Characterization and *In Vivo* Testing of a Heterogeneous Cationic Lipid-DNA Formulation

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Purpose. To identify characteristics of lipid-DNA complexes that correlate with *in vivo* expression data.

Methods. DOTIM:cholesterol liposomes (1:1 mole ratio) and DNA expressing chloramphenicol acetyl transferase (CAT) were complexed at a 4.2:1 mass ratio (cationic lipid:DNA). Complexes were fractionated by density gradient centrifugation, analyzed for particle size and zeta potential and quantitated using HPLC methods. The unfractionated complexes, "purified" fractions of the complexes, and purified complexes supplemented with liposomes were administered to mice by intravenous injection (IV) and intratracheal instillation (IT) and their ability to express gene product was assessed.

Results. Centrifugation separated two distinct populations within complexes one consisting of free liposomes and the other of lipid complexed with DNA. The vesicle diameter and zeta potential among separated fractions varied from 113 to 354 nm, and +24 to +34 mV respectively. Re-centrifugation of the 'purified' fractions containing the lipid-DNA population produced a single band. CAT expression in lung tissue 24 hr post-IV was observed with the unfractionated complex, but not the purified form. Some activity was 'restored' with the liposome-supplemented complexes. In contrast, the same series of complexes administered by IT resulted in no significant difference in lung expression ($p = 0.16$ ANOVA).

Conclusions. An uncomplexed liposome population exists within DOTIM:cholesterol-DNA complexes that influences the expression of complexes administered IV but not IT.

KEY WORDS: liposomes; gene therapy; DNA; centrifugation.

INTRODUCTION

The structure and mechanisms of action of lipid-DNA complexes are poorly understood despite their success in transfecting DNA *in vitro* and *in vivo* (1–4). Formulations of cationic lipid-DNA complexes have been shown to contain particles that differ with respect to size, shape and density (5–7). Heterogeneity has been described in electron micrographs of "spaghetti and meatball-like" structures (5), and AFM images of irregularly sized and shaped globules (6) or compacted, irregularly shaped branches and rods (6) consisting of 3β-(N-(*N'*,*N'*-dimethylaminoethane)carbamoyl) cholesterol (DC-chol)/DOPE-DNA complexes, N-[1-(2,3-dimyristyloxy) propyl]-N,N-dimethyl-N-(2-hydroxyethyl)ammonium bromide (DMRIE)/DOPE-DNA complexes and βAE/DMRIE-DNA complexes respectively. These heterogeneous formulations may not be optimal for long term stability, and are likely to contain a number of sub-populations that could vary in biological activity. Thus it is appropriate to define and characterize the sub-populations and their ability to transfect DNA. Size, shape and density determine the mobility of a particle within a density gradient during centrifugation. This technique is routinely used to separate liposomes that differ in size and number of lamella (8), and has been used to analyze N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP)-DNA complexes (9) and DC-chol liposomes/poly (L-lysine)/DNA complexes (10). Thus, it is feasible to separate cationic lipid-DNA complexes by this methodology.

Studies were carried out to characterize 1-[2-(9(Z)-Octadecenoyloxy)ethyl]-2-(8(Z)-heptadecenyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM):cholesterol-DNA complex populations isolated by density gradient centrifugation. A nominal mass ratio of 4.2:1 (cationic lipid:DNA) was used throughout, as this ratio has been shown to be optimal for *in vivo* CAT expression from DOTIM:cholesterol formulations delivered IV (11). Two populations were identified and isolated. These populations underwent physico-chemical characterization, and were also tested *in vivo* for marker gene expression in the lung following intravenous or intratracheal administration in mice.

MATERIALS AND METHODS

Preparation of Lipid-DNA Complexes

Plasmid DNA containing the marker gene chloramphenicol acetyltransferase (CAT) was used in all experiments (12). DOTIM:cholesterol liposomes were prepared via rotary evaporation of chloroform to a thin film, which was rehydrated with 5% (w/v) dextrose forming a final concentration of 20 mM of each lipid. Complexes were prepared as described previously (11), producing a final concentration of 1.875 mM cationic lipid, plus 0.3125 mg/ml plasmid DNA. This combination represents a theoretical charge ratio of approximately 2:1, and has been shown to produce high levels of marker gene expression in lung tissue following IV delivery (11). Complexes were used immediately after formation.

Fractionation of Lipid-DNA Complexes by Centrifugation

Linear 5–25% (w/v) dextrose gradients were formed and equilibrated to 5°C prior to use. To calculate the dextrose concentrations throughout the gradient, pre-run gradients loaded with 3 ml 5% (w/v) dextrose were fractionated and the fractions were assayed for osmolarity by vapor pressure osmometry (Wescor 5500, Logan, UT). The resulting values were adjusted

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ABBREVIATIONS: CAT, chloramphenicol acetyltransferase; DC-chol, 3β-(N-(*N'*,*N'*-dimethylaminoethane)carbamoyl)cholesterol; DMRIE, N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl)ammonium bromide; DOPE, phosphatidylethanolamine; DOTAP, N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; DOTIM, 1-[2-(9(Z)-Octadecenoyloxy)ethyl]-2-(8(Z)-heptadecenyl-3-(2-hydroxyethyl)imidazolinium chloride; DOTMA, *N*-[1-[2,3-bis(oleoyloxy)]propyl]-*N*,*N*,*N*-trimethylammonium chloride; HCMV, human cytomegalovirus; PCS, photon correlation spectroscopy; SV40, Simian virus 40.

