A Novel Series of Amphiphilic Imidazolinium Compounds for in Vitro and in Vivo Gene Delivery†

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ABSTRACT: We have developed three catioinic amphiphiles based on the structure 1-[2-(acyloxy)ethyl]-2-alkyl(alkenyl)-3-(2-hydroxyethyl)imidazolinium chloride. Although these three compounds differ only in the structure of the hydrophobic acyl chains, they differ greatly in their ability to mediate in vivo and in vitro gene delivery. Moreover, in vitro efficiency is not predictive of in vivo efficiency. The myristoyl form is the most effective compound in vitro, and the oleoyl form is the most effective compound in vivo. The compounds readily form suspensions in aqueous media, both in the pure form and as mixtures with either cholesterol or dioleoylphosphatidylethanolamine. These suspensions can be sonicated to produce smaller particles. Particle size, electron microscopy, and the ability to capture glucose suggest that these lipids form liposomes on suspension in aqueous media. When mixed with plasmid DNA, the lipid particles appear to fuse and form larger particles. Fusion is maximal at the critical DNA:lipid ratio where extensive aggregation and precipitation are observed. Therefore, these compounds behave similarly to other cationic liposome-forming lipids upon interaction with DNA.

Cationic lipids capable of forming positively charged liposomes are known to be valuable tools for formation of DNA−lipid complexes, which can be used for gene delivery to mammalian cells in vitro and in vivo (Barthel et al., 1993; Behr et al., 1989; Brigham et al., 1989; Felgner et al., 1987; Hug & Sleight, 1991; Ito et al., 1990; Philip et al., 1993; Stribling et al., 1992; Zhu et al., 1993). Molecules of these cationic amphiphiles consist of both a hydrophilic polar head group and lipophilic aliphatic chains. However, the potential toxicity and nonbiodegradability of the majority of these lipids places limits on their use for gene delivery. Consequently, the development of novel cationic structures that may be nontoxic and biodegradable cationic lipids is of great importance. The cationic moiety of most previously synthesized cationic lipids is usually either a quaternary ammonium compound (Felgner et al., 1987; Leventis & Silvius, 1990) or a primary, secondary, or tertiary amine (Behr et al., 1989; Gao & Huang, 1991). In order to expand the possible choices of lipids for gene delivery, we have chosen to investigate other quite different cationic groups for their utility in this regard.

Fatty acid derivatives of 2-imidazolines (4,5-dihydroimidazoles), and especially their salts, are known to be surface-active compounds and are used as emulsifiers and adhesive agents (Ferm & Riebsomer, 1954; Glankler, 1979; Schwitzer, 1979). Such properties make these compounds promising for transfection and gene delivery. However, the direct synthesis of these compounds from fatty carboxylic acids and 1,2-diamines requires reaction conditions employing high temperatures and extended incubation times (Chitwood & Reid, 1935; Riebsomer, 1948). This is necessary because of the formation of intermediates, *N,N'*-diacyl-1,2-ethylenediamines, which are difficult to cyclize (Butler et al., 1976, 1978, 1983). More readily cyclizable monoacylated 1,2-ethylenediamines (Perillo & Lamdan, 1970) are much less readily produced, because their formation involves the use of a large excess of the corresponding diamine (Hill & Aspinall, 1939; Aspinall, 1939), and the product has only one fatty acid chain residue attached to the 2-imidazoline molecule. The latter property makes it unlikely that such compounds would form bilayers, a structural feature that seems to be essential for gene delivery (Hug & Sleight, 1991). None of these methods has been shown to give directly a 1,3-dialkylated imidazolinium salt, which would have better chemical stability in aqueous suspension than a nonalkylated imidazoline (Gary, 1990).

In this paper, we describe a novel synthesis for imidazolinium compounds based on intramolecular aminolysis. We have prepared three closely related compounds that differ only in the structure of the hydrophobic acyl chains. We find that this small change in structure can considerably affect the ability of the compound to promote DNA delivery, and there is little correlation in this regard between in vivo and in vitro delivery.

MATERIALS AND METHODS

Reagents. All reagents for organic synthesis were purchased from Aldrich Chemical Co., unless otherwise indicated, and were used without further purification. Dioleoylphosphatidylethanolamine (DOPE), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), and

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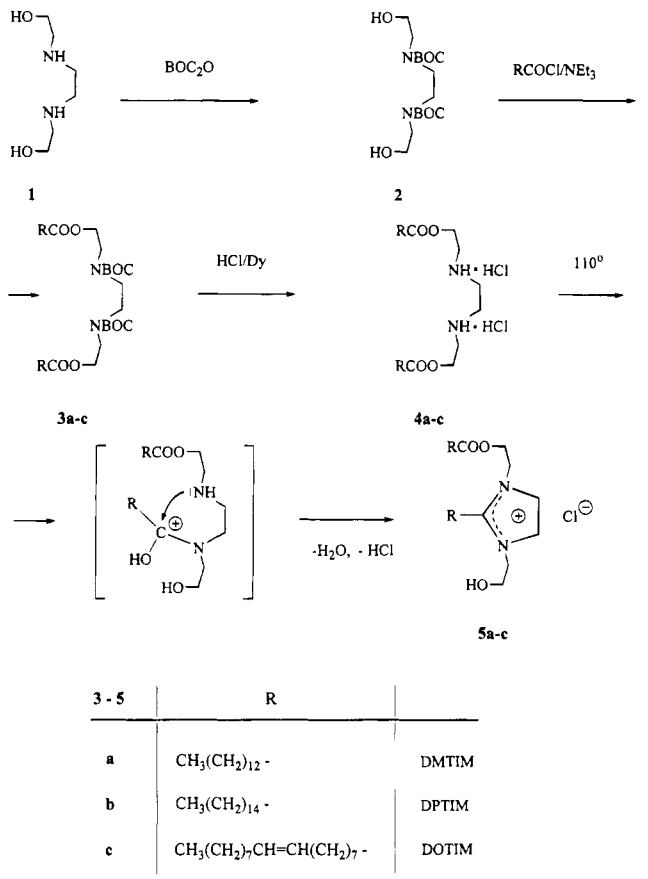

3 - 5	R	
a	$CH_3(CH_2)_{12}$ -	DMTIM
b	$CH_3(CH_2)_{14}$ -	DPTIM
c	$CH_3(CH_2)_7CH{=}CH(CH_2)_7$ -	DOTIM

FIGURE 1: Scheme for synthesis of cationic lipids. Abbreviations: BOC_2O, di-*tert*-butyl pyrocarbonate; NEt_3, triethylamine; HCl/Dy, a 4 M solution of HCl in dioxane.

