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Systemic Cationic Lipid-DNA Complex Mediated Gene Delivery: A Mechanistic Study

by

Lee Barron

B.Sc. Animal Science, Univ. of B.C.
M.S. Animal Science, Univ. of Calif., Davis

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

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by

Lee Barron

To my mother who provided me with the vision of higher goals and to my father who gave me the integrity to achieve them.

Acknowledgements

This has been a long journey with both pleasant and trying waypoints. My ability to savour the former and endure the latter was facilitated by many people. I am grateful to Dr. Frank Szoka for his guidance, his intellectual and financial support and, most importantly, for providing a challenging environment in which I feel I have progressed from student to scientist.

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Abstract

Cationic lipid-DNA complexes (lipoplexes) are synthetic DNA delivery vectors used for gene therapy. They are formed through electrostatic interactions when a cationic lipid is mixed with anionic DNA. I investigated the mechanisms involved in their preferential uptake and transfection of lung endothelial cells after positively-charged lipoplexes are injected in the tail vein of mice. Studies were conducted in *in vivo* models to determine the mechanism by which this occurs with the hypothesis being that lung transfection is dependent on electrostatic binding between the lipoplex and negative residues either on the endothelium or plasma proteins. To remove anionic glycosaminoglycans from the lung endothelium, I injected heparinase and found that transfection was substantially decreased. I also showed that cationic lipoplexes bind to albumin and activate complement proteins; however, when I injected lipoplexes into albumin- and complement-depleted animals, lipoplex-mediated transfection was not decreased. These results indicate that neither albumin nor complement plays a significant role in the transfection mechanism.

The observation that IV-injected cationic liposomes retained subsequently injected plasmid DNA in murine lungs suggested that the lipid maintained the ability to bind to anionic molecules via electrostatic interactions after exposure to blood. The importance of charge interactions on lung uptake is demonstrated by the fact that coating the lipoplexes with polyanionic D-polyglutamate neutralized the charge on the lipoplex and reduced both lipoplex uptake and transfection.

I defined the time window for transfection by disruption of lipoplexes with anionic liposomes at increasing time-intervals post-lipoplex injection. Lung uptake of lipoplex occurs within 60 minutes of IV injection. This raised the question if the principal role of the cationic lipid was to act as a controlled release matrix. To test this hypothesis, I infused transfectionally-active plasmid DNA to high steady-state concentrations for 60 minutes and found no significant transfection in the lung or any other organ. This shows that the cationic lipid component of the lipoplex serves in a function beyond simply cross-linking plasmid DNA to the endothelium and releasing it as the active transfection agent. Thus, the DNA is most probably internalized as a complex with cationic lipids.

In summary, cationic lipid-mediated transfection in the lung is a mediated by electrostatic interactions with anionic components of the vascular compartment. These findings more clearly define the *in vivo* mechanism of action and can be used to develop strategies to reduce toxicity of cationic lipoplex gene delivery.

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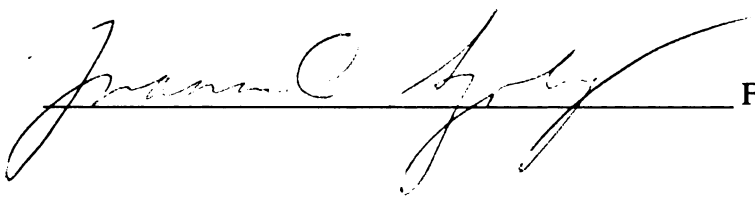
Thesis Committee Chairman:



Francis C. Szoka, Jr., Ph.D.

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List of Abbreviations

BPE	p-hydroxybenzamidine phosphatidylethanolamine
CHOL	cholesterol
Cl	clearance
CMV	cytomegalovirus
C_{ss}	steady-state concentration
CVF	cobra venom factor
DC-Chol	dimethylaminoethanecarbamoyl cholesterol
DDAB	dimethyldioctadecylammonium bromide
DMPE	dimyristoylphosphatidylethanolamine
DOPC	dioleoylphosphatidylcholine
POPG	dioleoylphosphatidylglycerol
DOPE	dioleoylphosphatidylethanolamine
DOSPA	dioleoylsperminocarboxamidodimethylpropanaminium trifluoracetate
DOTAP	dioleoyltrimethylammoniumpropane
DOTMA	dioleoyltrimethyl ammonium chloride
EM	electron microscope(y)
Ga	gauge
hAT	human alpha-1-antitrypsin
HDL	high-density lipoprotein
HUVEC	human umbilical vein endothelial cells
IA	intraarterial
IL-2	interleukin-2
IP	intraperitoneal
IT	intratracheal(ly)
IV	intravenous(ly)
LUV	large unilamellar vesicles
MOI	multiplicity of infection
ODN	oligodeoxynucleotide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethylenimine
pI	isoelectric point
RES	reticuloendothelial system
RLU	relative light units
RSV	Rous sarcoma virus
S.D.	Sprague-Dawley
SUV	sonicated unilamellar vesicles
SAXS	small angle x-ray scattering
SCID	severe combined immunodeficient
T_m	transition temperature

Chapter I: Introduction

Numerous studies have demonstrated that cationic lipid-DNA complexes, when injected via the intravenous route into mice are preferentially distributed to and transfect the lungs. This phenomenon was dependent on the establishment of a net positive charge on the complex by virtue of a molar excess of cationic lipids versus plasmid DNA nucleotides. The mechanism by which these phenomena occur is not clear.

1. Research Aim:

To understand the mechanistic and temporal factors governing the delivery of transcriptionally-active DNA to the lung by cationic lipid-DNA complexes.

2. Research Hypothesis:

Interactions between cationic lipid-DNA complexes and anionic components of the vascular compartment are significant factors affecting gene expression observed in the lung following systemic administration of the complex.

In this chapter, the literature pertaining to the application of cationic lipid-DNA complexes as gene delivery vectors *in vitro* and *in vivo* is reviewed. The first section is a summary of cationic lipid structure and cationic lipid-DNA complex formation. The second section reviews the literature concerning various steps of *in vitro* transfection in which cationic lipids are involved. The third section of this chapter summarizes the literature relevant to the various *in vivo* delivery routes, as well as the toxicity associated with *in vivo* delivery of cationic lipid-DNA complexes. The final section will discuss the scope of this thesis work.

3. Cationic Lipids

Gene delivery to somatic cells is a clinical strategy for the treatment of genetic and acquired diseases through the alteration of the patient's genetic repertoire. Two distinctive technologies have arisen to achieve this goal: viral and non-viral.

Viral gene-delivery vectors typically consist of replication-deficient viruses in which a portion of the viral genome has been replaced by a recombinant therapeutic sequence. Retroviral vectors have been extensively studied as gene delivery vehicles but have the inherent disadvantage that they are suitable for the transfection of actively dividing cells only. Furthermore, retroviruses, in the course of transduction, randomly integrate their genetic material into that of the host cell. This phenomenon has the potential to activate oncogenes or alter desirable host-gene function. Adenovirus-based vectors have also been employed *in vivo* but tend to be very immunogenic thus limiting opportunities for repeated administration which, given the transient gene expression characteristic of adenoviral vectors, may be necessary to achieve a therapeutic effect. Adeno-associated viral vectors have strong potential as gene delivery vehicles but existing problems with low viral titres and limited gene capacity must be surmounted.

Cationic lipid–mediated gene transfer, a principal non-viral system, has important advantages over viral approaches; cationic liposome-based vectors tend to be less immunogenic than viral systems and can accommodate larger recombinant DNA constructs. In addition, the ease with which cationic lipid-DNA complexes can be prepared is an important technical advantage, particularly upon scale-up.

Felgner and colleagues conceived the use of cationic lipids for gene delivery

(Felgner et al., 1987). This group formed a complex between N-(2,3-(dioleoyloxy)propyl)-N,N,N-trimethyl ammonium chloride (DOTMA) liposomes and plasmid DNA and observed that the cationic lipid-DNA complex or lipoplex (Felgner et al., 1997) successfully transfected several cell-lines with a reporter gene. Lipoplexes were first shown to transfer genes *in vivo* by Brigham and colleagues (Brigham et al., 1989). Since then, a number of groups (Gao and Huang, 1995; Lee et al., 1996), using a variety of cationic lipids, have shown gene transfer in animal models after administration of lipoplexes. This has led to the use of several cationic lipid-based gene delivery protocols in clinical trials (Caplen et al., 1995; Chadwick et al., 1997; McLachlan et al., 1996; Nabel et al., 1994; Nabel et al., 1993; Sorscher et al., 1994; Stopeck et al., 1997; Table 1).

Cationic Lipid Structure

The prototypical cationic lipid is comprised of an hydrophobic anchor connected by a linker to a positively-charged headgroup (Table 2). The hydrophobic moiety generally consists of tandem aliphatic chains or a cholesterol ring. The principal lipids used in this research are dioleoyltrimethylammoniumpropane (DOTAP) dioleoylphosphatidylethanolamine (DOPE) and cholesterol (Figure 1). In spite of considerable research, attempts to correlate lipid structure with transfection activity have met with limited success. Akao and colleagues (Akao et al., 1991) synthesized a series of double acyl-chain ammonium amphiphiles and correlated the transfection efficiency of the resulting liposomes with their physicochemical properties. They found that lipids with phase transition temperatures (T_m) below 37°C were more efficient in *in vitro* gene

Table 1

In Vivo Gene Delivery Studies

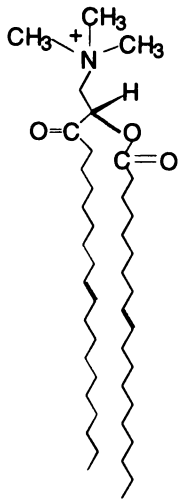
Cationic Lipid Formulation	Model	Route of Administration	Tissues Assayed	Reference
DOTMA/DOPE	Mouse	I.V.	Lung, liver & kidney	Brigham et al., 1989
DOTMA/DOPE	Mouse	I.V.	Various Tissues	Zhu et al., '93
DOTMA/DOPE	Mouse	I.V.	Lung, liver & kidney	Brigham et al., 1993
DDAB:CHOL	Mouse	I.V.	Many	Liu et al., 1995
DOTMA/DOPE	Rabbit	I.V. & Aerosol	Pulmonary endothelium & epithelium	Canonico et al., 1994
DOTMA/DOPE & DMRIE/DOPE	Rat	I.T. Instillation	Pulmonary Endothelium	Logan et al., 1995
Imadazolinium – based lipids	Mouse	I.V.	Lung, heart & liver	Solodin et al., 1995
GAP-DLRIE/DOPE	Pig	I.A.	Arteries	Stephan et al., 1996
DOTAP/Protamine Sulfate	Mouse	I.V./Intraportal	Heart, Lung Liver, Spleen & Kidney	Li & Huang, 1997
DOTMA/Tween 80	Mouse	I.V.	Heart, Lung Liver, Spleen & Kidney	Liu et al., 1997
DOTIM/CHOL & DOTIM/DOPE	Mouse	I.V.	Heart, Lung Liver & Spleen	Liu et al., 1997
DOTAP:CHOL, DOTAP/DOPE, DOTMA/CHOL & DOTMA/DOPE	Mouse	I.V.	Heart, Lung, Liver, Spleen & Kidney	Song et al., 1997
DOTIM/CHOL	Mouse	I.V.	Various Tissues	McClean et al., 1997
DOTAP:CHOL	Mouse	I.V.	Various Tissues	Smyth-Templeton et al., 1997
DOTAP:CHOL	Mouse	I.V.	Heart, Lung, Liver & Spleen	Barron et al., 1998