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to % (w/v) dextrose concentration by comparison to dextrose calibration data. A 3 ml sample of freshly prepared lipid:DNA complex was layered onto each gradient. Gradients were centrifuged at 15,000 RPM (17,600 × g), at 5°C in a JA 20 (Beckman, Palo Alto, CA) rotor for 8 hr. Assays were carried out as described below on 1.5 ml fractions collected by continuous aspiration from the bottom of each tube. DOTIM absorbs at a wavelength of 237 nm, masking the 260 nm value for DNA within lipid-DNA complexes. The contribution of the DNA absorption to this peak is insignificant due to a comparatively low molar extinction coefficient, and therefore by reading the absorbance at 237 nm, the DOTIM distribution within the dextrose gradient was obtained. Dilution of the fractions prior to optical density readings eliminated any background turbidity associated with light scattering from the vesicles. DNA concentration was determined following separation of the lipid and DNA components (see below).

Assay of Fractionated Lipid-DNA Complexes

Fractionated lipid-DNA complexes were assayed at room temperature. The optical densities at 237, 260 and 400 nm were measured using a Beckman DU 640 spectrophotometer. Photon correlation spectroscopy was used to measure the hydrodynamic diameter of particles. Size and ξ measurements (13) were made on samples diluted with 5% w/v dextrose using a Brookhaven (NY) Zeta-sizer with a 50 mW solid state laser and standard Brookhaven software. A minimum of 5 cycles were obtained for each ξ value and subsequently averaged. The ξ was automatically calculated from the electrophoretic mobility based on the Smoluchowski formula.

DNase I Assay and Serum Stability

Samples containing 30 μg of DNA, with or without liposomes, were incubated at 25°C in a buffer containing 2 units of DNase I per microgram of DNA, 0.1 mM $MgCl_2$ and 10 mM Tris-HCl pH 7.4. These conditions were chosen for rapid analysis of DNA degradation. Aliquots of 6 μg of sample were removed at 0, 1, 5 and 15 minutes and the DNA was extracted immediately by ethanol precipitation. One tenth sample volume of 3 M sodium acetate pH 5.2 was added to each sample and vortexed prior to addition of 2.5 volumes of ice cold 100% ethanol and re-vortexing. Samples were stored at −20°C for 1 hr, then centrifuged at high speed for 15 minutes and the supernatant removed. The pellet was washed with ice-cold 70% ethanol and subsequently dried under vacuum. The DNA pellet was resuspended in 10 mM Tris-HCl pH 8.0 and then subjected to electrophoresis on a 0.9% agarose gel in TBE buffer and 0.5 μg/ml ethidium bromide according to standard techniques (14). DNA was visualized by photography with UV transillumination.

Original and purified complexes (described below) were incubated in water, or 5% Fetal Bovine Serum (HyClone Labs. Inc., Logan, UT) at concentrations that produced an intensity reading in the optimal range (200–250 KHZ) for the Submicron Particle Sizer (Nicomp, Santa Barabara, CA).

Separation of Lipid and DNA Components

Separation of the DNA and lipid within each fraction was carried out according to a modified method of Bligh and Dyer (15). The DOTIM/cholesterol-DNA complexes were diluted with 4 times their volume of isotonic saline and solubilized in a Bligh-Dyer monophase consisting of chloroform/methanol/water (1.5/1/1). The samples were mixed vigorously by vortexing and centrifuged at 3,000 RPM for 1 min. at room temperature. The lower organic phase was removed and dried in a Speed Vac Plus (Savant, Holbrook, NY) at room temperature. The dry lipid was rehydrated in methanol. The amounts of DOTIM in methanol, and DNA in the aqueous phase were determined via optical density at wavelengths of 260 (DNA) and 237 nm (DOTIM) respectively. Conversion to mg/ml quantification was calculated for the DNA and DOTIM (DOTIM extinction coefficient in methanol is 7262.5 $M^{-1}cm^{-1}$). In control experiments, the overall ratio of lipid to DNA was found not to influence the yield of either component, therefore the DNA yield was normalized to 100%. Separation of the lipid and DNA components was determined not to induce degradation of either material. To examine lipid integrity and to ensure that the molar ratio of DOTIM to cholesterol was not altered in the fractions obtained after centrifugation, HPLC analysis was performed on the lipids in the organic phase of the extracted samples. The cholesterol and DOTIM were separated and well resolved by injecting 10 μl samples, containing ~0.2 mg/ml lipids onto a C8 reverse phase HPLC column (Alltima C8 5μ, 250 mm × 4.6 mm, Alltech, Deerfield, IL) held at 37°C. A mobile phase of methanol:acetonitrile:water (65:30:5) and 0.1% (v/v) trifluoroacetic acid running isocratically at 1 ml/min was employed. The system was controlled using Shimadzu EZ Chrom software. UV detection was fixed at a wavelength of 215 nm and the ELSD was positioned in-line after the UV-Vis detector.