N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were obtained from Avanti (Alabaster, AL). Cholesterol (Sigma, St. Louis, MO) was recrystallized four times from methanol. Purified cholesterol, DOPE, fluorescently labeled lipids, and all three synthesized cationic lipids were dissolved in chloroform and stored under argon at −20 °C.

Analytical. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. The IR and ^1H and ^{13}C NMR spectra of all compounds were consistent with their assigned structure. ^1H NMR spectra were recorded using a Bruker AM300 300 MHz spectrometer. Chemical shifts were reported in parts per million (δ) relative to tetramethylsilane. IR spectra were recorded using a Perkin-Elmer 599B IR spectrometer. Column chromatography was performed with the use of EM Separations Technology silica gel 60, 35−70 μm. Thin-layer chromatography was performed on Merck silica gel 60 F254 (0.2 mm thickness) glass-backed plates. Spots were visualized with UV illumination or by spraying the plates with a 4% w/v solution of phosphomolybdic acid in ethanol and heating at 200 °C for 10 min.

Synthesis of Cationic Lipids. In order to examine the possibility of synthesis of 1,3-dialkylated imidazolinium derivatives through the well-known reaction of intramolecular aminolysis, we first prepared *O,O*-diacylated derivatives of bis(2-hydroxyethyl)ethylenediamine **4a**−**c** (Figure 1). Prior to acylation, the amino groups of bis(2-hydroxyethyl)-ethylenediamine, **1**, were first protected using the acid labile butyloxycarbonyl (BOC) protective group. After acylation of the hydroxyl groups with the appropriate acyl chloride, the resultant ester, **3a**−**c**, was deprotected by treatment with a 4 M solution of hydrogen chloride in dioxane to form the dihydrochloride, **4a**−**c**. After a solution of the dihydrochloride, **4a**−**c**, in ethyleneglycol was heated at 110 °C for 30 min, the imidazolinium derivatives, **5a**−**c**, were obtained with yields of 72−78%. With heating, the *O,O*-diacylated derivatives of bis(2-hydroxyethyl)ethylenediamine **4a**−**c** are capable of intramolecular migration of an acyl group from the oxygen to the amino group as shown (Figure 1). We propose that this migration followed by loss of the elements of water is the most likely mechanism for the production of the imidazolinium compound.

*N,N′-Dibutyloxycarbonyl-bis(2-hydroxyethyl)ethylenediamine (**2**).* To a solution of 1.48 g (0.01 mol) of *N,N′*-bis-(2-hydroxyethyl)ethylenediamine, **1**, in 100 mL of $CHCl_3$ were added 4.57 g (0.021 mol) of di-*tert*-butyl pyrocarbonate and 5 mL of saturated aqueous $NaHCO_3$. After the mixture was stirred at room temperature for 5 h, the organic layer was separated, washed with water (25 mL × 2), dried over $MgSO_4$, and evaporated on a rotavapor. The white crystals thereby obtained were washed with 2 × 25 mL portions of hexane on a filter, crystallized from ethyl acetate/hexane, and dried under vacuum to give 2.8 g (80% yield) of product **2**: mp 140−141 °C; ^1H NMR ($CDCl_3$) δ 1.44 (s, 18 H, $2C(CH_3)_3$, 3.40 (s, 4 H, NCH_2CH_2N), 3.45 (bs, 4 H, $2NCH_2$-CH_2O), 3.74 (bs, 4 H, $2NCH_2CH_2O$, 4.80 (bs, 2 H, 2OH).

*General Procedure for the Preparation of Compounds **3a**−**c**.* To a solution of 1.0 g (0.0029 mol) of **2** in 50 mL of dichloromethane at 0 °C was added 1.0 mL (0.007 mol) of triethylamine. The solution was stirred for 15 min, and 0.006 mol of the appropriate fatty acid chloride was then added. The mixture was stirred at 0 °C for 30 min and then at room temperature for 45 min. The resulting solution was diluted with 50 mL of chloroform, washed with 2 × 50 mL portions of a 10% aqueous solution of citric acid, followed by 2 × 50 mL portions of 10% aqueous sodium bicarbonate, and dried over $MgSO_4$ for 2 h. The dried solution was filtered, the filtrate was evaporated with a rotavapor, and the residue was purified by chromatography on a silica gel column using 0−15% ethyl acetate/hexane as the eluant.

*N,N′-Dibutyloxycarbonyl-N,N′-bis[2-(tetradecanoyloxy)-ethyl]ethylenediamine (**3a**)* was prepared in 90% yield as a colorless oil: ^1H NMR ($CDCl_3$) δ 0.89 (m, 6 H, $2CH_2CH_3$), 1.26 (s, 40 H, $2(CH_2)_{10}$), 1.46 (s, 918, $2(CH_3)_3C$), 1.61 (m, 4 H, $2CH_2CH_2CO$), 2.32 (m, 4 H, $2CH_2CH_2CO$), 3.37 (m, 4 H, NCH_2CH_2N), 3.49 (m, 4 H, $2NCH_2CH_2O$), 4.17 (m, 4 H, $2NCH_2CH_2O$).

*N,N′-Dibutyloxycarbonyl-N,N′-bis[2-(hexadecanoyloxy)-ethyl]ethylenediamine (**3b**)* was prepared in 91% yield as a colorless oil: ^1H NMR ($CDCl_3$) δ 0.87 (m, 6 H, $2CH_2CH_3$), 1.27 (s, 48 H, $2(CH_2)_{12}$), 1.46 (s, 18 H, $2(CH_3)_3C$), 1.60 (m, 4 H, $2CH_2CH_2CO$), 2.30 (m, 4 H, $2CH_2CH_2CO$), 3.36 (m, 4 H, NCH_2CH_2N), 3.45 (m, 4 H, $2NCH_2CH_2O$), 4.16 (m, 4 H, $2NCH_2CH_2O$).

*N,N′-Dibutyloxycarbonyl-N,N′-bis[2-(9(Z)-octadecenoyloxy)-ethyl]ethylenediamine (**3c**)* was prepared in 94% yield as a colorless oil: ^1H NMR ($CDCl_3$) δ 0.88 (m, 6 H, $2CH_2CH_3$), 1.26, 1.29 (each s, 40 H, $2(CH_2)_6$ and $2(CH_2)_4$), 1.46 (s, 18 H, $2(CH_3)_3C$), 1.60 (m, 4 H, $2CH_2CH_2CO$), 2.0 (m, 8 H, $2CH_2CH{=}CHCH_2$), 2.29 (m, 4 H, $2CH_2CH_2CO$), 3.37 (m, 4 H, NCH_2CH_2N), 3.45 (m, 4H, $2NCH_2CH_2O$), 4.15 (m, 4 H, $2NCH_2CH_2O$), 5.35 (m, 4 H, 2CH=CH).

