Mol. Cells, Vol. 10, No. 2, pp. 142–147

Molecules and Cells

© Springer-Verlag 2000

In Vivo Gene Transfer to the Mouse Nasal Cavity Mucosa Using a Stable Cationic Lipid Emulsion

Tae Woo Kim[1,2], Hesson Chung[1], Ick Chan Kwon[1], Ha Chin Sung[2], and Seo Young Jeong[1,]*

[1] *Biomedical Research Center, Korea Institute of Science and Technology, Seoul 136-791, Korea;*

[2] *Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea.*

(Received on November 3, 1999)

We evaluate a new cationic emulsion as a mucosal gene carrier and elucidate the relationship between the transfection efficiency and the stability of the carrier/DNA complex. A cationic lipid emulsion was formulated with soybean oil and 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP) as major components and was used to transfer genes to the epithelial cells of the mouse nasal cavity via intranasal instillation. Correlation between the transfection efficiency and the stability of the carrier/DNA complex was investigated by measuring the carrier size changes and by observing the degree of DNA protection against DNase I digestion in the presence of heparin. The cationic emulsion showed at least 3 times better transfection activity than the liposomal carriers in nasal mucosae. The cationic emulsion was stable in the presence of heparin whereas the liposomal carriers became very unstable. Unlike DNA in liposome/DNA complexes, DNA in the emulsion/DNA complex was resistant to heparin exchange and DNase I digestion. The cationic emulsion was more effective in delivering DNA to nasal mucosae than commercially available liposomal carriers. The transfection activities of the lipid carriers in nasal cavity mucosae are in agreement with the stability of the lipid carriers and their complexes with DNA.

Keywords: DNA Delivery; Heparin; *In Vivo* Transfection; Liposome; Nasal Mucosa; Physicochemical Property.

Introduction

Mucosal gene transfer, with the aid of viral or non-viral vectors, is of great interest in the fields of gene therapy and DNA vaccine development (Davies *et al*., 1998; Kim *et al.*, 1997; Klavinskis *et al*., 1999; McCluskie *et al*., 1998). Since the early days of gene therapy, cystic fibrosis transmembrane conductance regulator (CFTR) cDNA has been delivered to airway epithelia to correct the defects associated with cystic fibrosis (Davies *et al*., 1998). Mucosal immunization by means of DNA vaccines that encode antigens can elicit the humoral and the cellular immune responses against mucosal pathogens, especially viral pathogens (Klavinskis *et al*., 1999; Okada *et al*., 1997). In the case of viral vectors, the host immune system generates a series of immune reactions against the vector that creates problems, such as vector-induced hypersensitivity and inflammation. To overcome these problems, many non-viral vectors have been developed and used in *in vivo* gene delivery to the respiratory system (Gao and Huang, 1991; Wilson,1993). Cationic liposomes, one of the most potent non-viral vectors, still have a low gene transfer efficiency in the respiratory system (Davies *et al*., 1998; McCluskie *et al*., 1998).

There are many barriers in the mucosal region that prevent successful gene transfer. The thick secretions of anionic mucus and glycosaminoglycan are likely to prevent access of carrier/DNA complexes to the epithelial cell surface, and may dilute and alter the composition of the complexes. There are other barriers including mechanical hindrance by cilia and the glycocalyx. In the presence of many mucosal barriers, liposome/DNA complexes can lose their physical integrity and can be destabilized by negatively charged materials. The latter causes DNA to dissociate from the complex (McCluskie *et al*., 1998; Xu and Szoka, 1996; Zelphati *et al*., 1998). Because of these problems, the use of liposomes for *in vivo* mucosal gene transfer has been limited.

* To whom correspondence should be addressed.
Tel: 82-2-958-5902; Fax: 82-2-958-5909
E-mail: syjeong@kist.re.kr

Abbreviations: DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane.