Isolation of Purified Lipid-DNA Complex and *In Vivo* Studies

Lipid-DNA complexes were centrifuged and fractionated as described and the OD at 237 nm read for each fraction. Fractions falling within the 11.5 to 16% (w/v) dextrose region of the gradient, which had a narrow range of size and lipid:DNA ratio (Fig. 4) were pooled, and designated "purified." For animal studies, the purified material was concentrated by re-centrifuging onto a 20% (w/v) dextrose cushion for several hours at 13,000 RPM (JA-20 rotor) and dialyzing against 5% (w/v) dextrose for 16 hours at 5°C. Two hundred microliters of test sample containing 62 μg of DNA was administered by tail-vein injection to test groups each containing five ICR mice. Samples contained either lipid-DNA complexes, their purified counterparts, or purified material with additional liposomes (to replace the material lost by centrifugation, as determined by extraction and quantitation of the lipid). A control group of animals were administered 200 μl 5% (w/v) dextrose solution. A similar study was also completed by targeting the lungs via intratracheal instillation. Here, Balb/c mice were administered 100 μl of sample by intratracheal injection. In each experiment, lung tissue was removed at 48 hours and assayed for soluble protein and CAT content (11).

CAT ELISA Assay

Lung samples were harvested from treated animals, frozen on dry ice and stored at −70°C until processed. Samples were

homogenized in a Savant FP120 FastPrep bead mill (BIO101, Vista CA) in lysis buffer containing 25mM Tris pH.8.2, 5mM EDTA, 10ug/ml Aprotinin, 0.1mM TPCK and 0.1mM TLCK. After homogenization, the samples were clarified by centrifugation in a microcentrifuge at 10,000 RPM for 10 minutes. The supernatants containing the soluble protein fraction were transferred to storage tubes and stored at −70°C until assayed. The total protein concentration of each sample was determined using a BCA microplate assay and BSA protein concentration standards (BCA Reagent and BSA standard: Pierce, Rockford, IL).

The concentration of CAT protein in each sample was determined by analysis in a CAT specific ELISA with a quantitation range between 250 and 2000 pg/ml. The assay is a sandwich ELISA utilizing a rabbit polyclonal antibody for capture and a sheep polyclonal antibody for detection. Details are as follows: Medium binding Corning EIA/RIA plates (VWR, West Chester, PA) were coated overnight at 2–8°C with 50 μl per well of a polyclonal rabbit anti-CAT antibody (5 Prime-3 Prime, Boulder, CO) diluted to 12 μg/ml in 50 mM NaHCO3, pH 9.0. The plates were blocked for 1 hr at room temperature with 200 μl Blotto (5% w/v Dry milk (PGC Scientific, Gaithersburg MD), 0.02% v/v Tween 20 (Sigma, St. Louis, MO) in 1X PBS) per well. The plates were washed four times with 425μl per well of 0.2% Tween 20, PBS. Purified CAT enzyme (Boehringer Mannheim), to be used as a quantitation standard, was prepared in Blotto and serially diluted to generate a standard curve covering a concentration range of 2000–250 pg/ml CAT enzyme. Unknown samples were also diluted in Blotto and 100 μl of each standard and unknown was added per well. Samples were incubated on the plates at 37°C for one hour. The plates were washed four times with 425 μl per well of 0.2% Tween 20, PBS. Digoxigenin labeled sheep anti-CAT polyclonal antibody (Boehringer Mannheim, Indianapolis IN) was diluted 1:100 in Blotto and 50 μl of this solution was added per well. The plates were incubated at 37°C for 45 minutes. The plates were washed four times with 425 μl per well of 0.2% Tween 20, PBS. Anti-digoxigenin Fab'2 antibody conjugated with peroxidase (Boehringer Mannheim, Indianapolis IN) was diluted 1:400 in Blotto and 50 μl of this solution was added per well. The plates were incubated at 37°C for 45 minutes. The plates were washed four times with 425 μl per well of 0.2% Tween 20, PBS. 0.5 mg/ml 2,2'-Azino-di-(3-ethylbenz-thiazoline sulfonic acid; ABTS, Sigma, St. Louis, MO) was prepared in 1X ABTS Substrate Buffer (Zymed, South San Francisco, CA) and 150 μl of this substrate solution was added per well. The plates were incubated at room temperature for 30–35 min and the optical density (OD) of the samples was measured with a dual wavelength program of 405 nm–490 nm using a Spectramax 250 plate reader and Softmax Pro software (Molecular Devices, Sunnyvale, CA). Spiking experiments were performed to determine accuracy and reproducibility of the assay. Replicate spiked samples (n = 72) assayed on 9 plates over three different assay dates returned a %CV of 15%. Control serum and lung homogenate samples spiked with CAT enzyme have been assayed and return values within 20% of the predicted value. Criteria for data release include the following: 1) OD readings for standard curve points must be within one standard deviation of a mean OD reading calculated from >50 replicates performed using identical lots of assay reagents. This qualification range is recalculated as needed when reagent lots change. 2) The squared correlation coefficient for the standard curve on each assay plate must be > 0.99. 3) Duplicate determinations of each sample must be performed and may not differ by more than 10%. CAT protein values are reported as pg of CAT enzyme per mg of total protein in the sample.