*General Procedure for the Preparation of Compounds **4a**−**c**.* To 0.001 mol of *N,N′*-dibutyloxycarbonyl ester **3a**−**c**

in a 100 mL flask under argon was added 12 mL of a 4 M solution of hydrogen chloride in dioxane, and the mixture was stirred at room temperature for 2 h. After completion of the reaction, as determined by TLC, dioxane was evaporated on a rotavapor, and the solid residue was suspended in 50 mL of dry ethyl ether. The suspension was filtered to collect the white crystals, which were washed with two additional 50 mL portions of ethyl ether. The white crystals were dried under vacuum (5 mmHg) for a few hours.

*N,N'-Bis[2-(tetradecanoyloxy)ethyl]ethylenediammonium dichloride (**4a**)* was prepared in 96% yield as white crystals: mp 163−168 °C; ^{1}H NMR ($CDCl_3$) δ 0.89 (m, 6 H, $2CH_2CH_3$), 1.28 (s, 40 H, $2(CH_2)_{10}$), 1.62 (m, 4 H, $2CH_2CH_2CO$), 2.39 (m, 4 H, $2CH_2CH_2CO$), 3.42 (m, 4 H, $2NCH_2CH_2O$), 3.74 (m, 4 H, NCH_2CH_2N), 4.38 (m, 4 H, $2NCH_2CH_2O$), 9.90 (bs, 4H, NH_2^+).

*N,N'-Bis[2-(hexadecanoyloxy)ethyl]ethylenediammonium dichloride (**4b**)* was prepared in 94% yield as a fine white powder: mp 165−170 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (m, 6 H, 2 CH_2CH_3), 1.29 (s, 48 H, $2(CH_2)_{12}$), 1.62 (m, 4 H, $2CH_2CH_2CO$), 2.42 (m, 4 H, $2CH_2CH_2CO$), 3.43 (m, 4 H, $2NCH_2CH_2O$), 3.72 (m, 4 H, NCH_2CH_2N), 4.45 (m, 4 H, $2NCH_2CH_2O$), 9.90 (bs, 4 H, NH_2^+).

*N,N'-Bis[2-(9(Z)-octadecenoyloxy)ethyl]ethylenediammonium dichloride (**4c**)* was prepared in 95% yield as a fine white powder: mp 150−155 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (m, 6 H, $2CH_2CH_3$), 1.29, 1.30 (each s, 40 H, $2(CH_2)_6$ and $2(CH_2)_4$), 1.63 (m, 4 H, $2CH_2CH_2CO$), 2.03 (m, 8 H, $2CH_2CH{=}CHCH_2$), 2.46 (m, 4 H, $2CH_2CH_2CO$), 3.43 (m, 4 H, $2NCH_2CH_2O$), 3.75 (m, 4 H, NCH_2CH_2N), 4.49 (m, 4 H, $2NCH_2CH_2O$), 5.37 (m, 4 H, 2CH=CH), 9.90 (bs, 4 H, NH_2^+).

*General Procedure for the Preparation of Compounds **5a−c**.* To 0.0015 mol of ester **4a−c** was added 3 mL of ethyleneglycol, and the reaction mixture was stirred at 110 °C in an oil bath for 30 min. The solution obtained was diluted with 150 mL of chloroform and washed several times with 100 mL portions of a mixture of 5% w/v aqueous NaCl: MeOH (2:1). The chloroform layer was separated, dried over $MgSO_4$, and evaporated on a rotavapor, and the residue was purified by chromatography on silica gel using 5−20% methanol in $CHCl_3$ as the eluant.

*1-[2-(Tetradecanoyloxy)ethyl]-2-tridecyl-3-(2-hydroxyethyl)imidazolinium chloride (**5a**, DMTIM)*[1] was prepared in 72% yield as a white waxy solid: ^{1}H NMR ($CDCl_3$) δ 0.88 (m, 6 H, $2CH_2CH_3$), 1.26 (s, 40 H, $2(CH_2)_{10}$), 1.59 (m, 4 H, CH_2CH_2CO and $CH_2CH_2C^+_{cycl}$), 2.31 (m, 2 H, CH_2CH_2CO), 2.77 (m, 2 H, $CH_2CH_2C^+_{cycl}$), 3.52 (m, 2 H, NCH_2CH_2OH), 3.77 (m, 2 H, NCH_2CH_2OCO), 3.86 (m, 2 H, NCH_2CH_2OH), 4.09 (s, 4 H, NCH_2CH_2N), 4.33 (m, 2 H, NCH_2CH_2OCO), 6.00 (bs, 1 H, OH); IR ν 1620 cm^{-1} (imidazolinium); UV_{max} 238 nm, UV_{1cm} 8032 nm.

*1-[2-(Hexadecanoyloxy)ethyl]-2-pentadecyl-3-(2-hydroxyethyl)imidazolinium chloride (**5b**, DPTIM)* was prepared in 78% yield as a white waxy solid: ^{1}H NMR ($CDCl_3$) δ 0.88 (m, 6 H, $2CH_2CH_3$), 1.26 (s, 48 H, $2(CH_2)_{12}$), 1.60 (m, 4 H, CH_2CH_2CO and $CH_2CH_2C^+_{cycl}$), 2.31 (m, 2 H, CH_2CH_2CO), 2.76 (m, 2 H, $CH_2CH_2C^+_{cycl}$), 3.52 (m, 2 H, NCH_2CH_2OH), 3.77 (m, 2 H, NCH_2CH_2OCO), 3.87 (m, 2 H, NCH_2CH_2OH), 4.09 (s, 4 H, NCH_2CH_2N), 4.32 (m, 2 H, NCH_2CH_2OCO), 6.05 (bs, 1 H, OH); IR ν 1620 cm^{-1} (imidazolinium); UV_{max} 237 nm, UV_{1cm} 8161 nm.