In our previous study, we developed a gene carrier in the form of an oil-in-water (o/w) type cationic lipid emulsion (Yi *et al*., 2000). A stable cationic lipid emulsion was formulated with soybean oil and DOTAP, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly (ethylene glycol) 2000] ($PEG_{2000}PE$) as emulsifiers. This cationic emulsion successfully delivered a reporter gene into cells *in vitro* in the presence of up to 90% serum. The cytotoxicity of cationic emulsion was comparable to or less than commercially available Lipofectamine. It is well known that a small amount of serum (~10%) can dramatically reduce the transfection activity of liposome/DNA complexes because it contains anionic materials (Xu and Szoka, 1996; Zelphati *et al*., 1998). Unlike liposomal carriers, the cationic emulsion that we developed retained its physical integrity when complexed with DNA. *In vitro* DNA release tests showed that our emulsion/DNA complex is strong, and is unaffected by an anionic polyion, poly-L-aspartic acid (Yi *et al*., 2000).

In this report, we quantified the efficiency of the cationic emulsion in transferring genes to the epithelial cells of the mouse nasal cavity via intranasal instillation. The results show that the transfection activity of emulsion is at least 3 times higher than those of Lipofectamine and Lipofectin, which are commercially available liposomal carriers. We also investigated whether the cationic emulsion, alone or when complexed with DNA, maintains its integrity in the presence of heparin, a polysaccharide of the glycosaminoglycan family, in mucosa. In addition, the relationship between transfection activity in nasal mucosae and the stability of the lipid carriers or the carrier/DNA complexes against heparin and a nuclease was established.

Materials and Methods

Materials Soybean oil was purchased from Sigma Chemical Company (St. Louis, MO). DOTAP, DOPE and $PEG_{2000}PE$ were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Lipofectamine, Lipofectin, fetal bovine serum (FBS) and heparin sulfate sodium salt were from Gibco BRL/Life Technologies (New York, NY). Deionized distilled water was produced by using a Milli-Q Plus water purification system (Millipore Corporation, Bedford, MA). All other chemicals and reagents used were of tissue culture grade.

Plasmid DNA The plasmid pCMV-CAT encoding the chloramphenicol acetyl transferase driven by the human cytomegalovirus immediate-early promoter was purchased from Invitrogen (Groningen, Netherlands). The plasmid pCMV-CAT was amplified in the *E. coli* DH5-α strain and purified by using a Qiagen mega-kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instruction. DNA purity was determined by agarose gel electrophoresis and by measuring optical density. DNA having $OD_{260}/OD_{280} \geqslant 1.8$ was used in this study.

Preparation of cationic lipid emulsion The cationic emulsion gene carrier was formulated as described previously (Yi *et al*., 2000). Briefly, the emulsion was prepared by first making the oil and aqueous phases separately. The oil phase consisted of 2 g soybean oil, 0.16 g DOTAP, 0.08 g DOPE and 0.08 g $PEG_{2000}PE$. The aqueous phase contained 0.45 g glycerol in 20 ml water. These two solutions were stirred separately at *ca*. 70°C to solubilize the components completely. The solutions were mixed with a rotor/stator mixer (T 25 Ultra-Turrax, Janke & Kunkel GmbH & Co KG, Staufen, Germany) at 8,000 rpm for 10 min at room temperature. The mixture was passed 10 times through a Microfluidizer® M110S (Microfluidics Co., Newton, MA) with an inlet air pressure of 80 psi. The lipid emulsion prepared was stored at 4°C prior to use.

Cationic emulsion and liposome size measurement The average droplet size of the lipid carriers (liposome and emulsion), or carrier/heparin mixtures was measured by using photon correlation spectroscopy (Malvern Zetasizer®, Malvern Instruments Ltd., England). The emulsion and liposomes were diluted 300 and 3 times, respectively, for the measurement, and the mean value reported represents the average of three measurements on a single sample. The size distribution follows a log-normal distribution (Orr, 1983). In this paper, error bars in Fig. 3 represent the standard deviation of this size distribution function. To quantify the magnitude of size changes induced by heparin, 10 µg heparin was added to the diluted lipid carrier solutions. The size was measured after a 10 min incubation of the diluted lipid carriers or the lipid carrier/heparin solutions at room temperature.