Table 2

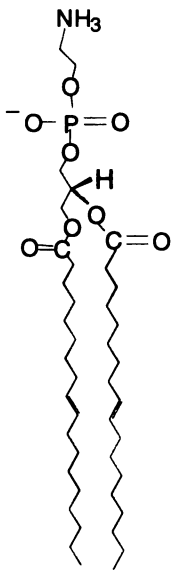
Structure-Function Relationship Between Cationic Lipids and Gene Transfer

	Types	Effects
Cationic Lipid	• # of positive charges (1,2,3,4,5)	•Charge density
	•Types of amino group (primary, secondary, tertiary, quaternary)	•Hydrogen bonding
	•Other helper groups (hydroxyl)	•Membrane fusion
Linker Group	•Amide •Ester •Carbamate •Ether •Phosphate	•Length •Hydrophilic property •Flexibility •Hydrogen bonding
Hydrophobic Region	•Saturated •Unsaturated •Fused ring	•Length •Transition temperature •Flexibility

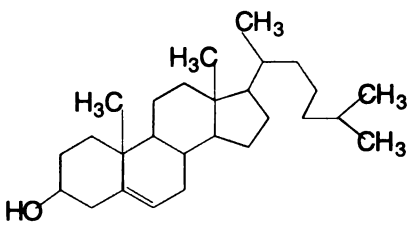
Figure 1 Lipid Structures



Dioleoyltrimethylammoniumpropane



Dioleoyphosphatidylethanolamine



Cholesterol

delivery than those with a T_m above 37°C. Similarly, the Felgner group (Felgner et al., 1994) found that as the length of the acyl-chain associated with a given polar headgroup was decreased from 18 to 14 carbons, yielding a lower T_m, the *in vitro* transfection activity of the corresponding lipoplex increased. A similar pattern of *in vitro* transfection was observed by Solodin and colleagues with cationic amphiphiles comprised of alkylated imidazolinium salts (Solodin et al., 1995). The longer alkyl-chain length elevates the T_m and bilayer stiffness of ensuing vesicles (Felgner et al., 1994). Lee and coworkers (Lee et al., 1996) also reported greater transfection activity *in vivo* upon decreasing chain length for a series of cationic lipids with a constant polar headgroup. Solodin and coworkers noted that while the saturated 14 carbon imidazolinium derivative was the most effective lipid for *in vitro* transfection, the optimal *in vivo* counterpart consisted of 18 carbon, *cis* unsaturated alkyl groups. Similarly, Wang and colleagues, (Wang et al., 1998) in studying a series of carnitine ester-based lipids, noted that the optimal hydrophobic moiety for *in vitro* gene delivery was a 14 carbon alkyl acyl group while the most efficient *in vivo* was an 18 carbon derivative with 1 *cis*-unsaturation. The incorporation of a double-bond has an effect similar to that of shortening acyl chain length in that it decreases the rigidity of the resulting bilayer.

The lack of correlation between *in vitro* and *in vivo* transfection results has been commented on before (Lasic et al., 1997; Lee et al., 1996; Solodin et al., 1995). Balasubramaniam and colleagues identified a cell-type-dependency for optimal T_m and transfection and suggested that cationic lipid structures would require optimization for a given cell-type (Balasubramaniam et al., 1996). This group also demonstrated that asymmetric hydrophobic domains transfected as well as or better than their symmetric

counterparts. Gao and Huang and later Lee and coworkers (Gao and Huang, 1991; Lee et al., 1996) showed that the complete alteration of the hydrophobic domain from that of paired acyl-chains to a cholesterol ring structure increased transfection activity in some contexts.

The linker group which connects the hydrophobic anchor to the polar headgroup also influences transfection activity (Lee et al., 1996). For the compounds studied, substitution of amide, amine or urea linkages in place of the existing carbamate function decreased transfection activity *in vivo;* however, this effect may be dependent on the lipid context (Lee et al., 1996). An important parameter associated with the linker group is its chemical stability and biodegradability. A lack of chemical stability can manifest itself in reduced transfection efficiency, as may be the case in the study by Lee and colleagues, where the substitution of less stable amide and urea linkers for carbamate yielded lower transfection activity. Reduced chemical stability may also limit the practical potential of a given formulation by decreasing its shelf-life (Gao and Huang, 1995).

The positively-charged headgroup of most cationic lipids consists of one or more amine groups subject to varying degrees of substitution. The functions of this domain in gene delivery include facilitating electrostatic interactions with negatively-charged DNA to form the lipoplex, as well as mediating interactions between the lipoplex and target cells. Typical amine group substitution is carried out via methylation, but Felgner and associates have shown that hydroxyethylation of the terminal amine of DOTMA yielded compounds which mediated greater *in vitro* transfection activity than the parent compound (Felgner et al., 1994). These authors suggested that the role of the hydroxyethyl group is to preserve bilayer integrity through the maintenance of membrane

hydration.

The ability of polyvalent headgroups to form more compact lipoplexes has been implicated in the observation that amphiphiles containing this motif frequently mediate higher degrees of gene delivery than their monovalent counterparts (Behr et al., 1989; Remy et al., 1994; Zhou et al., 1991). Lee and associates have observed that cationic lipids with multiple protonatable amines, when coupled to their respective lipid anchors in a T-shaped structure, yielded greater transfection activity than the linear construct (Lee et al., 1996). The authors suggested two roles for the T-shaped motif, first, it may promote better complexation with plasmid DNA and second, it might resemble a ligand for a particular cell-surface receptor, although no evidence was presented to support either of these conjectures.

Role of Helper Lipid

A typical cationic liposome formulation contains, in addition to the cationic lipid, a neutral helper lipid. The most common helper lipid employed *in vitro* is dioleoylphosphatidylethanolamine (DOPE) (Figure 1). This lipid is necessary for the establishment of a bilayered vesicle morphology with some cationic lipids (Gao and Huang, 1995). In addition, the fusogenic capacity of DOPE (Hui et al., 1981) enhances lipoplex-mediated transfection efficiency when compared to DOPC (Farhood et al., 1995; Wrobel and Collins, 1995) and several DOPE structural analogs (Zhou and Huang, 1994). It has been suggested that the augmentation of gene delivery is a reflection of increased endosomal DNA release into the cytoplasm as facilitated by DOPE-mediated endosomal membrane disruption. (Zhou and Huang, 1994; Farhood et al., 1995).

Cholesterol has proved to be a more effective helper lipid than DOPE for gene delivery in the context of systemic administration (Solodin et al., 1995; Song et al., 1997; Smyth-Templeton et al., 1997) and following intratracheal administration in mice (Meyer and coworkers, manuscript in preparation). The role of cholesterol *in vivo* is apparently a reflection of the increased stability of cholesterol-containing lipid bilayers in circulation (Senior and Gregoriadis, 1982) thus the lipoplex remains stable for a sufficient period of time to facilitate transfection.

Lipoplex Formation

The initial step in the formation of cationic lipid-DNA complexes is mediated by spontaneous electrostatic interactions between the positively-charged headgroup of the cationic lipid and the negatively-charged phosphate backbone of the nucleotide. The lipids serve to condense DNA into chemically and physically diverse structures (Gershon et al., 1993) described as multilamellar globules. The precise character of these aggregates depends both qualitatively and quantitatively on the lipid components of the liposome, on the method of lipid preparation including ionic strength of the buffers employed, the method of mixing, and on the elapsed time of incubation (Gershon et al., 1993; Sternberg et al., 1994; Yang and Huang, 1997).

Gershon and colleagues have demonstrated that as the lipid concentration increases relative to that of DNA, concomitant membrane fusion and DNA condensation rapidly occur (Gershon et al., 1993). These phenomena yield a complex that protects the DNA from DNase I digestion and ethidium bromide intercalation although, as Eastman and co-workers state, the latter may simply reflect compaction rather than encapsulation

(Eastman et al., 1997). Investigations using small angle x-ray scattering (SAXS) (Rädler et al., 1997; Lasic et al., 1997) have revealed that liposomes, upon mixing with DNA, undergo significant rearrangements resulting in the formation of multilamellar structures with a periodicity of 6.5nm. This measurement corresponds with a model of a hydrated DNA helix with a diameter of 2.5nm, intercalated between a cationic bilayer 4nm thick.

Analysis of lipoplex structure using electron microscopy, revealed amorphous aggregates with diameters in the sub-micron range (Gershon et al., 1993; Gustafsson et al., 1995; Sternberg et al., 1994). Gershon and colleagues, utilizing Kleinschmidt metal rotary-shadowing electron microscopy, observed that DOTMA-DOPE lipoplexes consisted of vesicles arranged along DNA strands at low lipid-to-DNA ratios, but as the amount of lipid was increased, the complexes assumed rod-like structures in which the DNA was presumably entirely encapsulated.

Sternberg and coworkers (Sternberg et al., 1994), through freeze-fracture electron microscopic imaging techniques, observed that both tube-like structures and globules formed upon mixing DC-cholesterol lipids with DNA and coined the term 'spaghetti and meatballs' to describe these moieties. Based on the diameter of the tubular structures (10nm), it was suggested that they consisted of single DNA strands ensheathed in cationic lipid membranes. These tubules were found both freely suspended and also associated with the 'meatball' components of the preparations.

Gustafsson and colleagues (Gustafsson et al., 1995) generated cryo-transmission electron micrographs of cationic detergents and lipids combined with DNA exhibiting oligolamellar structures which appeared to entrap DNA upon its addition to cationic liposomes.

In summary, cationic lipid-DNA complex formation is characterized by rapid charge neutralization of DNA upon addition of lipid accompanied by thorough lipid mixing. The lipids and DNA arrange themselves into multilamellar formations consisting of DNA aligned in strata between lipid bilayers with a periodicity of 6.5nm yielding the large globules that are apparent in electron micrographs. In some instances, the formation of tubular structures with a diameter of 10nm occurs (Gershon et al., 1993; Sternberg et al., 1994). These tubules appear to form when DOPE is included in the lipid formulation, and may be attributed to the ability of DOPE to accommodate structures of a high radius--of-curvature upon phase separation (Seddon, 1990). In support of this point, formulations prepared without DOPE tend not to yield lipoplexes with extensive tube-like structures (Eastman et al., 1997; Lasic et al., 1997; Xu et al., 1998). Although colloidal properties do not vary considerably between different lipid compositions, lipoplex aggregates tend to become larger and more polydisperse as the charge equivalency point is approached (Mahato et al., 1995; Rädler et al., 1997; Xu et al., 1998). The issue of lipoplex size is considered important in that the endocytotic process, whether receptor or fluid-phase-mediated, is limited to particles of approximately 200nm or less, although larger particles may be phagocytosed by some cell-types. This may be due to a co-operative endocytotic event that results in the internalization of large diameter lipoplexes that become associated, through electrostatic interactions, with multiple coated pits.