*1-[2-(9(Z)-Octadecenoyloxy)ethyl]-2-(8(Z)-heptadecenyl)-3-(2-hydroxyethyl)imidazolinium chloride (**5c**, DOTIM)* was prepared in 75% yield as a yellowish waxy solid: ^{1}H NMR ($CDCl_3$) δ 0.88 (m, 6 H, $2CH_2CH_3$), 1.27, 1.30 (each s, 40 H, $2(CH_2)_6$ and $2(CH_2)_4$), 1.60 (m, 4 H, CH_2CH_2CO and $CH_2CH_2C^+_{cycl}$), 2.03 (m, 8 H, $2CH_2CH{=}CHCH_2$), 2.31 (m, 2 H, CH_2CH_2CO), 2.63 (m, 2H, $CH_2CH_2C^+_{cycl}$), 3.51 (m, 2 H, NCH_2CH_2OH), 3.77 (m, 2 H, NCH_2CH_2OCO), 3.87 (m, 2 H, NCH_2CH_2OH), 4.09 (s, 4H, NCH_2CH_2N), 4.32 (m, 2 H, NCH_2CH_2OCO), 5.20 (m, 4 H, 2CH=CH), 6.09 (bs, 1 H, OH); IR ν 1615 cm^{-1} (imidazolinium); UV_{max} 238 nm, UV_{1cm} 6820 nm.

Plasmid Construction and Preparation. Plasmid pCMVβ was constructed by MacGregor and Caskey (1989) and was obtained from Clontech (Palo Alto, CA). pCMVβ contains the human cytomegalovirus (HCMV) immediate early 1 promoter enhancer element linked to the β-galactosidase gene. p4233 was created by first digesting pCMVβ with *Not*I + *Cla*I, filling in the blunt ends, and purifying the fragment. Then, the *cat* gene in a *Hind*III-*Bam*HI fragment, derived from pSV2-cat (Gorman et al., 1982), was inserted by blunt end ligation to give p4233. Plasmid p4119 contains a composite HCMV immediate early 1 promoter enhancer element, the *cat* coding sequence from pSV2-cat, and an intron from the rat preproinsulin gene 5′ to the *cat* gene (Zhu et al., 1993). All plasmids were purified by alkaline lysis and ammonium acetate precipitation (Sambrook et al., 1989). p4233 was further purified by PEG extraction (Sambrook et al., 1989). Plasmid concentration was measured by UV absorption at 260 nm.

Formation of Aqueous Suspensions. Cationic lipids were prepared as aqueous suspensions in the following way. Cationic lipid (5−20 μmol) in chloroform, either alone or mixed in a 1:1 molar ratio with Chol or DOPE, was dried down using a rotary evaporator in a 16 × 125 mm screw-capped tube to give a thin film. For lipid-mixing experiments, the lipid film also contained 1 mol/100 mol each of NBD-PE and Rh-PE. To the lipid film was added 1 mL of 5% w/v glucose. The tube was gently agitated until all of the lipid was suspended. The temperature of agitation was room temperature or higher. If higher temperatures were required, the tube was suspended in a water bath at the appropriate temperature. The suspension was then subjected to sonication by suspending the tube in a round ultrasonic bath (Lab Supplies, Hicksville, NY) and sonicating the contents for 5−20 min. Sonication was usually carried out at the same temperature as was required for swelling.

The ability of DOTIM to capture glucose was demonstrated in the following way. A sonicated suspension was prepared from 40 μmol of 1:1 DOTIM:Chol as described above. A 1 × 15 cm column of sephadex G50 was prepared in distilled water. The suspension was applied to the column, which was then eluted with distilled water. Fractions were collected, with a volume of 0.5 mL, and each fraction was analyzed for lipid content and glucose content. The lipid was measured on the basis of the absorbance of the imidazolinium compound at 240 nm. The glucose content was determined using a hexokinase assay kit (Sigma, St. Louis, MO).

[1] Abbreviations: DMTIM, 1-[2-(myristoyloxy)ethyl]-2-myristyl-3-(2-hydroxyethyl)imidazolinium chloride; DOTIM, 1-[2-(oleoyloxy)-ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride; DPTIM, 1-[2-(palmitoyloxy)ethyl]-2-palmityl-3-(2-hydroxyethyl)imidazolinium chloride; Chol, cholesterol.

Resonance Energy Transfer (RET) Fusion Assay. DNA-induced fusion was determined with the RET lipid-mixing assay by using a Perkin-Elmer MPF-4 fluorescence spectrophotometer. Labeled vesicles, containing 1 mol/100 mol each of NBD-PE and Rh-PE, were mixed with unlabeled vesicles at a 1:4 ratio and a total lipid concentration of 50 μM (25 μM cationic lipid) in 5% w/v glucose. DNA (0−25 μg) in 0.5 mL of 5% w/v glucose was added to 0.5 mL of 50 μM liposome suspension with rapid vortexing. Five minutes after mixing, the fluorescence emission of NBD-PE in the sample was measured. The excitation wavelength was 475 nm, and the emission wavelength was 530 nm, with the monochromator slit set at 6 nm. Vesicles containing 0.2 mol/100 mol of lipid of each probe were used for the measurement of NBD-PE fluorescence in vesicles where mixing had reached 100% (control). The results shown below are obtained from individual experiments. Companion studies designed to assess the reproducibility of the technique revealed that the standard deviation for percent fusion did not exceed a value of 7% fusion.

Particle Size Distribution. Particle size distributions of the vesicle−DNA complex were determined by dynamic light scattering using a NICOMP Submicron Particle Sizer Model 370 instrument (NICOMP Particle Sizing Systems, Santa Barbara, CA). From the intensity-weighted particle distribution, a volume-weighted distribution (relative particle volume vs diameter) was obtained. For 5% w/v glucose, viscosity and index of refraction were set at 1.066 cp and 1.340, respectively (*CRC Handbook of Chemistry and Physics*, 67th ed.). The scattering cell temperature was constant at 23 °C. Latex standards (Eastpor Microparticles, Bangs Laboratories, Inc., Carmel, IN) were used for instrument calibration.

For the measurement of particle size in lipid suspensions, 1 mL of 5 mM lipid in 5% w/v glucose was used. For the measurement of particle size after complex formation, 0.5 mL of 800 μM lipid in 5% w/v glucose was mixed wth 40−200 μg of DNA in 0.5 mL of 5% w/v glucose. The mixture was vortexed immediately and allowed to stand at room temperature for 5 min before measurement of particle size was begun. Typically, the collection of sufficient data for accurate sizing took 30 min. Values shown are the mean ± standard deviation taken from the Gaussian particle size distributions.

Electron Microscopy. Liposome suspensions or DNA−lipid complexes were diluted to 400 μM lipid and were placed on Formvar coated grids (EM science). After a few minutes, the sample was drained off with a filter paper, and the grids were stained with a drop of 1% w/v uranyl acetate. Samples were observed in a JEOL 100ZX electron microscope.