Plasmid DNA release from the lipid/DNA complexes by adding heparin The emulsion/pCMV-CAT and Lipofectamine/pCMV-CAT complexes were prepared by mixing 1 mg of pCMV-CAT with 2 µl of the cationic emulsion (16 mg of total emulsifiers/ml) and the Lipofectamine (2 mg of total lipids/ml) solutions, respectively, in 20 µl of PBS. The complexes were incubated for 20 min at room temperature. Increasing concentrations of the aqueous heparin solutions were added to carrier/DNA complexes to equivalency values of 5 to 500, where equivalency is defined as the negative charge ratio between heparin and DNA at pH 7.4. After incubation at room temperature for 1 h, the samples were analyzed by gel electrophoresis in 1% agarose gel including 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at 5 V/cm in TEA buffer (0.04 M Tris-acetate with 1 mM ethylenediamine tetraacetic acid), and DNA was visualized under UV light. To test the protection of DNA in the complexes against nuclease digestion in the presence of heparin, plasmid DNA, emulsion/DNA complex and Lipofectamine/DNA complex were incubated with 0.5 U/ml of DNase I in 50 mM Tris buffer (pH 7.4) containing 0.9 mM Mn^{2+} for 10 min at 37°C. The DNA from the samples was extracted twice with phenol : chloroform : isoamyl alcohol (50:49:1 by volume) prior to gel electrophoresis to remove lipids and oil.

***In vivo* gene transfer and CAT assay** To prepare DNA/carrier complexes, the DNA solutions containing 50 µg pCMV-CAT and diluted lipid carrier solutions at different DNA-to-carrier ratios were mixed by inversion. Each solution was diluted with 75 µl PBS before mixing. The mixture was incubated at room

temperature for 20 min. The DNA/carrier complex was administered to female Balb/C mice weighing approximately 20–25 g (6–8 weeks old) by intranasal instillation. Animal experiments adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). After 48 h, the mice were sacrificed, and nasal cavity samples from the nose were removed and homogenized. After homogenization, cells were lysed by three freeze/thaw cycles, and lysates were incubated at 65°C for 10 min to inactivate CAT-like and other esterase activities in the nasal lysates (De Maio, 1990). One portion of the lysate was assayed for protein concentration by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, CA) based on the Bradford method (Bradford, 1976). CAT enzyme activity was determined as reported (De Maio, 1990). To quantify CAT activity in lysates, the radioactivity of [^{14}C]chloramphenicol (Amersham Life Science Ltd., England) and its acetylated derivatives from thin-layer chromatograms (TLC) on an image plate (BAS-MP 2040P, Fuji Photo Film Co., Japan) was measured with a Bio-imaging analyzer (BAS 2000, Fuji). The CAT activity is expressed as relative activity based on the percent conversion of acetylated [^{14}C]chloramphenicols in total [^{14}C] chloramphenicols.

Results and Discussion

The *in vivo* transfection activity was evaluated for cationic emulsion at different emulsion-to-DNA ratios after an intranasal instillation in mice. Lipofectamine and Lipofectin were also used as gene carriers for comparison. The ratio between cationic lipid and DNA determines the net surface charge on the complex. This can alter the ability of the complex to bind and to enter cells and can change the stability of the complex against potential charged destabilizers in mucosae. Therefore, the ratio between the cationic lipid and DNA in the DNA/carrier complex is one of the critical variables in the optimization procedure for the *in vivo* gene transfer (Egilmez *et al*., 1996; Zhu *et al*., 1993).