RESULTS

Density Gradient Centrifugation

The average concentration profiles throughout 5–25% (w/v) dextrose gradients for uncomplexed DNA, the DOTIM within free liposomes, and the DOTIM within lipid-DNA particles are compared in Fig. 1. The concentration profiles among gradients (n = 3) were reproducible (Fig. 1). Sedimentation profiles of free liposomes and plasmid DNA centrifuged individually showed that neither sedimented far into the dextrose gradients (7.5% and 9% (w/v) dextrose respectively; Fig. 1). By comparison, two distinct DOTIM populations within the DOTIM-DNA complexes were distinguishable using this methodology. One population formed a broad band from the 16% boundary to approximately 11.5% (w/v) dextrose (Fig. 1). A second population of DOTIM containing material was found at around 5% (w/v) dextrose. These populations were further characterized with respect to their physical composition and content.

Physico-Chemical Characterization of Complexes

The vesicle size of the complexes throughout the gradients gradually declined from 354 nm to 113 nm as the dextrose concentration decreased, with a slight increase in size to 128 nm at the minimum dextrose concentration (5% w/v; Fig. 2). This was in contrast to the two peaks in the DOTIM profile obtained from the same samples. In addition, ξ was measured for most fractions (Fig. 3). At dextrose concentrations from 18.5 to 16% (w/v), a ξ of +24 mV was measured. As the dextrose concentration decreased from 16 to 7.5% (w/v) the ξ increased to +28–30 mV. At the lowest concentration dextrose, (5 to 7% w/v), at the very top of the gradient, ξ measurements around +34 mV were obtained. This value was representative of the ξ values obtained for free liposomes (typically ~+35 mV).

Composition of Fractionated Lipid-DNA Populations

Concentration profiles for the extracted DNA and DOTIM were obtained after separation of the lipid from DNA within individual fractions (Fig. 4). The concentration profile for the separated DOTIM correlated with that obtained for the DOTIM present in the intact lipid-DNA complex fractions (Fig. 1). However, the DNA concentration throughout the gradient was quite different. The greatest concentration of DNA was found within the higher density dextrose band, at approximately 11.5 to 16% (w/v) dextrose. As the dextrose concentration decreased towards the top of the gradient, the DNA concentration also decreased, indicating that the DOTIM peak at 5% (w/v) dextrose consisted primarily of free lipid (see Fig. 1, comparing the mobility of free liposomes and lipid-DNA complexes). Although the original formulation was mixed at a nominal mass ratio of 4.2:1 (cationic lipid:DNA), very few particles were found at this ratio. These particles were present within the shoulders of the complexed lipid-DNA peak (Fig. 4). Therefore, much of the complexed material formed multiple ratios of lipid

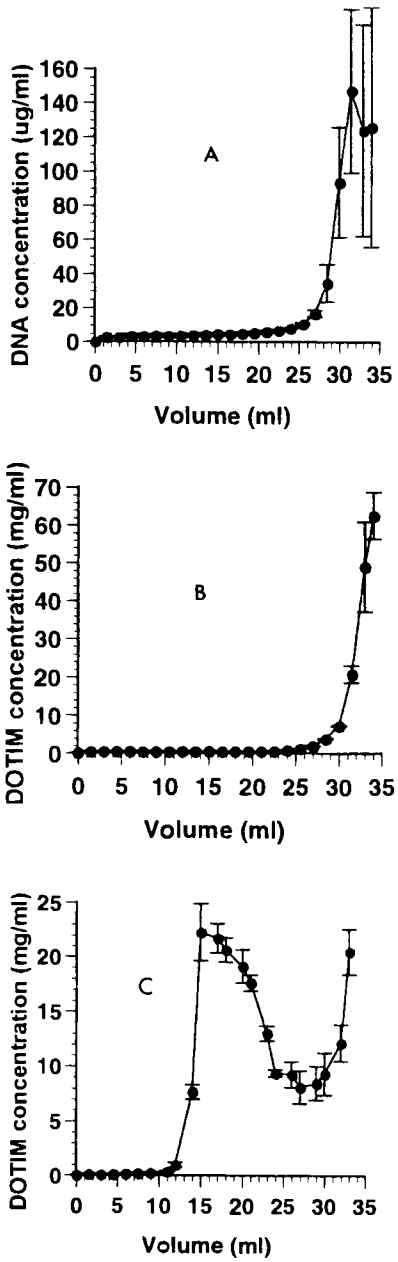

Fig. 1. Sedimentation profile of DOTIM/Cholesterol lipid-DNA complexes. Samples were loaded and centrifuged at 15,000 RPM (JA-20 rotor) for 8 hours, on 5–25% (w/v) dextrose gradients, fractionated into 1.5 ml fractions and the optical density was obtained at 237 or 260 nm to analyze DOTIM or DNA distribution respectively. Data points represent the mean (± standard deviation) of three runs. (A) plasmid DNA; (B) DOTIM/cholesterol liposomes at 1:1 ratio; (C) DOTIM within DOTIM/cholesterol-DNA complexes.