Cell Transfection. CV1-P, an African green monkey kidney cell line, was obtained from P. Berg, Stanford University (Palo Alto, CA). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/mL penicillin and streptomycin (Life Technologies, Inc., Grand Island, NY) and in a humidified 7% CO_2 incubator. Cells were plated at 150 000 cells per well in six-well plates (Corning, Corning, NY) and incubated overnight. DNA−lipid complexes were formed, 30 min prior to addition to cells, by addition of 20 μL of 0.5 mg/mL pCMVβ (10 μg of plasmid) to sterile polystyrene tubes containing 10−60 nmol of cationic lipid in 1:1 cationic lipid:DOPE liposomes suspended in 60 μL of 5% w/v glucose. The tube was vortexed rapidly upon addition of the DNA. Upon treatment, the entire contents of each tube was transferred to the cell-containing well, which contained complete, serum-containing medium. Control wells were treated with 80 μL of 5% w/v glucose. Three wells were resuspended at the time of transfection to obtain the original cell concentration using a cell counter (Coulter, Hialeah, FL).

Cells were harvested after 48 h of incubation with complexes at 37 °C. For each sample well, 0.2 mL of cells was counted to obtain the final cell concentration. The percent growth was calculated as previously described (Heath & Brown, 1989). The remaining cells in each well were placed in a microcentrifuge tube, sedimented by centrifugation at room temperature for 5 min at 2000*g*, and lysed by being resuspended in 50 μL of 1% Triton X-100. Cell lysates were centrifuged at 4 °C for 30 min at 2000*g*, and the supernatants were used for the β-galactosidase assay.

β-Galactosidase Enzyme Assay. β-Galactosidase activity was measured according to the procedure of Miller (1972) with some modifications, which follow. Purified β-galactosidase from *Escherichia coli* (Sigma) was used to construct a standard curve. To 50 μL of either cell lysate sample, or standard containing up to 0.02 unit of β-galactosidase, were added 450 μL of 60 mM sodium phosphate buffer (pH 8), 1 mM $MgSO_4$, 10 mM KCl, and 50 mM dithiothreitol. The enzyme reaction was initiated by addition of 0.1 mL of 4 mg/mL *o*-nitrophenol β-D-galactopyranoside in H_2O, and reaction was allowed to proceed at 37 °C in a water bath for 30 min. The reaction was stopped by addition of 500 μL of 1 M Na_2CO_3. The activity of β-galactosidase was monitored spectrophotometrically at 420 nm.

In Vivo Gene Delivery. In vivo gene delivery was carried out as previously described with modifications (Zhu et al., 1993). Prior to the experiments described here, the optimal DNA:lipid ratio for each lipid with p4233 was determined (Zhu et al., 1993) and was found to be 0.1 μg/nmol for DOTIM, 0.091 μg/nmol for DMTIM, and 0.083 μg/nmol for DPTIM. Optimal complexes were prepared from sonicated lipid suspensions containing 1:1 cationic lipid:cholesterol and plasmid p4233 by addition of 360 μg of plasmid in 600 μL of 5% w/v glucose to a 1.7 mL sterile plastic centrifuge tube containing 600 μL of liposome suspension in 5% w/v glucose. The liposome suspension contained 3.60 μmol of DOTIM, 3.96 μmol of DMTIM, or 4.32 μmol of DPTIM. The mixture was mixed by rapidly pipetting the tube contents twice. The mixture turned cloudy, but no precipitates or aggregates were visible. Mice (25 g ICR female), four per experimental group, received 200 μL of complex (60 μg of DNA) within 30 min of complex preparation by tail vein injection. The control group received 60 μg of a luciferase plasmid, complexed to 600−720 nmol of the relevant cationic lipid. Mice were sacrificed 24 h after injection, and organs were collected and homogenized as described (Stribling et al., 1992). Protein concentrations were normalized for each set of tissues assayed, and a volume of each extract was added to 10 μL of a 20 mM acetyl-CoA stock solution in water (Sigma), containing 0.3 μCi of [^{14}C]-chloramphenicol (Amersham, Arlington Heights, IL). Homogenization buffer was added to adjust the final sample volume to 122 μL, and the mixture was allowed to react at 37 °C for 8 h. cat activity was then measured (Gorman et al., 1982), and cat units were determined as previously described (Philip et al., 1993).

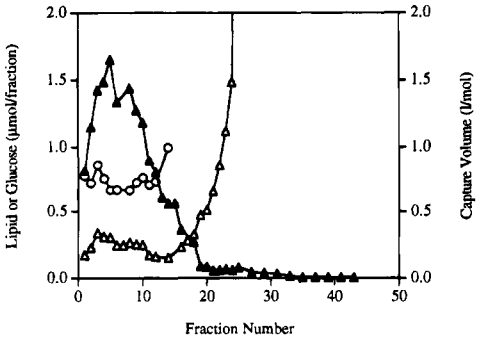

FIGURE 2: Glucose capture using DOTIM:Chol. Profile shows the lipid concentration (▲), the glucose concentration (△), and the captured volume determined from them (○).

Table 1: Particle Size of Sonicated Suspensions of Imidazolinium Compounds[a]

lipid	mean particle diameter (nm)		
	pure	1:1 with Chol	1:1 with DOPE
DOTIM	<5	48 ± 29	98 ± 73
DMTIM	<5	53 ± 26	49 ± 27
DPTIM	<5	72 ± 29	44 ± 30

[a] Suspensions of the three imidazolinium compounds were prepared in the pure form or mixed with either cholesterol or DOPE, and their size was measured by light scattering as described in Materials and Methods.

RESULTS

Suspension Preparation. DOTIM was found to suspend readily at room temperature to give a somewhat cloudy, viscous suspension, which, upon sonication at room temperature, did not scatter light and was not viscous. DOTIM:Chol (1:1) or 1:1 DOTIM:DOPE gave suspensions that behaved similarly, although the suspensions visibly scattered light. DOTIM:DOPE (1:1) was the most highly scattering after sonication.

DMTIM also suspended readily at room temperature, but the resultant suspension was very clear, even without sonication, and exhibited foaming. If the suspension was cooled to 4 °C, it became cloudy and viscous, but its clarity was restored upon being heated to room temperature once more. Sonication of DMTIM produced a suspension similar to DOTIM. Both 1:1 DMTIM:Chol and 1:1 DMTIM:DOPE produced a cloudy suspension, which clarified upon sonication for 5 min at room temperature. In appearance, the suspensions were similar to the corresponding DOTIM mixtures, and there was no evidence of foaming.

DPTIM required a temperature of 36 °C to induce swelling in aqueous suspension. Sonication at 36 °C produced a very clear suspension, with no evidence of foaming. DPTIM:Chol (1:1) and 1:1 DPTIM:DOPE behaved similarly to the corresponding DOTIM mixtures.