To prepare DNA/carrier complexes, 50 µg of plasmid DNA was complexed with different amounts of the emulsion or the liposomes in 150 µl PBS (Fig. 1). The expression level represents the relative CAT activity where the mean values in transfection efficiency of the naked plasmid is set to unity. It is interesting to note that the expression level of naked plasmid is significant when compared with that of the untreated nasal lysates (Fig. 2). As expected, each lipid carrier showed various transfection efficiencies at different carrier-to-DNA ratios. The mean transfection efficiencies of Lipofectamine, Lipofectin and the emulsion were 1.3, 1.6 and 4.1, respectively. Although the mean transfection efficiencies for the two liposome systems are slightly higher than that of naked DNA, the expression level of the CAT gene for the liposomal carriers were not statistically different from naked DNA (Fig. 1). In the case of the emulsion system, however, the expression level increased as the amount of the emulsion in the complex increased and was always greater than that of naked DNA. We could not increase the concentration of Lipofectamine and Lipofectin above 1.3 and 0.8 mg/ml, respectively, since they formed large insoluble aggregates when complexed with DNA beyond these concentrations. The highest transfection efficiency was obtained with the emulsion, which was about three times better than that of the liposomal carriers. It is worthwhile to note that the emulsion/DNA complex did not develop to an insoluble

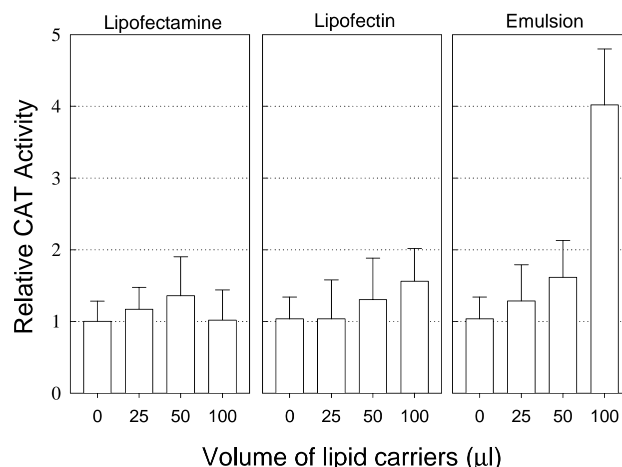

Fig. 1. Relative gene expression *in vivo* at different carrier-to-DNA ratios with 50 µg plasmid DNA. Each complex contains 50 µg plasmid DNA with different amounts of lipid carriers, each diluted in 150 µl of PBS. The complex was administered intranasally to groups of ten BALB/c mice under ketamine anesthetics. The efficiency of transfection was evaluated by the CAT assay.

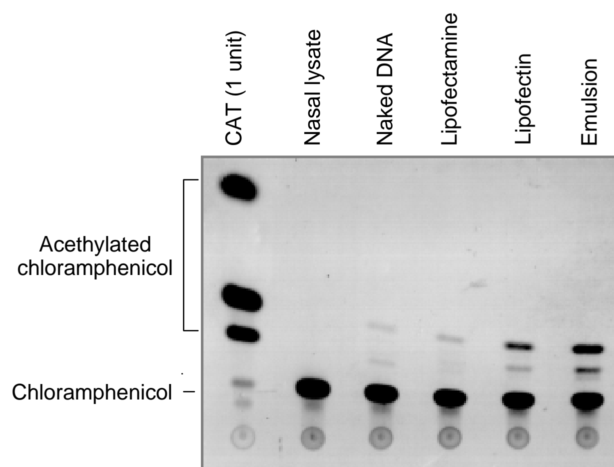

Fig. 2. TLC analysis of CAT activity in mouse nasal cavity with different lipid carriers to compare the transfection efficiencies. Carrier/DNA complex containing 50 µg of plasmid DNA and 50 µl of Lipofectamine, 100 µl of Lipofectin, or 100 µl of emulsion was administered to a group of ten BALB/c mice intranasally. Naked plasmid DNA (50 µg) was administered as a control.

aggregate with 50 μg of DNA in the emulsion concentration range studied.

TLC analysis of the CAT activities for different DNA/carrier complexes at their optimal ratios is shown in Fig. 2. When 50 μg of pCMV-CAT was used, different degrees of the transfection efficiency was observed for various lipid carriers, as evidenced by the presence of two acetylated chloramphenicol products. Among the lipid carriers, the emulsion had the highest level of acetylated chloramphenicol products, suggesting that it had the most potent transfection activity in nasal epithelial cells. In contrast, the liposomal carriers showed low levels of acetylated chloramphenicol products. This result illustrates visually that the cationic lipid emulsion has a superior transfection activity in epithelial cells in the nasal cavity.

