate the lipid/DNA complex from uncomplexed lipid. The other compelling observation was that the

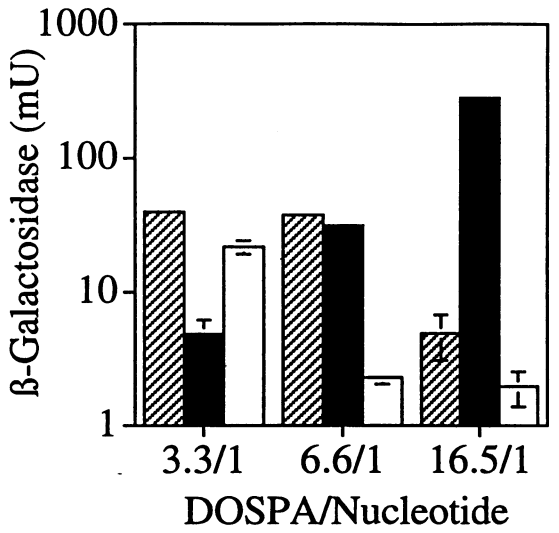

FIG. 4. Gene transfer of stable cationic lipid/DNA complex separated from uncomplexed lipid. Stable cationic lipid/DNA complexes were prepared at 3.3:1, 6.6:1, and 16.5:1 DOSPA to DNA nucleotide. The suspensions were centrifuged and the pellet (■), the supernatant (□), and the uncentrifuged suspension (▨) were assayed for gene transfer. DNA (0.2 μg) of the uncentrifuged suspension was added to 10^5 NIH 3T3 cells. The amount of pellet and supernatant added to the cells was based on an equivalent volume of the original suspension prior to centrifugation.

pellet for the 16.5:1 ratio gave higher levels of β-gal expression compared with the mixture, and the level was also greater than either the mixture or the pellet for the 6.6:1 ratio. Fig. 1 showed that a ratio greater than 10 resulted in cell death. This toxicity would explain the low level of expression of this mixture at this ratio.

To study this phenomenon further, β-gal expression was measured as a function of DNA concentration for the transient complex, the stable complex, the supernatant of the stable complex, and the pellet of the stable complex. The cell viability for each condition was also measured as a function of DNA concentration. The results are shown in Fig. 5 *A* and *B*. In Fig. 5*A*, the transient complex and the supernatant from the stable complex showed no β-gal expression. A low level of β-gal expression was observed for the stable complex; however, the expression level decreased as the DNA concentration increased. The resuspended pellet showed an increase in expression as the DNA concentration increased with the maximum level being 4-fold greater than the mixture. The cell viability of the transient complex, the stable complex, and the stable complex supernatant decreased with increasing DNA concentration with complete loss of cells at 1 μg/ml. The resuspended pellet showed no decrease at 1 μg/ml and only a marginal decrease at 10 μg/ml.

DISCUSSION

We initially used conventional liposome encapsulation procedures, such as reverse evaporation phase, extrusion, or dehydration–rehydration, to trap DNA into liposomes and obtained 30–40% trapping efficiencies. However, decreasing the diameter of the liposomes to 100 nm resulted in trapping efficiencies of less than 1%. To increase the trapping efficiency and maintain the liposome diameter below 100 nm, a method of preparation was developed that built the liposomes around the DNA rather than trying to force it inside the liposomes as they were being formed. The strategy was to bind lipid to the DNA in the absence of any liposome structure and coat the DNA/lipid complex with additional lipid. The challenge was to select a medium in which the lipid best existed as a monomer in solution and also maintained the DNA in solution. Detergent micelles were selected as the presentation medium because both lipid and DNA were soluble in this medium. Octylglucoside was selected because it is non-ionic and has a high critical micelle concentration.

The constraints on the molecular motion of the cationic lipid should be reduced in a mixed micelle compared with the packing constraints of a liposome bilayer, thus allowing the cationic lipid to interact with the DNA more freely. Secondly, the micelle is not a static structure with lipid being able to freely exchange between micelles (20). Thirdly, the size of the micelles is far less (6 nm diameter) than that of a liposome (100 nm diameter), thus increasing the accessibility of the cationic lipid to all binding sites on the DNA.

DOSPA was selected as the first lipid to be tested because of the spermine head group. Spermine has been shown to condense DNA into small compact structures (21, 22). Hence, binding of the DOSPA to the DNA in detergent should serve to condense the DNA and deposit a hydrophobic layer on the surface of the DNA on which additional lipid could be deposited. Dialysis of the detergent/lipid/DNA mixture produced a suspension that was able to transfect cells. This complex was shown to transfect several different cell types including HT1080 cells, HepG2 cells, mouse B16 melanoma cells, mouse renal cell carcinoma cells, mouse colorectal cancer cells, primary human fibroblasts, and primary human melanoma cells (results not shown).

Characterization of the transfection activity is shown in Fig. 1, where at low doses of DNA the stabilized complex yielded much higher levels of β-gal expression compared with the traditional transfection protocol with the same lipid. It also showed that as the DNA dose increased the optimal DOSPA/DNA nucleotide ratio decreased. A correlation was also observed between the level of β-gal expression and cell viability. Comparison of the stable and transient complexes showed that the cell viability did not decrease to the same extent as the transient complex as both the DNA dose and the DOSPA/DNA nucleotide ratio were increased. Hence, the reduction in β-gal expression with increasing DOSPA/DNA nucleotide is primarily due to toxicity.