Glucose Capture. Figure 2 shows the elution profile for a sonicated suspension of 1:1 DOTIM:Chol prepared in glucose. It is clear that glucose is associated with the lipid peak. Moreover, the peak fractions show a quite constant captured volume around 0.7 L/mol. This value is consistent with that seen for phospholipid SUV (Szoka & Papahadjopoulos, 1980) and suggests that this lipid is forming liposomes, with a mean diameter of approximately 50 nm.

Particle Size. Table 1 shows the particle size for suspensions of the three imidazolinium compounds. All three compounds formed particles smaller than could be detected

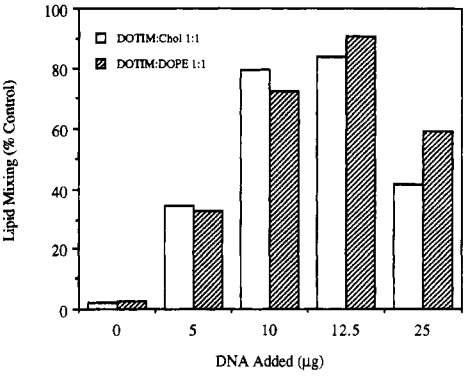

FIGURE 3: DNA-induced fusion of DOTIM vesicles. Lipid mixing for both 1:1 DOTIM:Chol and 1:1 DOTIM:DOPE induced by plasmid p4119 as measured by the RET assay.

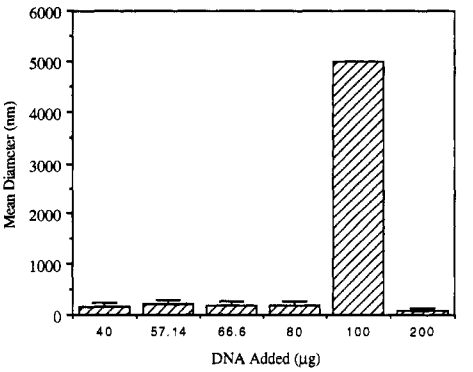

FIGURE 4: Particle size complexes of 1:1 DOTIM:Chol with pCMVβ. Particle size was measured by quasi elastic laser light scattering as described in Materials and Methods.

when they were suspended and sonicated in the pure form. Presumably, they existed as a micellar dispersion. When suspended and sonicated as mixtures with either cholesterol or DOPE, particles of sizes expected for highly sonicated liposomes were observed. The size of DOTIM:Chol liposomes as determined by light scattering agrees well with the size estimated from glucose capture.

DNA–Lipid Complex Formation. Figure 3 shows the percent lipid mixing of DOTIM vesicles upon formation of a complex with plasmid DNA. Maximum DNA-induced lipid mixing was observed at a DNA:cationic lipid ratio of 0.5 μg/nmol for 1:1 DOTIM:Chol and 1:1 DOTIM:DOPE, which corresponds to a DNA:lipid charge ratio of 1:1.5. Extensive lipid mixing was also observed at other ratios both higher and lower than the maximal, showing that the interaction between the DNA and the lipid occurs at all ratios. The maximum extent of lipid mixing is 80% for 1:1 DOTIM:Chol and 90% for 1:1 DOTIM:DOPE. These observations suggest that plasmid DNA induces fusion of the vesicles and that fusion is maximal when the ratio of DNA negative charge to the lipid positive charge is 1:1.5. This agrees well with similar observations made on other cationic lipids (Gershon et al., 1993).

The particle size obtained upon complexation of plasmid DNA with 1:1 DOTIM:Chol is shown in Figure 4. Flocculation and precipitation of the complex was found to occur at a ratio of 0.5 μg of DNA/nmol cationic lipid. The particle size at this ratio was greater than 5 μm, reflecting the precipitation that could be observed with the naked eye. This DNA:lipid ratio (0.5 μg/nmol) also corresponds to that at which lipid mixing was greatest. At DNA:lipid ratios both greater and smaller than 0.5 μg/nmol, the observed particle