4. *In Vitro* Transfection

Viruses have developed complex strategies in order to overcome numerous

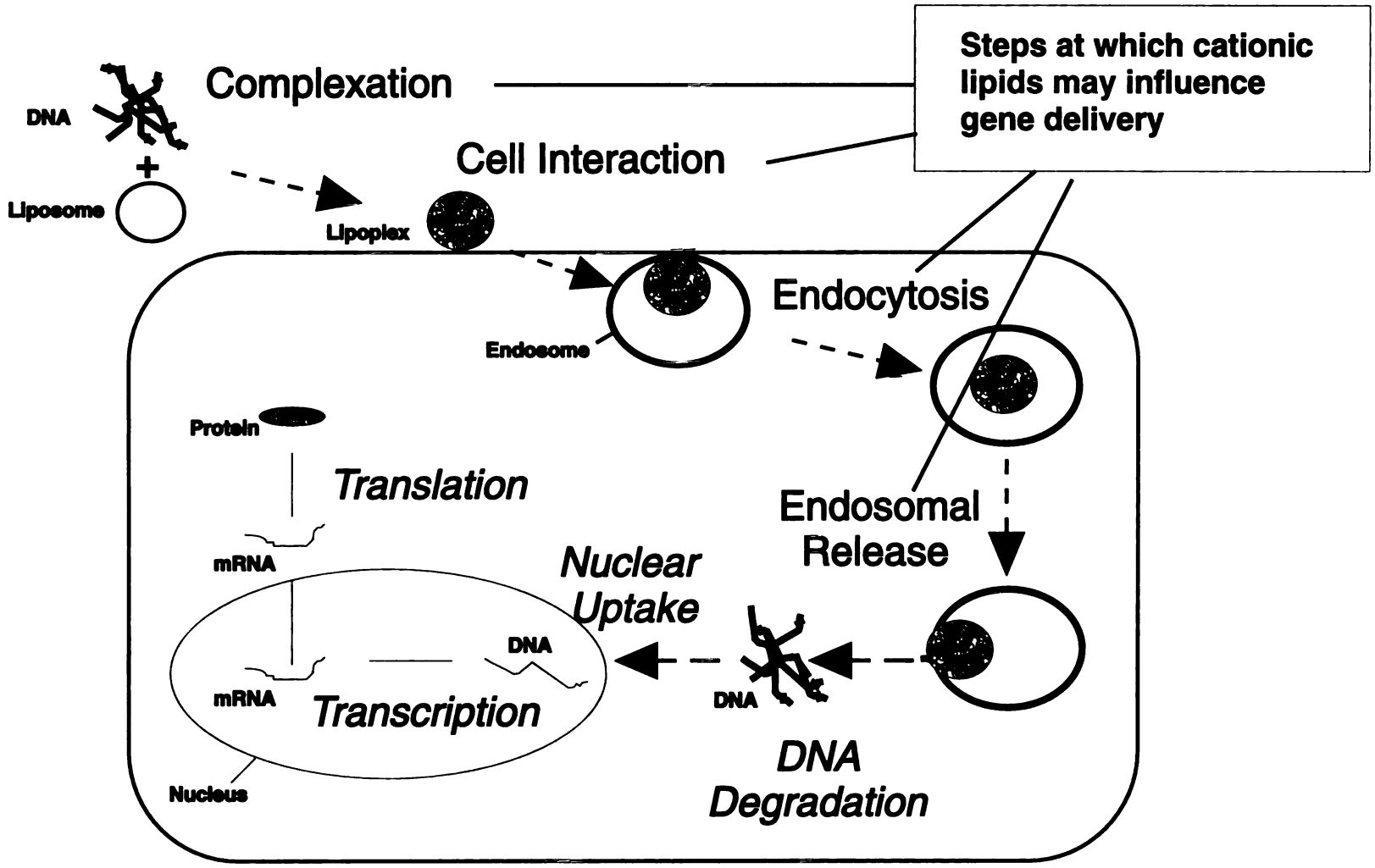
barriers to exogenous gene delivery and expression. These impediments include the initial interaction between the vector and the cell membrane, followed by internalization, endosomal release, transport into the nucleus and finally, transcription and translation of the therapeutic gene. It is reasonable to assume that the mediation of high transfection levels requires non-viral vectors to overcome the same series of barriers to transgene delivery and expression (Figure 2), therefore, multiple functions would be needed to overcome these barriers. Currently employed cationic lipoplexes add one, or at most two, components to the DNA so it is surprising that lipoplexes deliver genes as well as they do.

Cell Adhesion

The initial step for lipoplex-mediated transfection *in vitro* is thought to be the electrostatic interaction between the cationic lipid and the anionic plasma membrane (Felgner et al., 1987). As mentioned above, it is possible to manipulate the colloidal properties of the lipoplex by varying the ratio of cationic lipid to anionic nucleotide such that a charge excess of lipid is included in the lipoplex to neutralize the DNA. This yields a complex with a net positive charge, thus facilitating its interaction with the plasma membrane. The precise nature of this coupling is not clear; however, it may include binding of the lipoplex to negatively-charged membrane components such as sialylated glycoproteins or possibly through coupling with sulfated membrane-associated proteoglycans (Mislick and Baldeschwieler, 1996; Labat-Moleur et al., 1996).

Figure 2

In Vitro Gene Transfer Events



Route of Internalization

Following membrane association, the cellular uptake of cationic lipid-DNA complexes was originally thought to occur through fusion of the liposome and plasma membranes (Felgner et al., 1987; Felgner and Ringold, 1989). This mechanism was based on the observation that fluorescently–labeled DOPE distributed into the plasma and intracellular membranes of the target cells 4 hours after exposure to the lipoplex (Felgner et al., 1987). Moreover, it was demonstrated that cationic liposomes are capable of fusion with anionic liposomes (Felgner and Ringold, 1989; Düzgünes et al., 1989) although fusion activity was attenuated by pre-incubation with DNA (Leventis and Silvius, 1990).

More recently, two lines of evidence suggest that the lipoplexes gain entry to the cytoplasm of target cells via endocytosis: 1) enhancement of transfection by lysosomotrophic agents such as chloroquine (Legendre and Szoka, 1992; Felgner et al., 1994) and (2) morphologic studies which visually monitor intracellular lipoplex trafficking (Zhou and Huang, 1994; Zabner et al., 1995;Friend et al., 1996). Legendre and Szoka (Legendre and Szoka, 1992) have demonstrated that lysosomotrophic agents enhanced DOTMA/DOPE mediated transfection in some cell types. Similarly, Felgner and colleagues (Felgner et al., 1994), have shown that chloroquine influenced transfection mediated by both DORIE/DOPE and DMRIE/DOPE-based lipoplexes. The buffering capacity of chloroquine alters the trafficking of cationic lipid-based systems by preventing endosomal/lysosomal fusion, thus, the influence of this molecule on lipoplex-mediated transfection suggests the involvement of an endocytic uptake process.

Electron microscopy (EM) studies revealed electron-dense cationic lipopolylysine-containing liposomes in the endosomes of treated cells within 1 hour of

administration (Zhou and Huang, 1994). Similar *in vitro* studies by Zabner and colleagues (Zabner et al., 1995), showed the exclusive localization of gold-labeled DNA complexed with DMRIE/DOPE liposomes in cytoplasmic vesicles or endosomes. Friend and co-workers (Friend et al., 1996) found that the gold-labeled lipid component of the lipoplex associated with the clathrin coat assembly of the plasma membrane upon *in vitro* administration. This interaction was followed by the observation of the lipid labels in the endosomes. Unlike the gold-labeled DNA experiments mentioned above, these investigators noted extra-vesicular lipid label present in the cytoplasm. This phenomenon was attributed to the release of endocytosed material rather than the manifestation of a plasma membrane fusion event.

Although these data do not exclude the possibility that some lipoplex uptake occurs via membrane fusion, they suggest that the principal mode of cell uptake involves endocytosis.

Endosomal Release Mechanism

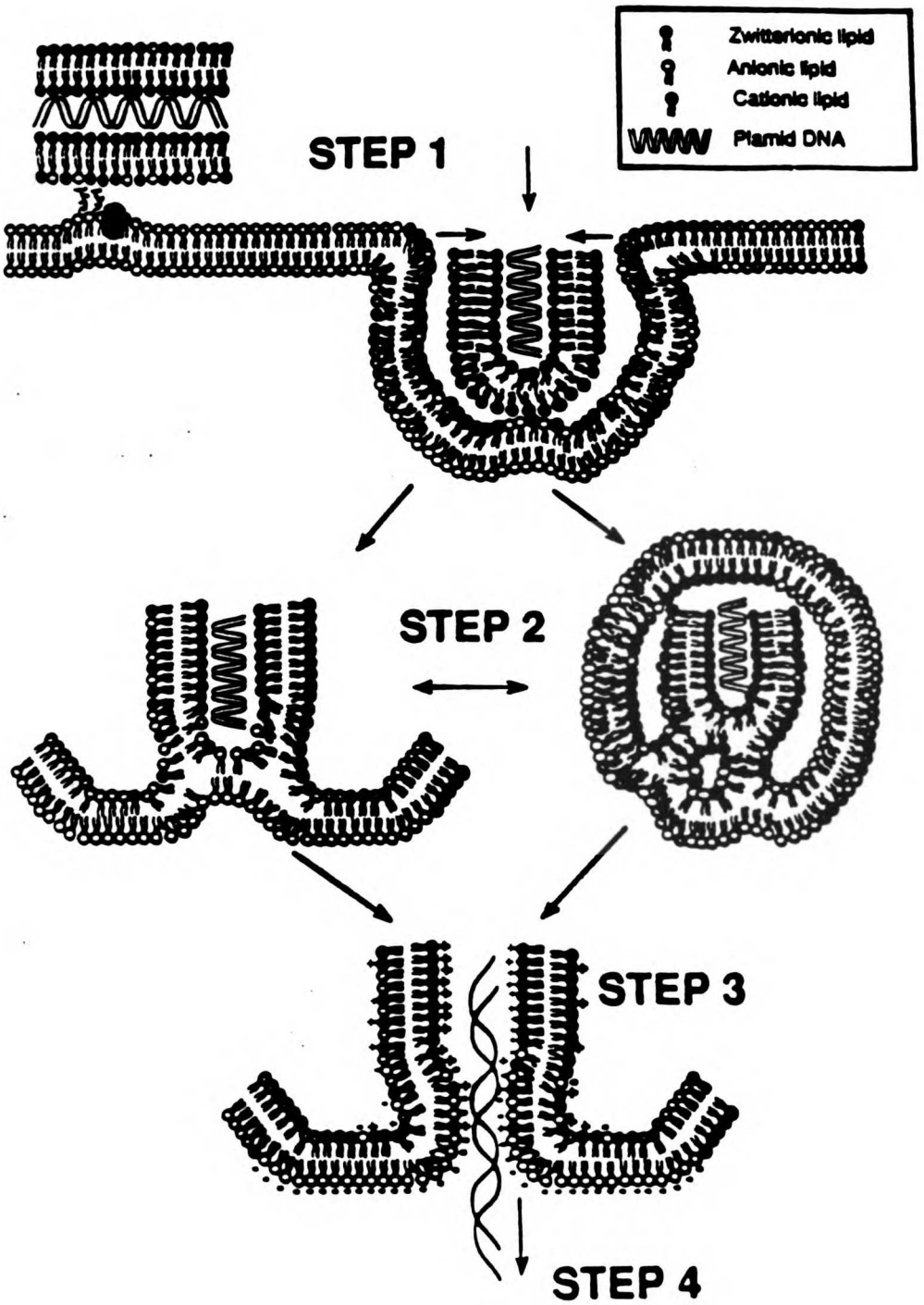
Zabner and colleagues have postulated that one of the salient factors limiting *in vitro* non-viral gene delivery is the release of free DNA from the endosome (Zabner et al., 1995). This conclusion was based on light microscopic visualization of fluorescently-labeled DNA and lipids, electron microscopic observation of gold-labeled DNA and quantitative analysis of cellular DNA uptake via dot blots (Zabner et al., 1995). Although DNA release from the endosome appears to be an inefficient process, transgene expression is realized upon administration of lipoplexes to cells demonstrating that a small amount of DNA gains access to the nucleus where it can be transcribed. Moreover, the DNA