to DNA most being well outside the nominal mixing ratio. The cationic and neutral lipid components of the complexes did not appear to redistribute. HPLC of the isolated fractions throughout the gradient revealed that the DOTIM and cholesterol were always present at a 1:1 molar ratio (data not shown).

DNA Protection and Stability of Purified Complexes

The sensitivity of DNA in lipid-DNA complexes to DNase I digestion has been used as an assay for exposure of the DNA

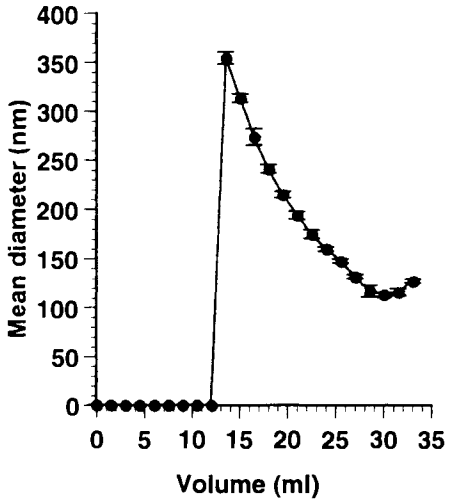

Fig. 2. Vesicle diameter of lipid-DNA complexes separated by centrifugation. Lipid-DNA complex was loaded onto 5–25% (w/v) dextrose gradients and centrifuged at 15,000 RPM (JA-20 rotor) for 8 hours, fractionated into 1.5 ml fractions and the mean vesicle diameters were obtained. Data points represent the mean (± standard deviation) of three runs.

in the complex (9,10). Under the digestion conditions used here, the uncomplexed DNA sample was rapidly digested within 1 min of exposure, as no evidence of intact plasmid was present following agarose gel electrophoresis (Fig. 5). Plasmid DNA within lipid-DNA complexes retained some protection to DNase I digestion for up to 15 min (Fig. 5). By comparison, DNA within complexes isolated from the high density sedimenting band, which contained less protective lipid, exhibited reduced protection against DNase I activity. The DNA within this sample had undergone complete degradation at 5 min (Fig. 5).

Similarly, the original and purified complexes were compared in serum. In both cases, incubation in 5% fetal bovine serum resulted in an 80% increase in mean particle size, and a shift from positive to negative ξ (original complex, +58 to −20 mV; purified complex, +54 to −25 mV). The ξ obtained

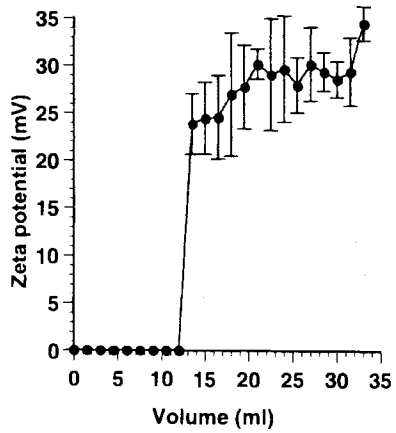

Fig. 3. Zeta potential of lipid-DNA complexes separated by centrifugation. Lipid-DNA complex was loaded onto 5–25% (w/v) dextrose gradients and centrifuged at 15,000 RPM (JA-20 rotor) for 8 hours, fractionated into 1.5 ml fractions and the mean ξ values were obtained. Data points represent the mean (± standard deviation) of three runs.

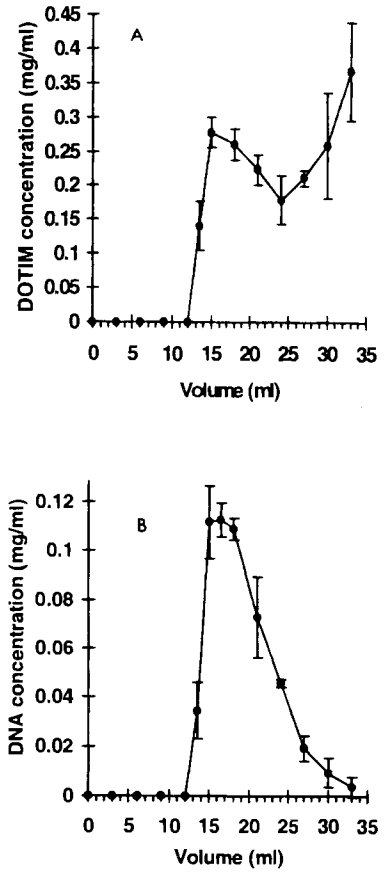

Fig. 4. Ratio of lipid to DNA for lipid-DNA complexes separated by centrifugation. Lipid-DNA complex was loaded onto 5–25% (w/v) dextrose gradients and centrifuged at 15,000 RPM (JA-20 rotor) for 8 hours, the gradients were then fractionated into 1.5 ml fractions. The lipid and DNA components of each fraction were separated, and the relative amounts of each quantitated as described in the Material and Methods. Data points represent the mean (± standard deviation) of three runs. (A) DOTIM; (B) DNA.

for the samples prior to incubation is higher than that obtained for centrifuged particles, as these samples were assayed in water, or water plus serum, rather than dextrose, to mimic conditions *in vivo*.