In order to act as an efficient mucosal gene carrier, the DNA/carrier complex must survive and deliver intact DNA to the target cells in the nasal mucosa (Xu and Szoka, 1996). In other words, one may presume that the DNA in this complex is resistant to inactivation by the components of nasal secretions. To verify this presumption, we evaluated the stability of the lipid carriers and the DNA/carrier complex using heparin, one of the anionic destabilizers in nasal mucosa.

Before testing the stability of the DNA/carrier complexes, the stability of the carriers themselves was evaluated by measuring the droplet size change in the presence of heparin (Fig. 3). Since heparin has a high anionic charge density associated with its sulfate and carboxylic groups, it could destabilize the cationic lipid carriers via electrostatic interaction and, as a result, could cause changes in carrier droplet size (Zelphati *et al.*, 1998). The droplet size measurement was made after a 10 min incubation of the heparin-carrier mixtures at room temperature. In the absence of heparin, the mean droplet sizes of Lipofectamine, Lipofectin, and the emulsion were 124.9, 113.5 and 202.4 nm, respectively. When the carriers were incubated in 3.3 μg/ml heparin solution, Lipofectamine and Lipofectin became unstable and underwent a 2–3-fold increase in average droplet size having wide distributions. Lipofectamine in particular showed the most drastic change in size. The size and its distribution of the emulsion, on the other hand, remained unchanged in the presence of heparin. This result indicates that, unlike liposomes, the cationic emulsion retained its physical integrity in the presence of an anionic polymer.

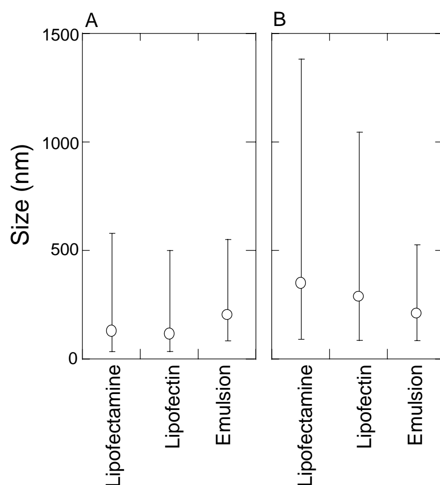

Fig. 3. Average particle size of the lipid carriers (**A**) in water and (**B**) in 3.3 μg/ml aqueous heparin solutions. Emulsion and liposomes were diluted 300 and 3 times, respectively, and were incubated for 10 min at room temperature in water or in heparin solutions before the measurements. The average particle size represents the average of three measurements on a single sample. The error bar represents the standard deviation of a log-normal distribution function (Orr, 1983).

The stability of the DNA/emulsion complexes was also investigated by observing the competitive exchange between heparin and DNA in the complexes. The complexes were incubated for one hour at room temperature after adding heparin solutions of varying concentrations (Fig. 4A). For the Lipofectamine/DNA complex, the plasmid began to dissociate as the equivalency of heparin increased above unity. The plasmid was released completely with a heparin equivalency of 50 or higher. A similar pattern was observed with Lipofectin (data not shown). In distinct contrast, the emulsion did not release its plasmid even with 50 equivalents of heparin. Thus, the complex between DNA and the emulsion is extremely stable and is resistant to competitive exchange by an anionic proteoglycan.

DNA released from the complex is vulnerable to nuclease attack. We demonstrated this by exposing heparin-treated liposome/DNA and emulsion/DNA complexes to DNase I digestion (Fig. 4B). In the case of the heparin-treated Lipofectamine/DNA complex, the released DNA was degraded by 0.5 U/ml of DNase I. The amount of digested plasmid increased as the equivalency of heparin increased. The plasmid was completely degraded at 50 equivalency of heparin. Not surprisingly, even at 50 heparin equivalents, the DNA in the heparin-treated emulsion complex remained intact. We could not observe any trace of degraded DNA or any decrease in the amount of intact DNA. From these results, we conclude that the stable emulsion/DNA complex provided a necessary protection against inactivation by nasal secretions and a higher accessibility of the active DNA to nasal epithelia than the liposome/DNA complex.