apparently enters the nucleus as a free molecule since nuclear or cytoplasmic microinjection of cationic lipid-DNA complexes does not express the encoded protein (Zabner et al., 1995; Pollard et al., 1998). In addition, the fact that T7 promoter-containing plasmid DNA is accessible to cytoplasmic T7 polymerase upon lipoplex delivery suggests that DNA is cytoplasmically released (Gao and Huang, 1993). Fluorescent confocal imaging of cultured cells exposed to cationic lipid-oligodeoxynucleotide complexes shows cytoplasmic dissociation of lipid and ODN with subsequent nuclear localization of the ODN while the lipid component of the complex remained in the cytoplasm (Zelphati and Szoka, 1996).

A model has been presented that explicates endosome release of DNA (Xu and Szoka, 1996; Zelphati and Szoka, 1997) and is consistent with the above observations: The paradigm describes the uptake of lipoplex by endocytosis followed by destabilization of the endosomal membrane (Figure 3). Destabilization results in the flip-flop of anionic lipids, located principally in the cytoplasmic monolayer, and their subsequent lateral diffusion into the complex where they form ion pairs with cationic lipids. Charge neutralization of the cationic lipids results in the release of DNA into the cytoplasm. Based on this mechanism, the cytoplasmic release of DNA is clearly dependent on the presence of sufficient numbers of anionic lipids to displace the DNA molecule. An approximation of the number of anionic lipids present on an endosomal vesicle monolayer of 150nm diameter was made based on the assumption that the monolayer has a surface area of 1.76×10^4 nm^2. In addition, it was assumed that the monolayer consists of 70% phospholipids of which 25% are negatively-charged and that each lipid has a surface area of $0.7nm^2$. Given these assumptions, the total number of anionic lipids

Figure 3

Mechanism of Release of Plasmid DNA from the Lipoplex



available for the plasmid DNA molecule to interact with is approximately 4.4 x 10^3. The complete dissociation of a 4 x10^3 base-pair plasmid DNA molecule would require circa 8,000 anionic residues, hence there may be an insufficient number of anionic lipids in the vesicle membrane of small endosomes to entirely release the plasmid DNA. This phenomenon may explain the lack of efficient cytoplasmic plasmid DNA release. However, the delivery of oligonucleotides appears to be more efficient (Zelphati and Szoka, 1996). The model can account for oligonucleotide release because the above-mentioned 4.4 x 10^3 interactions between anionic and cationic lipids would lead to the complete release of 220 20-mer oligonucleotides. This model has been described and rationalized in detail (Szoka et al., 1996; Zelphati and Szoka, 1997).

Nuclear Uptake

A second potentially rate-limiting step in lipoplex-mediated transfection is at the point of plasmid nuclear uptake (Zabner et al., 1995). As mentioned above, the cytoplasmic dissociation of the DNA from the cationic lipid appears to occur and, moreover, is necessary for transgene expression since nuclear injection of lipoplex results in little or no transfection (Zabner et al., 1995; Pollard et al., 1998). A detailed account of cytoplasmic trafficking and nuclear transport of nucleic acids has been compiled by Meyer and colleagues (Meyer et al., 1997).

Cell-Type Dependence of Transfection

In general, it has proved difficult to establish a firm correlation between cell-type and transfectability, indeed, Boussif and colleagues have found luciferase activity from

various transfected-cell extracts to span 5 orders-of-magnitude (Boussif et al., 1996). Commercial cationic-lipid transfection reagents have been used to transfect numerous different cell-lines but the literature from such companies is difficult to interpret in terms of the mechanism of action for a given cationic lipid. It does appear, however, that adherent cells are more amenable to lipoplex transfection than cells in suspension (Labat-Moleur et al., 1996; Boussif et al., 1996), but recently, Floch and colleagues have shown K562 cells, a non-adherent cell type, to be as transfectable as adherent cells (Floch et al., 1998).

Transfection data associated with a given synthetic delivery system *in vitro* is not generally predictive of data generated *in vivo*. Efforts to improve this correlation have involved the addition of serum in the cell culture medium to more closely resemble the *in vivo* gene transfer context. Although some cationic lipid-DNA complexes demonstrate limited resistance to the deleterious effects of serum (Hofland et al., 1996; Yang and Huang, 1998) the addition of serum to culture medium typically results in the reduction of transfection *in vitro* (Felgner et al., 1987). The decrease in transfection has been attributed to a reduction in cell-association due to charge neutralization of the complex (Yang and Huang, 1997) or through compromising the stability of the complex (Zelphati et al., 1998). The effects of blood components will be discussed in further detail below.

Dependence of Transfection on Cell-Division

There continues to be controversy concerning the reliance on cell-division for efficient lipoplex-mediated transfection. The dissolution of the nuclear membrane during mitosis may permit plasmid DNA access to nuclear transcriptional machinery, indeed,

Fasbender and colleagues have shown that actively-dividing airway epithelial cells are more readily transfected by lipoplex than cells which were not mitotically-active (Fasbender et al., 1997). Conversely, successful transfection by naked DNA of post-mitotic myotubes (Dowty et al., 1995) and neurons (Demeneix et al., 1990) has been documented indicating that cell division is not an absolute necessity for transfection. It is conceivable that the degree of dependence for transfection on the target cell's mitotic state may be governed by target cell-type. It is also possible that access to the nucleus is possible in the absence of cell-division but uptake is enhanced during mitosis.

In Vitro Summary

A net positive lipoplex surface-charge promotes significant lipoplex-plasma membrane binding through electrostatic interactions (Felgner et al., 1994; Gao and Huang, 1991; Zabner et al., 1995). Lipoplex-target cell association is a time-dependent phenomenon which appears to plateau between 6-12 hours post-administration (Felgner et al., 1987; Legendre and Szoka, 1992; Zabner et al., 1995). The amount of DNA associated with cells following lipoplex administration varies. Legendre and Szoka found a maximum of 4.5% of the administered dose to be taken up by cells after a vigorous washing procedure (Legendre and Szoka, 1992) whereas Zabner and colleagues and Labat –Moleur and colleagues found the same parameter to be 60% and 33%, respectively (Zabner et al., 1995; Labat-Moleur et al., 1996). The number of plasmid molecules internalized per cell was estimated to be 19,000 in the case of Legendre and Szoka's calculations and up to approximately $3x10^5$ as per the Zabner group's assessment. The variation in intracellular lipoplex-mediated delivery of plasmid DNA

may be a reflection of the different cell types or washing procedures used in these investigations. Based on these data, cell uptake may not be a significant rate-limiting step in *in vitro* gene delivery but rather downstream barriers such as endosomal escape and nuclear uptake appear to limit expression. Capecchi and colleagues have shown that, upon microinjection of plasmid DNA into the cytoplasm, less than 0.01% of injected cells express the encoded protein, whereas nuclear microinjection resulted in 50-100% of injected cells expressing the injected DNA (Capecchi, 1980). These observations were subsequently confirmed by Zabner and colleagues (Zabner et al., 1995). Pollard and coworkers, who also employed microinjection techniques, estimated that the efficiency of plasmid transit into the nucleus was 0.1% and that cationic lipoplexes failed to reach the nucleus in detectable amounts. (Pollard et al., 1998). It is clear that even if free plasmid DNA is released from the endosome (Xu and Szoka, 1996), major impediments to transgene expression exist. Dowty and colleagues have demonstrated that plasmid DNA, when microinjected near the nucleus is expressed more efficiently than more distally administered DNA (Dowty et al., 1995). This implies that the DNA is sequestered in the cytoplasm, perhaps by binding to cytoplasmic structures or by sieving through a cytoskeletal network. It is also possible that, prior to reaching the nuclear pore complex, DNase activity in the cytoplasm degrades free cytoplasmic DNA.

If gene delivery efficiency is expressed on a per-genome basis, viral vectors are much more effective than non-viral systems. Adenoviruses, for instance have been shown to be capable of quantitative transfection of cultured cells with as little as 10 MOI/cell (Lemarchand et al., 1992; Zabner et al., 1994). However, as mentioned above, when several thousand plasmids/cell are delivered via lipoplex *in vitro*, cells yield much lower

gene expression.

5. *In Vivo* Transfection

Cationic lipid-DNA complexes have been administered via many routes *in vivo*. Gene delivery occurs following delivery to the airways, by systemic injection and upon intraarterial administration via balloon catheterization (Table 1). Successful delivery of a lipoplex in an intact animal involves interactions with anatomical and physiological components located in the vasculature and extracellular space. These interactions with the biophase can alter lipoplex properties and/or prevent access to target cells. These phenomena will be discussed in more detail below.

Pulmonary Delivery

Gene delivery to the lung has applications in the treatment of genetic diseases such as cystic fibrosis and alpha-1-antitrypsin deficiency as well as acquired lung diseases where the expression of certain cytokines, surfactant proteins, antioxidants or mucoproteins have potential therapeutic value. The principal objective of these therapeutic strategies is transfection of the pulmonary epithelial cell, which can be targeted most directly via intratracheal (IT) instillation of lipoplex in solution (Brigham et al., 1989) or when administered as an aerosol (Stribling et al., 1992; Canonico et al., 1994). These approaches have the added benefit of limiting systemic exposure. The feasibility of direct administration to the airways has been demonstrated by the pioneering work of Brigham (Brigham et al., 1989) and the initial experiments have been reviewed by Gao and Huang (Gao and Huang, 1995). More recently, Meyer and

colleagues (Meyer et al., 1995) showed that the complexation of plasmid DNA with DOTMA liposomes increased the amount of large molecular weight DNA present in the mouse lung following intratracheal injection, but DNA expression was not enhanced relative to DNA alone. The fact that naked DNA transfects as effectively as lipoplex-delivered DNA in the rodent lung is also observed for lipoplexes made from DOTAP and dimethyldioctadecylammonium bromide (DDAB) (Tsan et al., 1995). Lipoplexes have been applied to the nasal epithelium of cystic fibrosis patients in several clinical studies (Caplen et al., 1995; Gill et al., 1997; Porteous et al., 1997; Zabner et al., 1997). Only partial, transient correction of gene function was realized. Interestingly, GL-67:DOPE-based lipoplexes which proved 1000-fold more effective than naked DNA in transfecting murine lung tissue (Lee et al., 1996) were no more effective than naked DNA when applied to human nasal epithelium (Zabner et al., 1997), emphasizing the complexity of extrapolating from animal experiments to clinical studies. Meyer and coworkers have shown that instillation of DOTAP/Cholesterol (CHOL)-based lipoplex mediated higher gene expression in the lung than when DOPE was included as a helper lipid or when DNA was administered alone (Meyer et al., 1998). Cholesterol as a helper lipid might function to increase the stability of the complex in the lung thus facilitating expression.