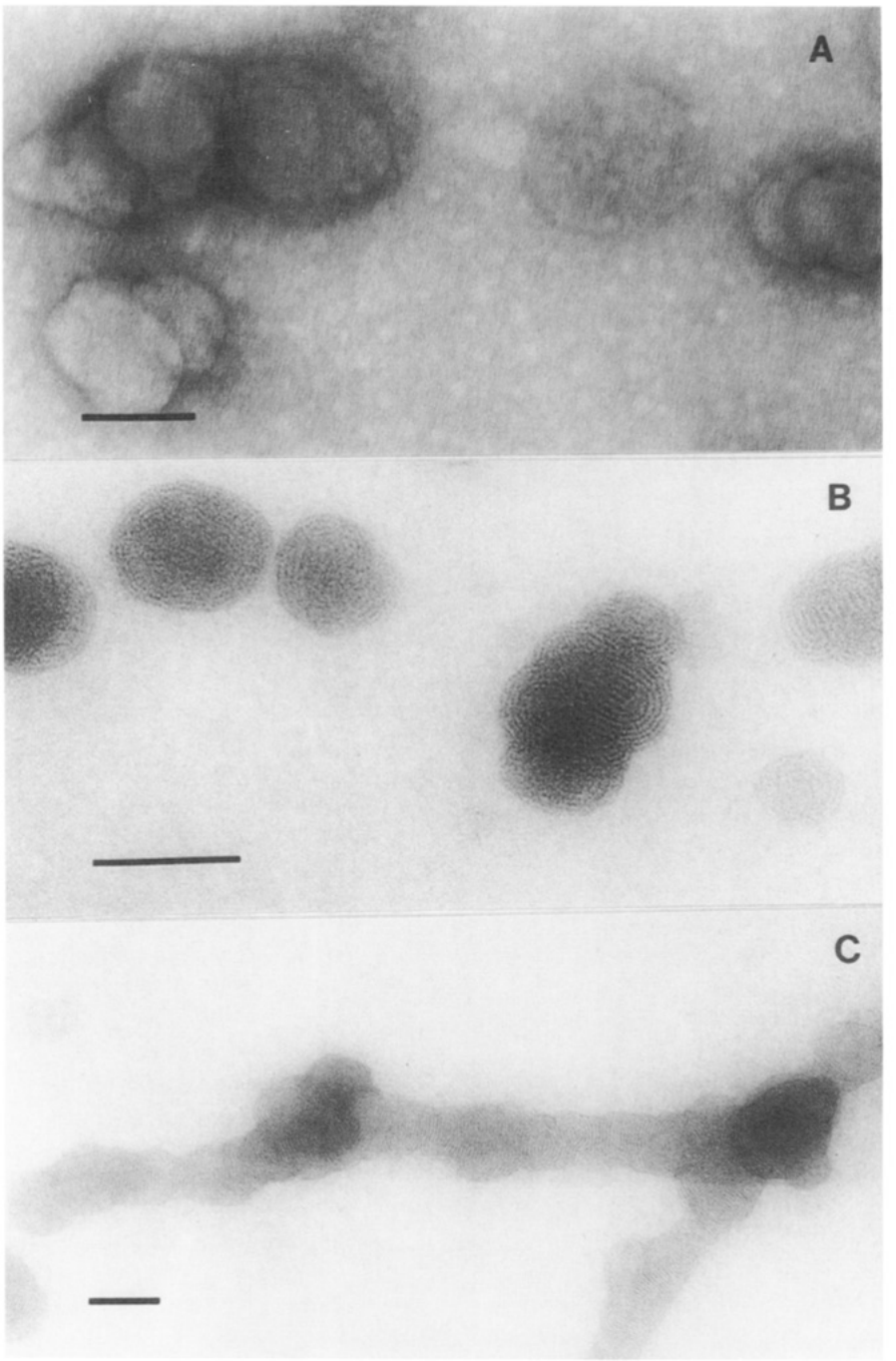

FIGURE 5: Electron microscopy of 1:1 DOTIM:Chol. (A) Sonicated lipid suspension. Owing to their vesicular appearance, the diameter of these particles in suspension was determined by dividing the observed diameter on the grid by $\sqrt{2}$. (B) Lipid suspension complexed with DNA at a ratio of 0.2 μg of DNA per nanomole of lipid. (C) Lipid complexed at 0.33 μg of DNA per nanomole of lipid. Size bars are 100 nm.

size was much smaller, and no precipitation or flocculation was observed. DNA−lipid complexes were larger at all ratios studied than the uncomplexed particles. Typically, complexes formed at ratios other than 0.5 μg/mol had a mean diameter of 200−400 nm.

Figure 5 shows the electron microscopic observations of 1:1 DOTIM:Chol before and after complexation with DNA. Before complexation, the suspension appears to contain particles similar to unilamellar liposomes, whose mean diameter is 70−84 nm. This value is consistent with those obtained from light-scattering measurements and glucose capture data described above. The presence of numerous smaller particles may be an artifact of staining or may indicate that a part of the lipid exists in the form of micelles. The latter would be consistent with the failure of the pure cationic lipids to form particles detectable by light scattering. Upon complexation with DNA, 1:1 DOTIM:Chol appears as 100 nm particles with a distinct multilamellar structure. These particles are isolated at a ratio of 0.4 μg of DNA/nmol of lipid but appear to form larger rodlike structures at higher ratios. Unlike the uncomplexed particles, the DNA−lipid complexes appear to take up the uranyl stain, appearing as dark structures against a paler background.

In Vitro Transfection. Figure 6 shows the transfection and growth inhibition of CV1-P cells by DNA−lipid complexes. Complexes containing these three lipids appear to inhibit cell growth at quite similar lipid concentrations, 50% growth inhibition occurring when between 30 and 40 nmol of lipid is added. All three lipids induce cell transfection, as indicated by β-galactosidase expression. Moreover, as with

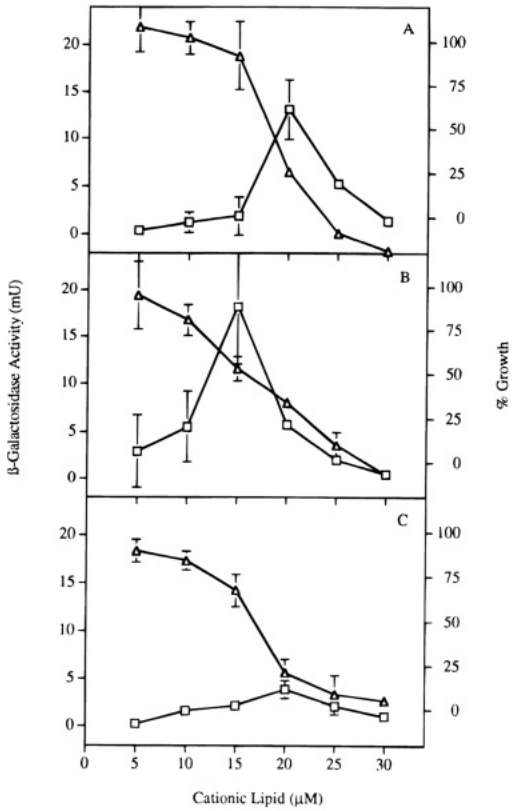

FIGURE 6: In vitro transfection (48 h) of CV1-P cells. Cell transfection (□) and growth inhibition (△) for complexes of pCMVβ with 1:1 DOTIM:DOPE (A), 1:1 DMTIM:DOPE (B), and 1:1 DPTIM:DOPE (C). Each curve is the mean of two separate experiments.

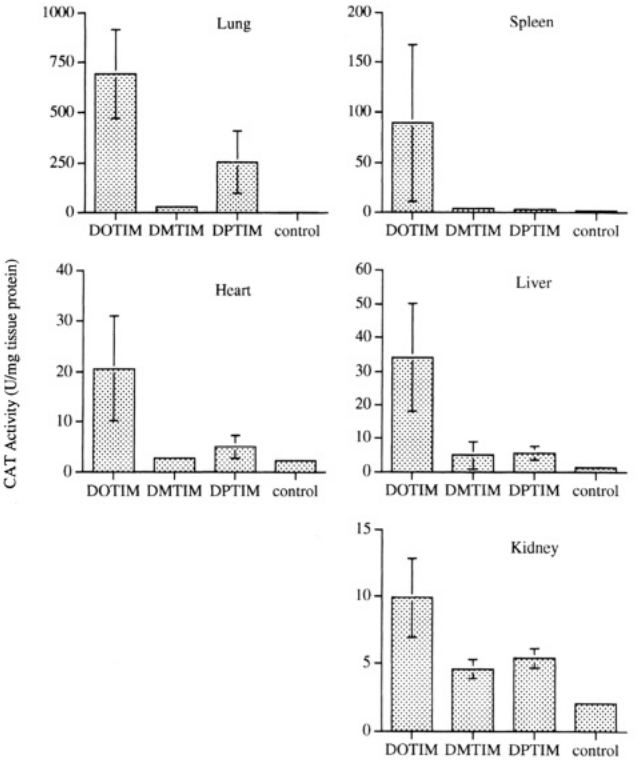

FIGURE 7: *cat* gene expression in ICR mice. Groups of four mice received 60 μg of p4233 complexed with either 1:1 DOTIM:Chol, 1:1 DMTIM:Chol, or 1:1 DPTIM:Chol. Activity is expressed as *cat* units per milligram of tissue protein. For each organ studied, each mean was compared to every other mean by *t* test. All values are at least significantly different from all other values ($p \leq 0.1$), except for 1:1 DMTIM:Chol and 1:1 DPTIM:Chol in the liver, which are not significantly different.