To determine whether the individual fractions containing complex populations were stable, a single fraction that sedimented at 13.5% (w/v) dextrose was taken and diluted to 5% (w/v) dextrose. This material was loaded onto three 5–25% (w/v) dextrose gradients and centrifuged as before. Analysis of the fractionated gradients revealed a distinct single band that could be reproducibly generated at approximately 13% (w/v) dextrose (Fig. 6).

In Vivo Activity of Purified Complexes

The original complex (4.2:1 nominal mass ratio) preparation resulted in a high level of CAT expression in lung tissue following IV administration (1651 ± 1068 pg/mg soluble protein; Fig. 7A). By comparison, the purified complex (2.4:1 nominal mass ratio) produced negligible expression levels (Fig. 7A). The addition of DOTIM/cholesterol liposomes to the purified complex resulted in the restoration of some CAT expression (630 ± 205 pg/mg soluble protein) in this test group. Intratracheal delivery of a similar set of test groups was also carried out to determine whether direct delivery of the complexes to the target organ would be effective. There was little difference in CAT production among the test groups (Fig. 7B) and statistical analysis by ANOVA confirmed that differences between groups were not significant ($p = 0.16$). The levels of expression following intratracheal delivery are lower overall than observed after IV injection. However, the complexes were not optimized for this route of delivery and were prepared solely for direct comparison with the IV formulation.

DISCUSSION

Two populations of particles were identified within a DOTIM/cholesterol- DNA formulation by density gradient centrifugation. One population, found at low density, had a high DOTIM content, negligible DNA content and physical properties similar to those of free liposomes. The second population contained the DNA and remaining cationic and neutral lipids. At the mass ratio used in this IV formulation (4.2:1), there was a large excess of positively charged lipid to DNA (a calculated 2:1 charge ratio). Thus, it appears that a population of 'free' or 'loosely' bound liposomes was present in the formulation. Cationic lipid-DNA complexes have previously been shown to contain free liposomes and complexed DNA populations (positive complex of N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP)-DNA complexes; (9)), or a single mobility population (DC-chol liposomes/poly (L-lysine)/DNA complexes; (10)), when centrifuged through 5–30% sucrose gradients.

Other forms of heterogeneity were identified among particles in the DNA-rich population including a significant spread in particle size and some variation in their zeta potential. It has been speculated that small particle sizes may be taken up preferentially by cells (16–18). However, size-specific functionality is difficult to substantiate when such a wide range of particle sizes is present within a complex dispersion. The heterogeneity described here is not surprising given that simple mixing of a DNA solution with liposomes is a turbulent mechanism governed by bulk transfer processes in combination with diffusional events. The actual rates of electrostatic association between DNA and lipid components are likely to be significantly faster than the rate of mixing. Thus, despite defining the conditions under which mixing will take place, a polydisperse product would still be expected. Collectively, these differences in physico-chemical and structural properties are likely to effect changes in the distribution and 'performance' of complexes when administered to animals.

The ξ among lipid-DNA particles varied from +25 to 34 mV among the fractions characterized. The highest ξ value (34 mV) was associated with the free liposome population, whereas the lipid-DNA complexes exhibited ξ values that ranged from +25 to 30 mV. It is notable that this narrow ξ distribution encompasses a range of lipid to DNA ratios and suggests that the DNA is oriented and bound to the lipids such that its capacity to neutralize the cationic charge is limited. Of further note is the observation that complexes will precipitate when combined at ratios approaching calculated charge neutrality (19). It has been postulated that the excess positive charge present on cationic lipid-DNA complexes aids complex uptake