Freimark and colleagues reported an increase in cytokines and an influx of macrophages and neutrophils in the lung following the instillation of DOTMA/CHOL-based lipoplex into the airways (Freimark et al., 1998). Generally, the immune responses were more profound for the lipoplex than for the lipid or DNA components administered separately. The authors attributed this to three possibilities, first, the individual components may elicit the release of co-stimulatory cytokines, second, augmented

induction of the immune response may be due to a lipid-facilitated increase in cell-uptake of DNA, or finally, the less rapid degradation of pulmonary-delivered DNA when complexed to cationic lipid (Meyer et al., 1995), may increase its persistence in the airways, thereby enhancing DNA's ability to stimulate the immune system.

Aerosol delivery of lipoplex to the lung provides a less invasive alternative to nasal or intratracheal instillation. A limitation of this approach has been the DNA shearing that transpires upon nebulization (Schwarz et al., 1996). In subsequent studies, Eastman and colleagues optimized a liposome preparation consisting of lipid GL-67 combined with dimyristoylphosphatidylethanolamine-polyethylene glycol (DMPE-PEG_{5000}) that prevented nebulization-based shearing of complexed DNA (Eastman et al., 1997; Eastman et al., 1997). Upon administration, this preparation yielded gene expression comparable to that of instilled lipoplex while invoking little or no inflammatory response (Eastman et al., 1997). Similarly, McDonald and colleagues, utilizing aerosolized lipoplex, have demonstrated delivery and expression of the CFTR gene with EDMPC/CHOL -based lipids to the apical epithelium of Rhesus monkeys without visible inflammation (McDonald et al., 1998). Chadwick and coworkers demonstrated the safety of aerosol delivery of lipid GL-67A to the lungs of healthy human volunteers (Chadwick et al., 1997). The lack of toxicity inherent in aerosolized preparations as compared to instilled lipoplex is presumably a reflection of more efficient distribution of the former, leading to less localized accumulation of lipoplex; consequently, aerosolization may provide a means of increasing gene expression in the lung through multiple dosing regimes.

Intravenous Delivery

The first example of successful intravenous (IV) lipoplex delivery was achieved by the Brigham group in 1989 (Brigham et al., 1989). Since that time, a variety of lipoplex formulations have been employed to effect systemic gene delivery (Table 1 and Gao and Huang, 1995). The fate of intravenously (IV) injected liposomes alone has been reviewed extensively (Patel, 1992; Semple and Chonn, 1996; Senior, 1987). Liposomes are cleared rapidly from circulation by the reticuloendothelial system (RES) unless they have a diameter of less than 100nm and a neutral surface charge or have a surface polymer to sterically stabilize them (Woodle et al., 1992). Virtually all lipoplex preparations used in systemic gene delivery are formulated with excess positive charge that ensures that they will be rapidly eliminated from circulation. The utility of this composition when administered IV, may be in its ability to facilitate beneficial interactions with negatively-charged cell surfaces and proteins. Alternatively, the excess positive charge may aid in DNA protection or, as Yang and Huang have suggested, could neutralize negatively-charged serum components that are deleterious to transfection (Yang and Huang, 1997). When formulated with excess positive charge cationic lipid-DNA complexes are eliminated from circulation, within 5 minutes of injection. Initially, as much as 85% of the lipid/DNA dose administered may collect in the lung (Barron et al., 1998) as quantitated by radiolabeling, and a qualitative evaluation of lipoplex distribution by confocal fluorescent microscopy demonstrated lipoplex delivery is principally limited to the pulmonary vasculature (McLean et al., 1997).

Interestingly, based on radiolabeled systemic distribution experiments, the ratio of lipid-to-DNA in the lung decreases versus that of the initially injected preparation

(Barron et al., 1998). This suggests that a portion of the excess lipid, is not entrapped in the lung as a part of the lipoplex and continues to circulate beyond the pulmonary microvasculature. The lipid-to-DNA ratio in the liver gradually increases which implies that the excess lipid accumulates in this organ.

Barron and coworkers demonstrated significant uptake of both lipid and DNA in the liver but, up to 24 hours post-injection, the percent of administered dose in the liver remained less than that of the lung (Barron et al., 1998). Similarly, other groups, have found the majority of the DNA localizing in the lung at early time points with the majority of the remaining label accumulating in the liver (Liu et al., 1997). McLean and colleagues, utilizing fluorescent imaging of the cationic lipid component of the lipoplex, found that relative to tissue mass, the lungs, lymph nodes, Peyer's patch and ovaries accumulate the greatest amount of lipid (McLean et al., 1997). The fluorescently-labeled lipid generally was confined to endothelial cells, macrophages and intravascular leukocytes. One exception to this circumstance was the observation of diffuse fluorescence in the spleen, probably attributable to extravasation of lipoplex through the discontinuous endothelium in that organ (Poste et al., 1982).

Transgene expression following IV lipoplex injection tends to reflect that of its distribution with the majority occurring in the lung (Barron et al., 1998; Liu et al., 1997; Liu et al., 1997). Also in accordance with distribution data, transgene expression was found primarily in endothelial cells and monocytes (Liu et al., 1997; Liu et al., 1997; Song et al., 1997). Some expression was noted in interstitial macrophages (Liu et al., 1997).

Increasing pulmonary retention time for plasmid DNA correlates with higher

reporter gene expression in the lung (Liu et al., 1997; Liu et al., 1997; Smyth-Templeton et al., 1997). The inclusion of cholesterol as opposed to DOPE in the liposome formulation tends to increase lung expression following IV injection (Solodin et al., 1995; Song et al., 1997; Smyth-Templeton et al., 1997) probably through increasing pulmonary retention (Smyth-Templeton et al., 1997). This phenomenon is apparently a reflection of the increased stability of cholesterol-containing lipid bilayers in circulation (Senior and Gregoriadis, 1982).

The manner in which liposomes are prepared also affects transfection efficiency. Song and coworkers (1997) found that DOTAP-based lipoplex transfection was dependent on the size of the initial liposome and ensuing lipoplex formed. As liposome size was increased so did that of the resulting lipoplex. Moreover, as lipoplex diameter increased beyond approximately 450nm, systemic transfection increased (Song et al., 1997). We have noted a similar effect in that lipoplex of circa 550nm diameter formed from extruded liposomes yielded 10-fold higher pulmonary transfection than lipoplex formed from sonicated liposomes that had a diameter of approximately 250nm (Barron and Szoka unpublished). Interestingly, Smyth-Templeton and co-workers have demonstrated that serial extrusion of liposomes through membrane filters of decreasing pore-size (down to 0.1μm) yielded lipoplex with transfection activity twice that of unextruded liposomes of the same composition. This protocol appears to yield a liposome of a unique vase-like structure which may explain its enhanced activity (Smyth-Templeton et al., 1997); however, levels of gene expression reported in this paper are not higher than expression levels observed with a traditional lipoplex preparation (Barron et al., 1998).

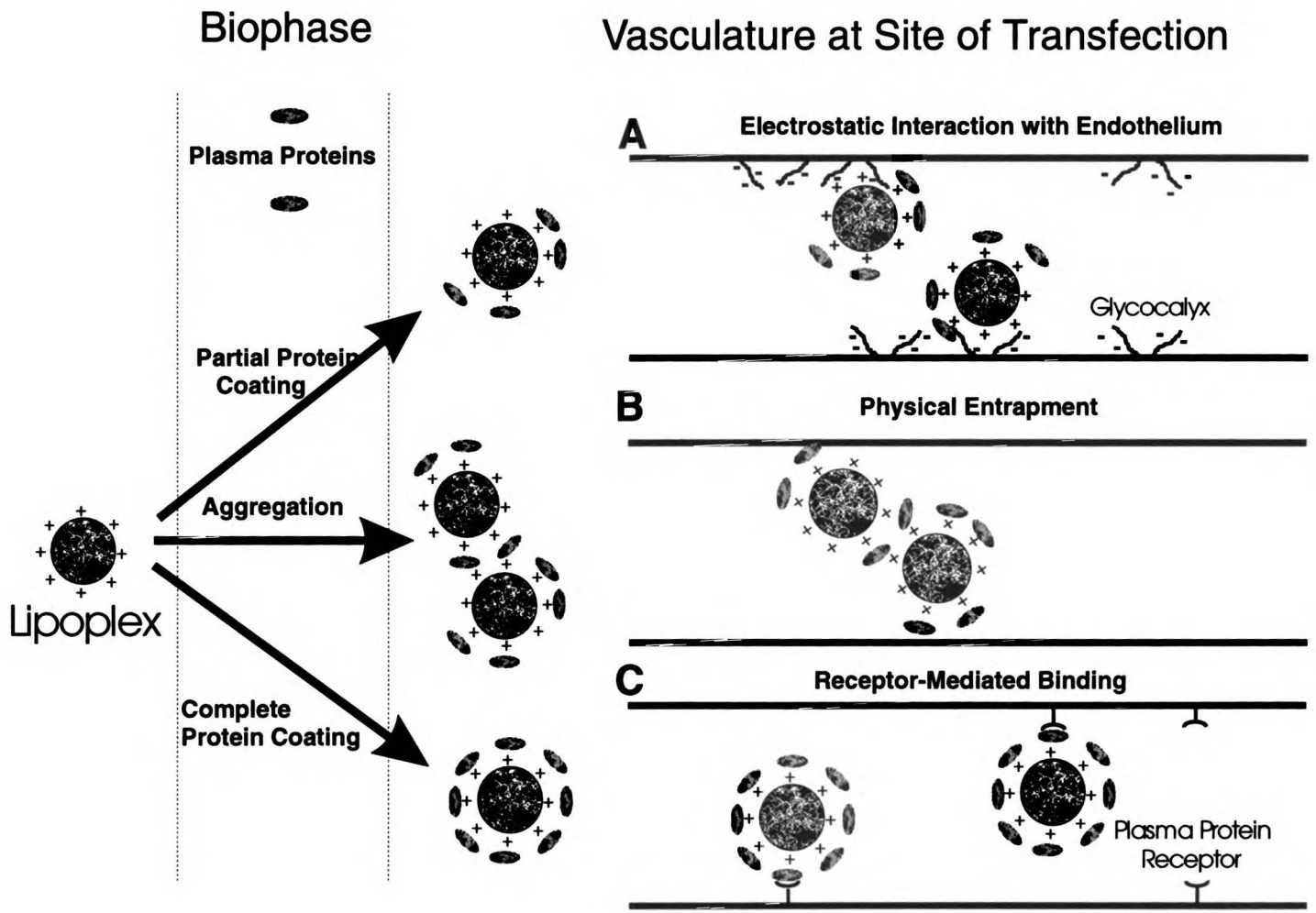
Although, as previously mentioned, a significant amount of the administered dose of lipoplex is delivered to the liver, transgene expression in this organ is generally low. This is likely a reflection of the fact that DNA is primarily internalized by non-parenchymal cells in the liver (Mahato et al., 1995; McLean et al., 1997). These cells include phagocytes such as Kupffer cells that likely degrade most of the DNA before it can be expressed.