many other cationic lipids, an optimal DNA:lipid ratio for transfection is observed (Barthel et al., 1993; Behr et al., 1989; Felgner et al., 1987; Felgner et al., 1994; Gao & Huang, 1991; Ito et al., 1990; Rose et al., 1991; Zhou et al., 1991; Zhou & Huang, 1994). This ratio is 0.33 μg of DNA/nmol of lipid for 1:1 DMTIM:DOPE and 0.25 μg of DNA/nmol of lipid for 1:1 DOTIM:DOPE and 1:1 DPTIM:DOPE. The difference between the lipids is not significant in this regard, as we have also observed lower optimal ratios for 1:1 DOTIM:DOPE in other experiments (not shown). Peak transfection levels are highest for 1:1 DMTIM:DOPE. DOTIM:DOPE (1:1) gives comparable transfection levels, while transfection levels with 1:1 DPTIM:DOPE are 5-fold lower. The results shown in Figure 6 are in each case the mean of two separate experiments. Therefore, we conclude that, for in vitro transfection, DMTIM is the most effective of the lipids, although DOTIM is comparable. In order to determine the effectiveness of these lipids relative to known materials, we have also examined 1:1 DOTMA:DOPE in our system. We find that the peak level of expression with 1:1 DMTIM:DOPE is approximately twice that observed with 1:1 DOTMA:DOPE.

In Vivo Gene Delivery. Figure 7 shows the in vivo *cat* expression induced by the administration of DNA−lipid complexes, using p4233 as a vector. In all cases, *cat* expression is highest in lung tissue, which agrees with previous observations using other cationic lipids (Zhu et al., 1993). DOTIM:Chol (1:1) is considerably better in all cases for induction of *cat* expression than either 1:1 DPTIM:Chol or 1:1 DMTIM:Chol. DPTIM:Chol (1:1) gives 2.7-fold lower lung expression ($p \leq 0.005$) and 1:1 DMTIM:Chol gives 23-fold lower lung expression ($p \leq 0.0005$) than DOTIM:Chol. Both 1:1 DPTIM:Chol and 1:1 DMTIM:Chol give considerably lower expression in the spleen, heart, and liver than 1:1 DOTIM:Chol. Kidney expression levels are about 2-fold lower for DPTIM and DMTIM than they are for DOTIM. In summary, DOTIM:Chol is a very effective lipid formulation for in vivo gene delivery. Of the three lipids, DOTIM is the best for in vivo expression, and this result could not be anticipated from the results of in vitro transfection studies.

DISCUSSION

The results described above show that a novel series of imidazolinium compounds can be highly effective for intracellular gene delivery. In vivo, DOTIM is the most effective of the three compounds. In our experience, DOTIM is among the most effective lipids that we have used for in vivo *cat* expression and has also proven to be very effective for delivery and expression of a number of other gene products in vivo, including luciferase and GM-CSF (unpublished observations).

Our study of these compounds for in vitro transfection demonstrates the limited capacity of in vitro studies in predicting the most effective compounds for in vivo gene delivery. While DMTIM is highly effective for transfection of CV1-P cells in vitro, it is the least effective of these three lipids for in vivo gene delivery. In addition, DPTIM, which is almost without effect in our in vitro test system, shows significant in vivo gene delivery and appears overall to be more effective in this regard than DMTIM. DOTIM is highly effective both for in vitro transfection and for in vivo gene delivery. However, our in vitro studies do not show

DOTIM to be more effective than many other cationic lipids (C.-Y. Chow and T. D. Heath, unpublished observation). The minimal correlation between in vitro transfection studies and in vivo gene delivery emphasizes the importance of primarily using in vivo testing to evaluate cationic lipids for their effectiveness in gene delivery.

The studies that we have described above show that these lipids, like many other cationic compounds that have proven to be effective for the delivery of DNA to cells, appear to form liposomes, although in the pure form, they may produce micellar aggregates. Evidence for their ability to exist as liposomes comes from a number of sources. The ability of 1:1 DOTIM:Chol to capture glucose clearly points to the presence of closed vesicles with an internal aqueous space. The viscous, more turbid character of unsonicated preparations is indicative that these suspensions contain larger particles. Light microscopic observation of such suspensions has shown the presence of highly refractile 20−50 μm spherical structures which clearly resemble large vesicles (not shown). The size and appearance of the sonicated particles as determined by electron microscopy and laser light scattering is also indicative that they are liposomal rather than micellar in their structure.

Physical studies of DNA complexes formed from DOTIM suggest that this lipid forms complexes similar to those formed from other cationic lipids. Of these lipids, the most extensively studied is DOTMA. Gershon et al. (1993) have shown, using a fluorescence assay similar to the one we have used here, that the interaction of DOTMA suspensions with DNA results in the mixing of lipids from different vesicles, presumably through the fusion of the vesicle membranes. Our observations with DOTIM show that, while the particle diameter can increase by as much as 100-fold over the size of the particles in the original lipid suspension, the extent of lipid mixing does not exceed 85% of the maximum possible. In our studies, 100% mixing would be observed if fusion minimally involved the combination of the lipid contents of five lipid particles because our unlabeled:labeled lipid ratio is 4:1. However, the creation of complexes of the size that precipitate requires the aggregation of many more than five lipid particles. Therefore, it seems likely that the formation of DNA−lipid complexes, while partly involving the fusion of lipid particles, may result from the formation of aggregates in which the lipid is present in discrete domains. Electron microscopy of DNA complexes with 1:1 DOTIM:Chol reveals the presence of strings of smaller particles, each particle being about 0.2 μm in diameter and appearing to consist of stacked lamellae. Such a structure is consistent with the physical data that we have obtained, although it should be pointed out that the observed level of fusion may be lower than what actually occurs if emitted light is scattered by the DNA−lipid complexes.

In conclusion, we have synthesized a series of novel cationic lipids that demonstrate the possibility of establishing precise relationships between the structure of cationic lipids and the ability of liposomes containing these lipids to mediate efficient in vivo gene transfer and expression. The importance of this approach is emphasized by the considerable change in gene transfer efficiency brought about by changing the acyl groups.

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