In this report, we have demonstrated convincingly that cationic lipid emulsion can be used as an effective mucosal gene carrier *in vivo*. There are a few studies where cationic emulsions have been used as a gene carrier (Liu *et al.*, 1996a; 1996b; Yi *et al.*, 2000). These studies, however, are limited to *in vitro* systems. To our knowledge, this is one of the first demonstrations of a cationic o/w emulsion as an

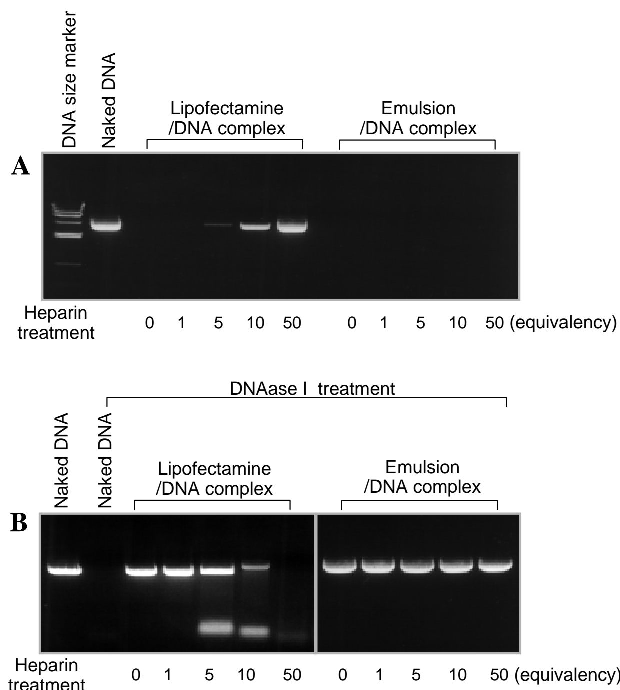

Fig. 4. Gel electrophoresis of (**A**) pCMV-CAT after an exchange reaction of the lipid carrier/pCMV-CAT complexes by heparin and (**B**) heparin-treated carrier/DNA complex after DNase I digestion. Carrier/DNA complexes were formed by mixing 1 μg of pCMV-CAT with 20 μl of the cationic emulsion (16 mg of total emulsifiers/ml) or Lipofectamine (2 mg of total lipids/ml) in 20 μl PBS. The complexes were incubated for 1 h at room temperature after adding varying concentrations of heparin solutions. The equivalency value ranged from 5 to 500. After the incubation, samples were analyzed by gel electrophoresis. The carrier/DNA complexes were mixed with different amounts of heparin (indicated by Heparin Equivalency in (**B**) for 1 h at room temperature. Heparin treated complexes as well as naked DNA were incubated with 0.5 U/ml of DNase I for 10 min at 37°C. DNA from samples was extracted twice with phenol:chlorofrom:isoamyl alcohol (50:49:1 by volume) prior to gel electrophoresis.

effective gene carrier *in vivo*. Here, we have shown that the cationic emulsion is superior to two commercially available liposome systems for delivering DNA to mouse nasal epithelia.

Though the cationic emulsion is superior to liposomes, the transfection efficiency of the cationic emulsion would appear to be low as indicated by the large fraction of unacetylated chloramphenicol in Fig. 2. Nonetheless, the cationic lipid emulsion should prove to be a potent mucosal gene delivery system since intact DNA in the emulsion/DNA complex has a higher accessibility to nasal epithelia. On the other hand, DNA in the liposome/DNA complex can be released from the complex and digested by nucleases in the presence of mucosal destabilizers such as heparin.