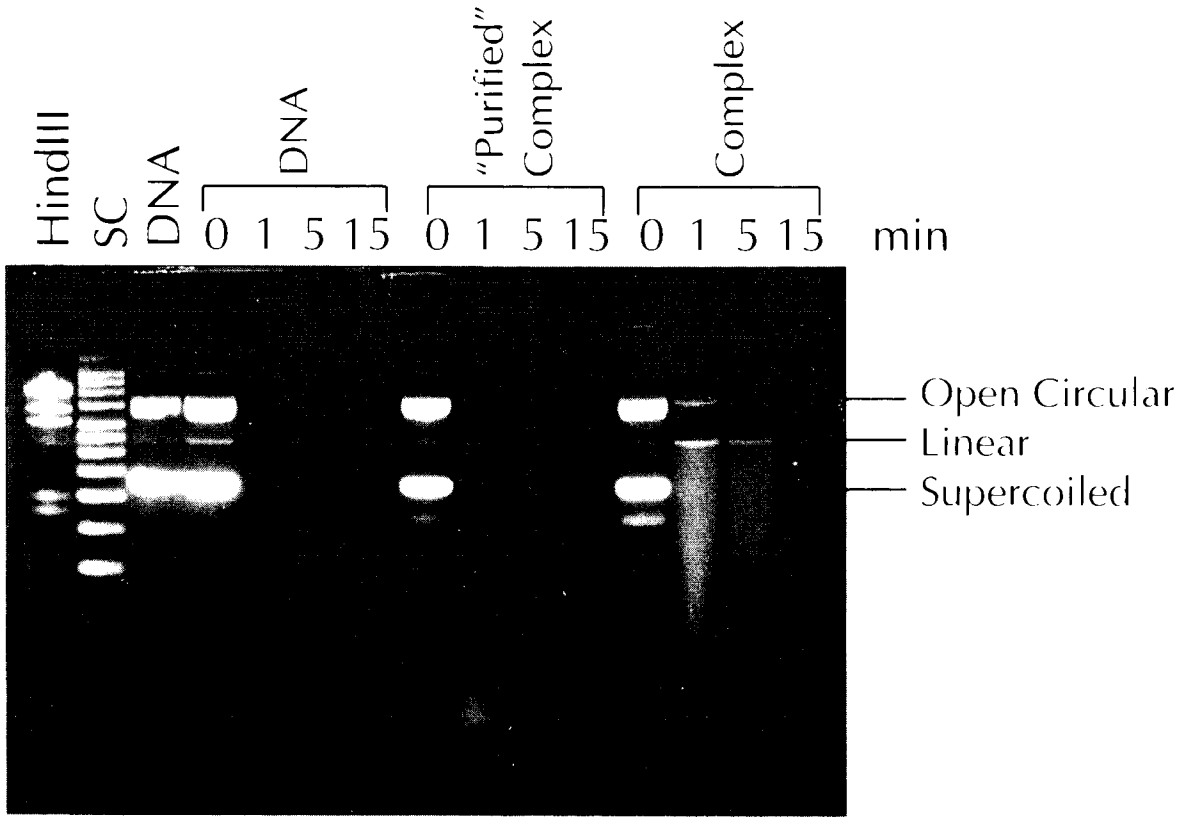

Fig. 5. Digestion of plasmid DNA by DNase I. DNA (naked or lipid-complexed) was subjected to DNase I digestion (2 units/μg of plasmid DNA) and aliquots removed at various time points for analysis. Lane 1, HindIII digest of Lambda DNA; lane 2, supercoiled DNA ladder; lane 3, original plasmid DNA standard; lanes 4–7, digestion of naked plasmid, times 0, 1, 5, 15 min; lanes 8–11, digestion of purified complex, times 0, 1, 5, 15 min; lanes 12–15, digestion of original complex, times 0, 1, 5, 15 min.

by the target cell (20) and can enhance specific tissue uptake *in vivo* (21). Therefore, ξ should be a good direct indicator of complex activity *in vivo*. However, the ξ of the particles will change immediately upon entering a physiological salt environment, and blood proteins are known to bind to cationic lipid containing particles (22–24). Here, incubation of purified and original complexes in serum resulted in an increase in size and shift in ξ from positive to negative, suggesting that protein binding took place. At best, the extent to which this binding occurs *in vivo* may be affected by the initial surface charge density of the injected particles. In this case, the original and purified samples had similar initial ξ.

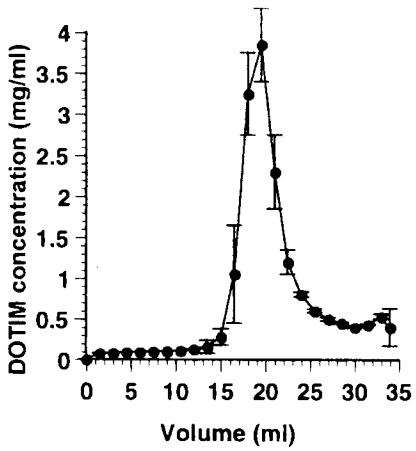

Fig. 6. Stability of lipid-DNA complexes separated by centrifugation. A single lipid-DNA complex fraction was taken from a centrifuged 5–25% (w/v) dextrose gradient, diluted to 5% (w/v) dextrose, and re-analyzed on a second, similar gradient. Data points represent the mean distribution of DOTIM (± standard deviation) from 3 runs.