The higher ratio of gene expression to DNA uptake in the lung versus that in the liver has been suggested to be due to the presence of transactivating factors in the lung which tend to promote transcription while such constituents are absent in the liver (Liu et al., 1997). Although most systemic studies employ CMV–based DNA constructs, the same expression pattern was observed with RSV-driven plasmid constructs (Thierry et al., 1997) suggesting that if the variance in expression is explained by a molecular mechanism, it is not limited to a single promoter type.

The preferential distribution of systemically administered lipoplex to the lung and subsequent transgene expression therein has been explained as a first-pass phenomenon since, upon tail-vein injection, this is the first capillary bed encountered by the complex (Brigham et al., 1989). This may happen by three mechanisms depicted in figure 4. Cationic lipid-DNA complexes are inherently reactive with negatively-charged blood proteins (Senior et al., 1991). If the lipoplex is not completely coated by proteins, some positive residues will be exposed. Upon encountering the surface of the small-caliber blood vessels of the pulmonary microvasculature, these positive domains may interact with the negatively charged residues present on the pulmonary endothelium (Mislick and Baldeschwieler, 1996; Mounkes et al., 1998) (Figure 4A). The extent of uptake would

Figure 4

Endothelial Lipoplex Binding



then be a function of the large surface area of the pulmonary capillary bed. Alternatively, we have speculated that the localization and expression profile of lipoplexes may also represent plasma protein-lipoplex interactions in which associated proteins act to influence lipoplex pharmacokinetics and subsequent cell uptake. (Barron et al., 1998). Although there is a paucity of studies addressing the interactions between cationic lipid-DNA complexes and blood proteins, Senior and colleagues have shown that upon incubating cationic lipids in serum, a dose-dependent increase in turbidity was observed, ultimately leading to the formation of clot-like masses (Senior et al., 1991). Further evidence of the inherent reactivity of positively-charged liposomes with negatively-charged plasma proteins was provided by Semple and Chonn who demonstrated that DOTMA-based liposomes accrue over 0.5g of plasma protein per millimole of lipid upon circulating *in vivo* for 5 minutes (Semple and Chonn, 1996). If the interaction between plasma proteins and the lipoplex results in cross-linking and resilient aggregate formation, these aggregates could be trapped in the pulmonary capillary beds thus passively targeting the lipoplex to the lung (Figure 4B); however, we would expect the formation of essentially lipoplex-protein emboli in the lung to yield some respiratory distress and morbidity upon administration and yet such reactions are not seen upon cationic lipoplex injection IV. In addition, McLean and coworkers did not find evidence of significant aggregate formation upon IV injection of lipoplexes and subsequent confocal microscopic analysis of the vasculature (McLean et al., 1997). Although we have not eliminated lipoplex-protein aggregation as contributing to transfection, we favor a model in which lipoplex-associated proteins may indirectly target the lipoplex through binding their cognate receptor on a particular endothelium such as that of the lung (Figure

4C). This latter phenomenon may also explain the relatively avid binding and efficient transgene expression on a per-gram-tissue protein basis, in the ovaries, lymph nodes and anterior pituitary (McLean et al., 1997). McLean and colleagues speculated that these lipoplex distribution and expression patterns may be a reflection of, as yet unelucidated, regional differences in the vascular endothelium. These variations may include the expression of specific receptors such as that for low-density lipoprotein in different regions of the endothelium (Nistor and Simionescu, 1986). Transgene distribution and expression profiles achieved using lipoplexes could reflect a combination of the above-mentioned mechanisms: Pulmonary gene delivery may be a manifestation of the first pass effect followed by receptor mediated uptake. In tissues 'downstream' from the lung, receptor-mediated uptake may be the dominant mechanism. There is support for the notion that surface modification of the lipoplex can lead to specific lipoplex targeting and transfection. Cheng associated transferrin with the lipoplex and showed increased transfection *in vitro* (Cheng, 1996). Smyth-Templeton and colleagues have shown that the electrostatic complexation of succinylated asialofetuin to lipoplexes partially redirects transfection from the lung to the liver although lung transfection remains significantly higher than liver transfection for the asialofetuin-modified complexes (Smyth-Templeton et al., 1997).

Lipoprotein-liposome interactions have been the subject of research since the early days of liposome technology (Senior, 1987). Although the interactions between cationic lipid-based complexes and lipoproteins has not been directly studied, observations published for liposomes may also apply to lipoplex. Generally, the most profound interactions involve phospholipid exchange with high-density lipoprotein

(HDL) culminating in the reduction of liposome integrity. Strategies to reduce HDL-mediated liposome damage involve the formulation of liposomes from cholesterol and lipids with relatively high transition temperature. The fact that the inclusion of cholesterol in liposomes acts to increase pulmonary vasculature retention and subsequent transgene expression may be attributable to the ability of cholesterol to stabilize vesicle structure against HDL-mediated damage (Smyth-Templeton et al., 1997). Given the potential for extensive lipoplex-lipoprotein interactions and the presence of lipoprotein receptors on the endothelium (Nistor and Simionescu, 1986) this topic warrants further study.

Upon airway delivery, reporter gene expression tends to peak at 2-3 days post-administration with residual activity detected up to 21 days post-administration. (Stribling et al., 1992; Meyer et al., 1995; Lee et al., 1996). The persistence of gene expression following I.V. administration tends to vary considerably. Zhu and colleagues (Zhu et al., 1993) were able to detect reporter gene activity for up to 21 weeks post-lipoplex injection while others have observed peak pulmonary expression within 10 hours of injection and then the activity declined to less than 1% of the peak value within 4 days (Liu et al., 1997; Song et al., 1997). As Song and collaborators suggest, the discrepancy in activity upon systemic injection may be based on the plasmid constructs and detection methods used (Song et al., 1997). This short duration of gene expression is a significant limitation of the lipoplex as a gene carrier.

In comparing optimal formulations for IT versus IV administration, we have found that pulmonary gene expression following IT administration is 10% of that realized upon systemic injection (Meyer, Barron and Szoka, unpublished results). The variation in

expression levels following IT and IV administration may be a reflection of their different distribution characteristics and the cell types to which the lipoplex is delivered. Upon histological staining for transgene product following administration by these two modalities we found expression to be the same at about 1% of the cells in a given field. However, gene expression from the IV dose was detected in all fields examined whereas expression following IT delivery was more regional with many fields showing no expression. As indicated by several groups, the principal cell-type transfected upon IT administration is the epithelial cell whether expression is detected in the upper airways (Canonico et al., 1994; McDonald et al., 1998) or in the alveolar regions (Gorman et al., 1997). Following IV injection, gene expression is found primarily in the endothelium but also in circulating monocytes and interstitial macrophages (Liu et al., 1997; Liu et al., 1997). Canonico and coworkers have detected human alpha-1-antitrypsin (hAT) gene expression in the airway epithelium upon IV injection of lipoplexes (Canonico et al., 1994). They explain that this observation may reflect the secretion of hAT from the endothelium and subsequent diffusion into the pulmonary epithelium. Lower gene expression following airway administration may also be explained by the mucin and surfactant-based milieu that covers the airways and alveoli, respectively. These media may limit intimate contact between lipoplexes and the epithelium. In support of this notion, it has been shown that *in vitro* transfection is decreased when lipoplex is exposed to natural and synthetic surfactants (Duncan et al., 1997; Tsan et al., 1997).

Intraarterial Delivery

Transgene expression in the arterial wall provides a strategy for the prevention

and treatment of cardiovascular disease such as restenosis following balloon angioplasty. Regional delivery of corrective agents may facilitate the exposure of intimal lesions to concentrations of the agent that are not tolerated systemically; moreover, localized gene expression could generate therapeutic levels of a protein at the site (Nabel et al., 1990). Initial gene delivery efforts involving the site-specific delivery of negatively-charged lipoplex containing DOTMA/DOPE by Nabel and collaborators have demonstrated transgene activity in the intima, media and adventitia of the iliofemoral arteries of pigs (Nabel et al., 1990). Administration of lipoplex was carried out via double balloon-catheter. Ectopic gene delivery was not detected upon PCR analysis of untreated tissues. Subsequent studies in dogs have established that lipoplex-mediated-transgene expression can be achieved in the coronary arteries through balloon-catheter administration (Chapman et al., 1992). Interestingly, naked DNA yielded similar expression to that of the negatively-charged DOTMA/DOPE-based lipoplex (Chapman et al., 1992). The authors explain this phenomenon may be a manifestation of the elevated pressure (4 atm) at which the intrarterial (IA) infusion is executed. The high pressure might damage the tissue and make transfection by naked DNA more probable. More recently, Stephan and colleagues found that by formulating lipoplex from GAP-DLRIE/DOPE and DNA at a positive molar charge ratio, as opposed to the negatively–charged preparations used in the above two studies, reporter gene expression in porcine arteries was approximately 15-fold higher than that of naked DNA (Stephan et al., 1996). Transgene delivery mediated by direct arterial administration may be compared to IV injection as both modalities appear to transfect the endothelium, but important distinctions exist between the two methods. IA administration via balloon catheter typically involves the perfusion of the

target arterial segment with buffer prior to infusion of DNA or lipoplex. This effectively renders the target-site serum-free, thus reducing the potential for lipoplex-protein interactions. In addition, infusion is carried out under pressures of 1.5 –4 atmospheres which may influence transfection by altering endothelial permeability.

Intraperitoneal Delivery

The intraperitoneal injection of negatively-charged lipoplex made from DDAB/DOPE and DOTMA/DOPE lipids into mice (Philip et al., 1993) has been shown to yield significant expression in the spleen and lymph nodes. Gene delivery to these tissues is likely a result of lymphatic drainage of the lipoplex from the peritoneum. Using DOTAP as the cationic lipid component of the lipoplex tended to decrease lymphoid tissue transfection while increasing that of the liver. A subsequent study has shown a broader distribution of gene expression in the spleen, liver, lung, heart, kidney and muscle upon IP injection of DOTMA/DOPE-based lipoplex formulated with a positive charge ratio (Yokoyama et al., 1996). It is possible that the difference in lipoplex charge ratio influenced the disparity in transfection observed between these two studies.