In summary, we have demonstrated that a cationic emulsion capable of delivering plasmid DNA *in vitro* in the presence of 90% serum also successfully delivered the plasmid to mouse nasal epithelial cells *in vivo*. The transfection efficiency of the emulsion carrier is approximately 3 times better than commonly used commercial liposome carriers. The increased transfection efficiency using the emulsion carrier may be ascribed to the increased probability of delivering intact DNA to the nasal mucosae since the complex is strong and insensitive to mucosal destabilizers.

Acknowledgments We wish to thank Dr. E. B. Lee for technical advice, S. W. Yi for providing the emulsion, and T. Y. Yune for technical assistance with the CAT assay. We also thank Drs. M. Caffrey and A. Cheng for valuable comments and for carefully reading the manuscript. This work was supported by the KIST-2000 project.

References

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Davies, J. C., Geddes, D. M., and Alton, E. W. (1998) Prospects for gene therapy for cystic fibrosis. *Mol. Med. Today* **4**, 292–299.

De Maio, A. and Buchman, T. G. (1990) Mammalian liver contains an activity which mimics bacterial chloramphenicol acetyltransferase. *Biochim. Biophys. Acta* **1087**, 303–308.

Egilmez, N. K., Iwanuma, Y., and Bankert, R. B. (1996) Evaluation and optimization of different cationic liposome formulations for *in vivo* gene transfer. *Biochem. Biophys. Res. Commun.* **221**, 169–173.

Gao, X. and Huang, L. (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.* **179**, 280–285.

Kim, Y. S., Lim, H. K., and Kim, K. J. (1997) Production of high-titer retroviral vectors and detection of replication-competent retroviruses. *Mol. Cells* **8**, 36–42.

Klavinskis, L. S., Barnfield, C., Gao, L., and Parker, S. (1999) Intranasal immunization with plasmid DNA-lipid complexes elicits mucosal immunity in the female genital and rectal tracts. *J. Immunol.* **162**, 254–262.

Liu, F., Yang, J., Huang, L., and Liu, D. (1996a) Effect of non-ionic surfactants on the formation of DNA/emulsion complexes and emulsion-mediated gene transfer. *Pharm. Res.* **13**, 1642–1646.

Liu, F., Yang, J., Huang, L., and Liu, D. (1996b) New cationic lipid formulations for gene transfer. *Pharm. Res.* **13**, 1856–1860.

McCluskie, M. J., Chu, Y., Xia, J. L., Jessee, J., Gebyehu, G., and Davis, H. L. (1998) Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA. *Antisense Nucleic Acid Drug Dev.* **8**, 401–414.

Okada, E., Sasaki, S., Ishii, N., Aoki, I., Yasuda, T., Nishioka, K., Fukushima, J., Miyazaki, J., Wahren, B., and Okuda, K. (1997) Intranasal immunization of a DNA vaccine with IL-12- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. *J. Immunol.* **159**, 3638–3647.

Orr, C. (1983) Emulsion droplet size data; in *Encyclopedia of Emulsion Technology*, Becher, P. (ed.), Vol. 1, pp. 369–404, Marcel Dekker, Inc., New York and Basel.

Wilson, J. (1993) Cystic fibrosis. Vehicles for gene therapy. *Nature* **365**, 691–692.

Xu, Y. and Szoka, F. C. (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **35**, 5616–5623.

Yi, S. W., Yun, T. Y., Kim, T. W., Chung, H., Choi, Y. W., Kwon, I. C., Lee, E. B., and Jeong, S. Y. (2000) A cationic lipid emulsion/DNA complex as a physically stable and serum-resistant gene delivery system. *Pharm. Res.* **3**, 313–319.

Zelphati, O., Uyechi, L. S., Barron, L. G., and Szoka, F. C. (1998) Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells. *Biochim. Biophys. Acta* **1390**, 119–133.

Zhu, N., Liggitt, D., Liu, Y., and Debs, R. (1993) Systemic gene expression after intravenous DNA delivery into adult mice. *Science* **261**, 209–211.