DNase I digestion experiments show that DNA within the purified complex is more sensitive to the enzyme than the original, liposome-rich material. However, it is surprising that there is such a disparity in protection between original and purified complexes, as there is still an excess of lipid in the 'pure' complexes. One explanation for the observation is that the DOTIM:cholesterol liposomes actively bind to DNase I or sterically inhibit DNase I activity independent of the protection endowed on individual lipid-DNA particles. The assumption is that the only significant difference between the original and purified complexes is the removal of liposomes and that no other significant dynamic changes have occurred within the complexes. This assumption may have some validity. The purified material was further characterized with respect to stability. Following storage for 12 hours under refrigerated conditions, pure complex was re-centrifuged and produced a single band sedimenting at approximately the same dextrose concentration as the original material. Thus, the purified fraction is not simply composed of a distribution of complexes but contains a comparatively homogeneous subset of complexes (of similar size and

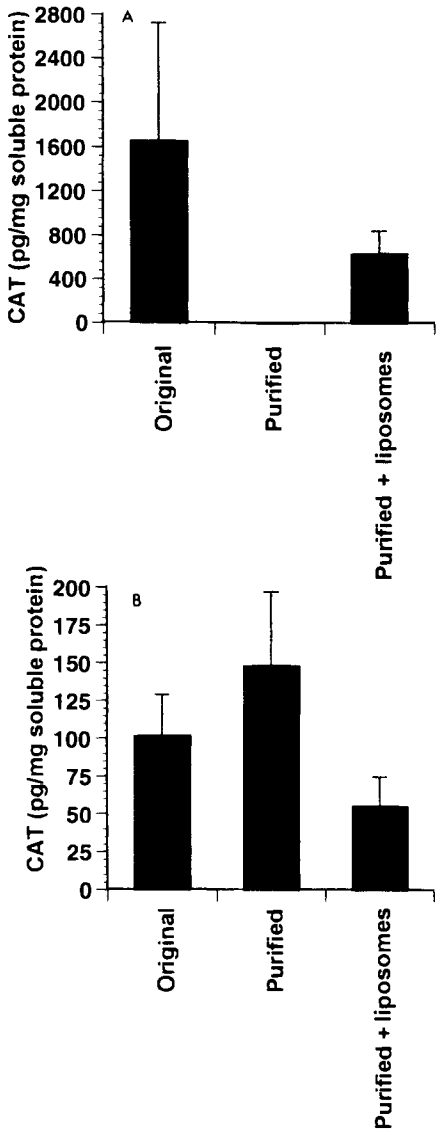

Fig. 7. In vivo comparison between lipid-DNA complexes and complexes purified by rate zonal centrifugation. ICR mice were administered intravenously or intra-tracheally with lipid-DNA complex, purified complex, or purified complex plus additional liposomes (to a final 6:1 mass ratio). Lung tissue was harvested at 48 hours and total protein and protein expression from the marker gene CAT were assayed. Values are expressed as mean picograms of CAT per milligram of total protein (± standard deviation) from 5 animals. (A) intravenous administration; (B) intratracheal instillation.

density) that does not obviously change over a short storage period.

Following *in vivo* testing the IV injected complexes resulted in distinctly different expression levels within the lungs of the mice (Fig. 7). The original, unmodified complexes, produced measurable CAT expression levels (1651 ± 1068 pg/mg soluble protein), whereas the purified complexes resulted in no detectable levels of expression. Addition of liposomes to the purified complexes resulted in a partial restoration of the "lost" activity (630 ± 205 pg/mg soluble protein). This result has been repeated in separate experiments (data not shown). The lack of activity of the pure complex, and the restoration of activity accompanying the re-introduction of lipid (to match the original concentration) suggest a number of mechanisms. The diminished expression achieved from the purified material may be due to its reduced protection of the complexed DNA in the circulation. For example, supercoiled plasmid degrades within 1 minute following IV injection in mice (25). This is presumably why free DNA delivered IV at doses similar to those described here, does not result in detectable levels of expression *in vivo*. Another explanation is that there is a loss of lung-delivery with purified complexes, regardless of the state of the DNA. The data suggest that the 'free' liposome component of the complexes contributes to lung uptake. Furthermore, confocal imaging of lung-tissue after IV injection of similar fluorescently-labeled complexes indicate that the site of uptake is the endothelium of the lung capillary bed (11), suggesting physical entrapment of aggregated complexes or liposomes. However, entrapment is unlikely to be the only mechanism involved, as it has also been shown that complexes can lodge in the high-endothelial *veins* of lymph nodes and completely bypass the capillary bed (11). This infers a more complicated mechanism of uptake than simple physical entrapment and a similar argument may be made with respect to lung uptake. A non-specific receptor mediated uptake mechanism (11) perhaps involving uptake of complement proteins bound to complexes (24) may contribute to the binding and internalization of complexes in the lungs.

Whatever the mechanism of 'targeting' to the lungs after IV delivery, it is not required when the complexes are administered directly to the lungs via the respiratory tract, as similar expression from all of the samples tested (original complex, purified, and purified plus lipid to original concentration) was observed.

In summary, cationic lipid-DNA complexes prepared by mixing resulted in a heterogeneous mixture. Populations varied with respect to their size, zeta potential and sedimentation in dextrose gradients. This heterogeneity manifested itself in changes in the expression of a CAT reporter gene in the lungs when administered to mice IV but no significant changes in expression were observed for the different complexes when administered intratracheally.

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