This point is more clearly illustrated in Figs. 4 and 5. Fig. 4 showed that the lipid/DNA complex could be separated from the uncomplexed lipid by centrifugation. The figure also showed that the resuspended pellet from the 16.5 DOSPA/DNA nucleotide ratio gave a 20- fold higher level of β-gal expression compared with the 16.5 DOSPA/DNA nucleotide mixture. This level of expression by the resuspended pellet was also greater that that obtained by the 6.6:1 ratio. The low level of expression by the 16.5:1 mixture can be explained by the results from Fig. 5. Fig. 5*B* showed that the amount of mixture

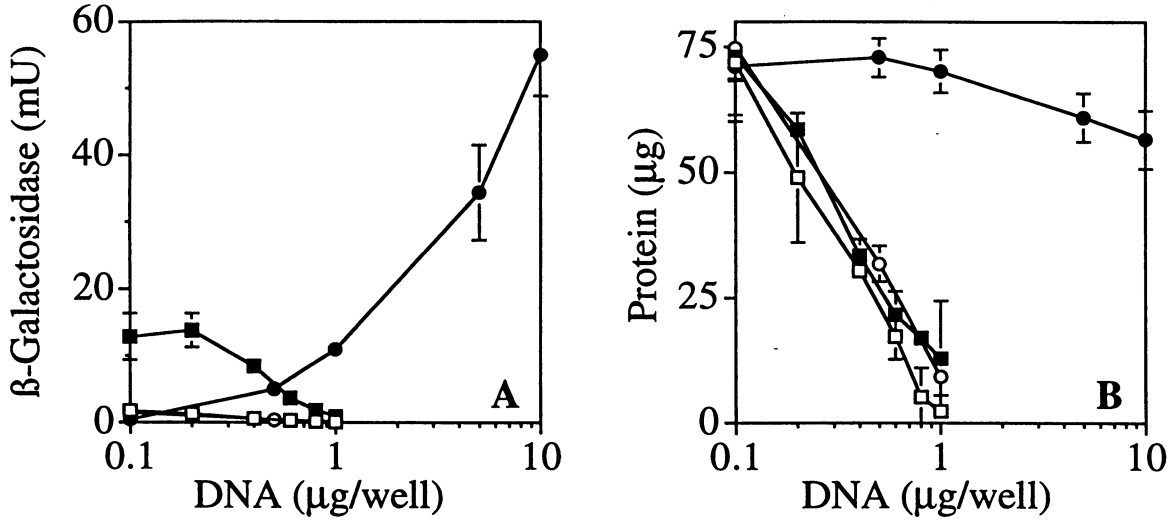

FIG. 5. Comparison of gene transfer and toxicity for the stable and transient cationic lipid complex and the pellet and supernatant of the stable complex. The stable (■) and transient (□) complexes were prepared at a DOSPA to DNA nucleotide ratio of 6.6:1. β-gal activity and cell protein were assayed as a function of DNA concentration. The pellet (●) and supernatant (○) were derived from centrifugation of the stable complex. The amount of pellet and supernatant added to the cells was based on an equivalent volume of the original suspension prior to centrifugation.

and supernatant at which 50% of the protein was recovered was between 0.3 and 0.4 μg of DNA, whereas the projected amount of the resuspended pellet with the same toxicity was at least two orders of magnitude greater. The detoxification of the complex by centrifugation has been repeated several times yielding the same result. However, recovery of the transfection activity in the pellet has varied from 30 to 90% of the original activity prior to centrifugation. The variable was the quality of the DNA. The precipitation step after polymixin B followed by dialysis increased transfection activity 4-fold compared with undialyzed DNA. Reproducibility was further improved by double banding the DNA with CsCl centrifugation.

The DNA concentration was found to be critical in forming the stabilized complex. Exceeding a 50 μg/ml DNA concentration when complexing the micellized lipid with the DNA resulted in a dramatic decrease in *in vitro* transfection efficiency. This is an important consideration for *in vivo* gene transfer. The pellet has been resuspended at concentrations of 1 mg/ml of DNA with no loss in transfection efficiency (results not shown). Hence, separation of the lipid/DNA complex from uncomplexed lipid dramatically decreased the toxicity and also allowed the DNA to be concentrated. Another important feature of the stable complex is the ability to transfect cells in the presence of serum. The transient complex clearly showed that 3% fetal calf serum completely inhibited gene expression, whereas the stable complex maintained 30% of the transfection efficiency up to 15% serum. There is no explanation for the initial drop in gene transfer from 0% to 2% serum other than the suspension may be composed of heterogeneous complexes, one of which was sensitive to inactivation by serum. This level of β-gal activity was significant because 10% of the cells were positive for 5-bromo-4-chloro-3-indoyly β-D-galactoside staining. The final redeeming characteristic of this formulation is the stability. It is not surprising that conditions can be identified for freezing or lyophilizing the lipid/DNA complex. However, storage of the complex as a suspension at 4°C for 90 days with no loss in gene transfer was a true demonstration of complex stability. The advantage of the shelf stability is realized in performing *in vivo* gene transfer. The experiments require a few weeks to process the results. This stability allows the same batch of lipid/DNA complex to be retested and used in subsequent experiments for comparison to other formulations.

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