Intratumoral Delivery

The first clinical trial employing cationic lipid-mediated gene delivery was conducted by Nabel and coworkers in 1993. Since then, other groups have initiated clinical trials against solid tumors (Gao and Huang, 1995). Intratumoral injection provides a means of maximizing lipoplex concentration at the tumor site while minimizing systemic exposure. This should increase gene delivery while minimizing

potential toxic effects elsewhere in the body. Egilmez and colleagues have injected DOTMA/DOPE and DC-Chol-based lipoplex into tumor xenografts established in SCID mice (Egilmez et al., 1996; Egilmez et al., 1996). Transfection efficiency, as measured by intratumoral β-galactosidase activity was low (< 0.3%); however, when plasmid DNA encoding IL-2 was administered there was significant tumor regression, and in some cases, complete ablation of the tumor occurred (Egilmez et al., 1996; Egilmez et al., 1996). These investigators observed partial tumor regression upon injection of lipoplex which did not contain a therapeutic gene. This phenomenon was attributed to lipid toxicity that was dependent on the complexation of DNA as lipid alone had no effect. Contrary to the above-mentioned findings, Yang and Huang found that intratumoral injection of lipoplex tended to yield lower expression than that of naked plasmid DNA injection (Yang and Huang, 1996). This effect may be tumor model-dependent as Egilmez and coworkers employed a lung squamous cell carcinoma model (2E9 cells) while Yang and Huang used a BL6 melanoma-based tumor. Sugaya and collaborators have shown tumor regression upon direct injection of lipoplex formed from DOSPA/DOPE liposomes and plasmid encoding thymidine kinase followed by acyclovir administration (Sugaya et al., 1996). Although this system resulted in only 1-2% transfection efficiencies, the considerable reduction in COLO320 DM and A-431 cell-based tumor burden was ascribed to the bystander effect characteristic of this enzyme/prodrug-based therapy. However, they did not consider the possibility that other endogenous genes such as those expressing cytokines might have been activated by the lipoplex and whose products might contribute to an anti-tumor effect such as has been demonstrated in the lung (Freimark et al., 1998). Nomura and coworkers found that

naked plasmid DNA mediated gene expression that was as high as that of DC-Chol-based lipoplex in a Walker 256 carcinosarcoma model, further emphasizing the necessity of naked DNA controls in evaluating transfection efficiency of different delivery vehicles (Nomura et al., 1997). Despite similar transfection activity, pharmacokinetic analysis of injected DNA revealed that when injected with cationic lipids, up to 90% of the injected dose of DNA was retained in the tumor after 2 hours, while in the absence of lipid, 51% of the DNA dose administered remained in the tumor (Nomura et al., 1997). The inability of cationic lipids to improve the efficiency of DNA expression was ascribed to the fact that the large size and profound positive charge of the lipoplex limits its capacity to distribute within the tumor (Nomura et al., 1998).

Intramuscular Delivery

The direct injection of naked plasmid DNA is an effective means of expressing transgene in muscle tissue (Wolff et al., 1990); surprisingly, complexation of plasmid DNA with cationic lipids decreased *in vivo* gene expression despite efficient *in vitro* transfection with similar lipoplex formulations. Intramuscular distribution studies indicate that cationic lipid-DNA complexes do not efficiently cross the external lamina and, therefore access to the target myofiber cells is limited whereas naked DNA is capable of passing through this barrier (Wolff et al., 1992), thus restricting lipoplex distribution and yielding limited gene expression in the muscle. This restricted distribution phenomenon is similar to that observed after intratumoral administration of lipoplexes.

Toxicity

The most common routes of administration for cationic lipids- IV and IT are not associated with profound toxic effects when the lipoplex is administered in moderate doses. Serum chemistry or cardiac activity were not adversely affected by IV injection of lipoplexes; moreover, a histological examination of the organs showed no abnormalities (Stewart et al., 1992). The doses tested in these studies were quite low. San and colleagues could detect no plasmid DNA in germ cells, at the level of PCR sensitivity, subsequent to IV injection of up to 50 μg of plasmid DNA complexed with 150 nmol of DMRIE/DOPE lipid (San et al., 1993). One cautionary note is the observation of complement activation *in vitro* and *in vivo* in rodent models which raises the potential for lipoplex toxicity due to complement activation in human subjects (Devine et al., 1994; Plank et al., 1996; Barron et al., 1998).

Canonico and colleagues have demonstrated that repeated IV or aerosol administrations of up to 500μg of plasmid DNA complexed with 2.5mg of DOTMA/DOPE lipids is possible in rabbits without eliciting inflammation in the lung. In addition, lung histology and function were unaffected (Canonico et al., 1994). Conversely, Scheule and collaborators have shown significant short-term toxicity in the lung following nasal instillation of up to 300nmol of lipid complexed with 400nmol of plasmid DNA (Scheule et al., 1997). This treatment resulted in multifocal lesions forming in the lung accompanied by inflammation and infiltrates of neutrophils, macrophages and leukocytes. This study underscores differences in toxicity in the same organ when lipoplexes are administered by two different techniques. It also points out the promise of aerosolization of lipoplexes, as it yields lower toxicity than instillation possibly due to a

more uniform pulmonary distribution (Eastman et al., 1997). Healthy human volunteers have had nebulized cationic liposomes delivered to their lungs in a dose proportional to that given to rodents (Chadwick et al., 1997). No untoward clinical effects were observed in the human patients. It should be emphasized, that the agent administered was the lipid component GL-67/DOPE/DMPE-PEG_{5000} only and not the lipoplex. The DMPE-PEG-based lipid possesses a polymer coat around the liposome that acts as a steric barrier between the cationic charge on the lipoplex and the cell membrane.

Song and colleagues (Song et al., 1997) have demonstrated that IV injection of lipoplexes can yield acute toxicity when doses in excess of 50-60 μg of DNA are complexed with lipid at high positive charge ratios. The effect of IV-administered lipoplexes may be more pronounced in larger animals since we have observed that the maximum tolerated dose of lipoplexes does not scale with body weight (unpublished data). Doses that are well-tolerated when administered in mice become lethal in rats when the dose is proportionally increased to body weight. The mortality can be very rapid, implying that the lipoplex may be aggregating upon interaction with serum proteins and occluding small-caliber pulmonary capillaries. This serves as a cautionary note for the IV administration of lipoplexes in humans and points out the need for additional toxicological evaluations in additional species prior to systemic use in humans.

The origin of toxicity is not understood, but cationic lipid-mediated toxicity may occur at the cell-signaling level. Bottega and Epand showed that cationic lipids consisting of quaternary amines and sterol hydrophobic groups (as opposed to tertiary amines conjugated to bilayer-stabilizing straight alkyl groups) are relatively potent inhibitors of protein kinase C activity (Bottega and Epand, 1992). The downstream effects of

influencing cell-signaling may be profound, and warrant additional studies.

There may be a correlation between transfection and toxicity; mechanisms that lead to cell transfection may, at marginally higher lipoplex doses lead to toxicity. The role that cationic lipids play at the cellular level in transfection is not clear, but if it does involve the alteration of plasma membrane dynamics or intracellular trafficking, it is easy to imagine that such cell function, may be disrupted as the dose increases.

In Vitro *Versus* In Vivo *Transfection of Endothelial Cells*

One of the limitations of non-viral gene therapy as acknowledged above, is the low efficiency of transgene expression realized with these systems. To gain some insight as to the amount of reporter gene expression in endothelial cells that could be expected under optimal conditions we transfected human umbilical vein endothelial cells (HUVEC) *in vitro.* Upon transfecting the endothelial cells with a DOTAP/CHOL-based lipoplex identical to that employed on our *in vivo* studies (Barron et al., 1998), the mean luciferase activity was calculated to be approximately 0.2 relative light units (RLU) per cell. We observe about 5×10^8 RLU/lung in mice after IV administration of this formulation. If we assume the number of endothelial cells in a 30 gram mouse is approximately 5×10^9 (Weibel, 1985) and activity was confined primarily to these cells, we obtain a value of 0.1 RLU/endothelial cell. We acknowledge that comparisons between *in vitro* and *in vivo* systems are not ideal; however, this comparison suggests that gaining access to and uptake into the lung endothelium may not be a rate-limiting process. Thus improvements in transfection may be realized through addressing downstream events such as endosomal release or nuclear uptake of DNA.

6. Scope of the Thesis

If lipoplex-mediated gene therapy is to become a widely used therapeutic modality, gene expression must be augmented and the persistence of that expression must be increased. With few exceptions, transgene expression is an ephemeral phenomenon-typically reaching a peak within days of expression and then declining to less than 5% of peak levels within a week. Although progress has been made in recognizing physiological, biochemical and cellular processes governing the efficacy of gene delivery vehicles, several questions remain to be addressed. Most studies involving the administration of cationic lipid-DNA complexes formulated with a strong net positive charge document marked lipoplex uptake and transfection in the lung. The accumulation and subsequent transfection in the lung may be a reflection of the fact that the pulmonary microvasculature is the first capillary bed encountered by the lipoplex upon administration. It is important to note, however, that in the study by McLean and collaborators (McLean et al., 1997) the ovaries and lymph nodes as well as the adrenal and pituitary glands, as a proportion of tissue mass, accrue a large amount of lipoplex and undergo significant transfection. Since these tissues are located downstream from the pulmonary capillary bed, an alternative explanation for lipid mediated transfection therein must exist.

The second chapter of this work emphasizes the importance of electrostatic interactions in mediating gene delivery. The central hypothesis being that the binding and uptake of the cationic lipoplex depends on interactions with anionic residues located on the endothelium or on plasma proteins such as albumin. In the third chapter, we show that

IV-injected cationic lipoplexes interact with and activate plasma complement proteins; however, we also demonstrate that this interaction does not influence lipoplex distribution or transfection. In chapter 4, we determined that the critical time window for binding and uptake of IV injected lipoplex in the lung is the first 60 minutes post-injection. In chapter 5 we show that the infusion of transfectionally-active plasmid DNA to high steady state concentrations over this time period did not yield appreciable systemic transfection. This implies that the cationic lipid component of the lipoplex serves in a function beyond that of a passive controlled-release matrix for plasmid DNA.

The objective of this body of work is to characterize the mechanisms by which current cationic lipid-DNA complex preparations mediate transfection. To this end, we have contributed information which may be applied in the design and application of more effective lipoplex formulations. We have shown the importance of electrostatic interactions between cationic lipoplexes and components of the vascular compartment Although the precise nature of this binding has not been determined, negative residues such as membrane phospholipids and proteoglycans may be involved. We showed that although lipoplex interacts with both albumin and complement, they are not critical factors in mediating gene delivery to the lung with cationic lipoplexes. Finally, we have demonstrated that the uptake of lipoplex in the lung occurs principally within 1 hour of administration and, since naked DNA alone is not capable of transfection, the lipoplex appears to be internalized relatively intact.

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Chapter II: Mechanism of Endothelial Binding of Cationic Lipoplex

1. Introduction

Cationic lipid-DNA complexes mediate high levels of gene expression in the lung relative to other organs following intravenous administration (Brigham et al., 1989). The mechanism by which this occurs is not clear, but certainly a principal event in the process of gene delivery is the binding of the lipoplex to the target cell prior to internalization. The goal of the experiments described in this section is to determine the nature of lipoplex binding to endothelial cells in the lung.

The particularly high level of lung expression relative to other organs may be a reflection of the fact that the pulmonary microvasculature is the first capillary bed encountered by IV-injected lipoplex. We have speculated that the lipoplex becomes associated with plasma proteins following injection into the blood and this ternary complex is then bound to and taken up by endothelial receptors for the lipoplex-associated proteins.

Mislick and Baldeschwieler have demonstrated the importance of proteoglycan-mediated binding in polyplex and lipoplex-based transfection *in vitro* (Mislick and Baldeschwieler, 1996). If this is so *in vivo*, proteoglycans expressed on the lung endothelium may electrostatically bind to lipoplexes and facilitate their uptake. In a recent publication, Mounkes and colleagues injected mice IV with heparinase and then injected lipoplexes via the same route. They observed a substantial decrease in systemic transfection. In view of the importance of cell surface proteoglycans in *in vitro* transfections (Mislick and Baldeschwieler, 1996) Mounkes and coworkers concluded that

proteoglycans are required for systemic transfection in animals (Mounkes et al., 1998).

To reconcile these findings with our plasma protein receptor hypothesis and explain the specificity of endothelial binding, we suggest that proteoglycan-mediated lipoplex uptake process also acts in unison with an additional, as yet unidentified, receptor and thus confers lung-specific binding. An example of this phenomenon is the proteoglycan-dependent uptake of lipoproteins and lipoprotein remnants which is enhanced by association with hepatic and lipoprotein lipase (Ji et al., 1997; Williams et al., 1992). Examination of fluorescently-labeled cationic lipoplexes using confocal microscopy, showed that cationic lipoplexes bound to and transfected the lung, anterior pituitary, adrenals and the ovaries while the brain and posterior pituitary accumulated little lipoplex (McLean et al., 1997). These observations suggest that a specific mechanism is involved in the transfection of certain tissues. The authors speculated that there may be tissue-specific receptors on the endothelial membrane responsible for lipoplex uptake. As presented in the introductory chapter, we also hypothesized that such receptors may govern the distribution of lipoplexes by binding to plasma-protein modified lipoplexes.

The possibility that plasma proteins that have bound to the lipoplex can target to cell-surface receptors is supported by the finding that transferrin which had been electrostatically associated with the lipoplex can target it to cultured cells (Cheng, 1996). This phenomenon has been demonstrated *in vivo* as hepatic gene expression was increased 7-fold by complexing a succinylated asialofetuin group to the lipoplex prior to systemic administration (Templeton et al., 1997). These experiments support the contention presented in the introductory chapter that anionic plasma proteins may interact

with cationic lipoplexes, thus, influencing their distribution and capacity to transfect specific tissues. The high plasma concentration of albumin, its low pI, and the presence of albumin receptors on the pulmonary endothelium (Galis et al., 1988; Ghitescu et al., 1986) led us to hypothesize that this protein may participate in lipoplex uptake and transfection seen in the lungs upon IV administration of lipoplexes. Our objective in the following experiments was to determine the manner by which lipoplexes associate with the pulmonary endothelium following IV administration.

2. Materials and Methods

Animals

Four- to six-week-old female ICR mice (Gilroy, CA) and Sprague Dawley rats (200-250 g body weight) were purchased from Simonsen. Breeding pairs of Nagase rats were obtained from Medizinische Hochschule Hannover, Hannover, Germany and a breeding colony was established at the University of California, San Francisco. The level of plasma albumin in the Nagase animals was examined via SDS polyacrylamide gel electrophoresis of plasma proteins followed by protein staining. There was no detectable staining corresponding to the molecular weight of albumin, thus confirming that the animals were albumin deficient. All animals were handled in accordance with protocols established by the National Institute for Health Guidelines for the Care and Use of Laboratory Animals, and with the approval of the Committee for Animal Research at the University of California, San Francisco. Animals were anesthetized with an intraperitoneal (IP) injection of ketamine (200 mg/kg), xylazine (10 mg/kg) and

acepromazine (2 mg/kg). Animals were sacrificed by anesthetization as above, followed by exsanguination via cardiac puncture.

Chemicals and Reagents

The cationic lipid 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids (Alabaster AL, USA). Cholesterol, D-polyglutamic acid (2,000-15,000 MW), protamine sulfate (Grade I), heparinase and bovine serum albumin (fraction V) were purchased from Sigma (St. Louis, MO, USA). Polyethylenimine (25,000 and 750,000 MW) was purchased from Aldrich (Milwaukee, WI).

Plasmid DNA

pCMVLuc plasmid DNA encoding the firefly *Photinus pyralis* luciferase gene was the generous gift of Dr. Keelung Hong (Hong et al., 1997) or GeneMedicine Inc., (The Woodlands, TX, USA). Plasmid DNA was amplified in DH5α sub-cloning efficiency *E. coli* (Gibco BRL, Gaithersburg, MD, USA), extracted by alkaline lysis and purified by centrifugation through two cesium chloride/ethidium bromide gradients according to standard laboratory procedures (Sambrook, 1989). Plasmid DNA was then dialyzed against 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and ethanol precipitated. The purity and identity of the plasmid DNA preparation was confirmed by absorbance measurements at 260 and 280 nm and through gel electrophoresis of plasmid restriction digest products. Endotoxin was reduced to less than 20 EU/mg plasmid DNA by passing the DNA through a polymyxin B column (Bio-Rad, Hercules, CA. USA). Endotoxin

levels were measured with a limulus amebocyte lysate assay kit (Kit QCL-1000, Biowhittaker, Walkersville, MD, USA).

Cationic Lipid-DNA Complex Preparation and Injection

The lipids, DOTAP and cholesterol were dissolved in chloroform at a 1:0.9 molar ratio then the chloroform solution was evaporated to dryness on a rotary evaporator. The lipid film was placed under vacuum overnight to remove the remaining solvent. The dried lipid film was hydrated in 10% glucose/10 mM Hepes buffer (pH 7.4) over a period of 30 minutes during which time the suspension was agitated on a vortex mixer for 30 second intervals about 10 times. Large unilamellar vesicles (LUV) were prepared by extruding the hydrated lipid suspension through a 0.2 μm polycarbonate membrane. Liposome-DNA complexes were formed at room temperature by injecting an equal volume of plasmid DNA diluted in dH_2O into the liposome preparation to yield a complex with a 5:1 charge ratio (+/-). The lipoplexes were allowed to stand for 15 minutes prior to injection. Each mouse received a 100 μL dose administered via tail-vein injection containing 900 nmol total lipid and 90 nmol (30 μg) plasmid DNA. In the case of mice receiving complex incubated with polyanions, each animal was dosed with 110 μL of the mixture. The IV lipoplex dosage for the rats was 160 μg (480 nmol) plasmid DNA complexed with 4.8 μmol lipid per animal in a volume of 530 μL. Sonicated unilamellar vesicles (SUV) for pre-injection experiments were prepared from the DOTAP:CHOL suspension described above but rather than extruding the lipids, they were sonicated for 15-20 minutes in a bath sonicator (Laboratory Supplies Company Inc., Hicksville, NY, USA) at 10°C until a translucent lipid dispersion was obtained.

Radiolabeling of Plasmid DNA

To study the distribution of the cationic lipid DNA complexes *in vivo,* radiolabeled DNA and liposomes were combined as above, injected into the tail-veins of mice and then tissue levels of the radiolabeled DNA were measured at stated time points.

Plasmid DNA was labeled with the Gibco BRL nick translation kit (Kit # 18160-010, Gibco BRL, Gaithersburg, MD, USA) and ^{125}I-dCTP (NEN Research Products, Boston, MA, USA). The nick translation procedure yielded high molecular weight DNA with a specific activity of 0.32 μCi/μg DNA (Meyer et al., 1995).

Luciferase Assay

Twenty-four hours following administration of cationic lipid/DNA complexes, animals were anesthetized with an intraperitoneal (IP) injection of ketamine (200 mg/kg), xylazine (10 mg/kg) and acepromazine (2 mg/kg). Approximately 1 mL of blood was removed via intracardiac puncture. One milliliter of phosphate buffered saline (PBS) was perfused through the right cardiac ventricle. The heart, lungs and a 300 mg portion of the liver were removed and reporter gene expression measured with the Luciferase Assay System (Promega, Madison, WI, USA). Tissues were placed in 2 mL polypropylene tubes containing 1.25 mL of lysis buffer (Promega, Madison, WI, USA) and homogenized utilizing a bead-mill (Mini-Beadbeater; Biospec Products, Bartlesville, OK, USA). Samples were then spun at 12,000 RPM in a microcentrifuge (7 minutes at 12,000x *g*) and 10 μL of the supernatant analyzed for luciferase activity as previously described (Meyer et al., 1995). A standard curve describing the correlation between relative light units and picograms of luciferase activity was constructed using luciferase

enzyme obtained from Analytical Bioluminescence Laboratory (Product #2400, San Diego CA, USA). Reporter gene activity was then expressed as picograms of luciferase activity per mg of tissue protein.

Pre-Injection of Polycations

Polycations were dissolved in buffer (5 mM Hepes/5% glucose, pH 7.4) to yield preparations of desired concentration. One hundred microliters of a given solution was injected into the tail vein of mice at the stated times prior to lipoplex injection. Tissues were assayed for luciferase activity 24 hours later as described above.

Protamine sulfate (PS) was prepared at a concentration of 900 μg/mL or 9 mg/mL. When injected in equal volume to lipoplex, these preparations corresponded to a charge equivalence or 10-fold charge excess relative to that of the cationic lipid component of the lipoplex, assuming a 5.0 μmol negative charge per mg PS.

PEI and liposomes were injected 5 minutes prior to naked plasmid DNA such that a 100 μL bolus of polycation constituted a nominal 450 nmol of positive charge. This dose constituted a charge equivalence to that of the cationic lipid component of a standard lipoplex. The quantity of PEI injected was based on a charge density of 5.4 μmol negative charge per mg of PEI. Liposomes (LUV and SUV) were prepared as outlined above. Distribution of radiolabeled DNA was carried out 5 minutes following DNA injection. Luciferase activity was measured 24 hours post-injection.

Pre-Injection of Heparinase

Heparinase was dissolved in 5 mM Hepes/5% glucose (pH 7.4) and a 100 μL

bolus containing 30U of heparinase was injected into the tail-vein either 5 minutes or 60 minutes prior to tail-vein injection of lipoplexes. Animals were sacrificed and luciferase activity was measured 24 hours later as described above.

Lipoplex-Coating Procedure

Commercially purified albumin (Sigma, fraction V) was further purified by gel filtration chromatography. Albumin powder was dissolved in buffer (10 mM Hepes and 0.15 M NaCl, pH 7.4) yielding a final protein concentration of 30 mg/mL. The sample was then loaded onto a column (100.0 cm x 3.0 cm) containing Sephacryl S-100 HS column matrix (Pharmacia, Piscataway, NJ) at a rate of 36 mL/hr. Fractions containing albumin only were dialyzed against 5 mM Hepes buffer (pH 7.4). Purified albumin was lyophilized and stored at 4°C. Lyophilized albumin was dissolved in buffer (5 mM Hepes/5% glucose, pH 7.4) yielding a final concentration of 240 mg/mL prior to experiments. D-polyglutamate was dialyzed against 10 mM $NaHCO_3$ buffer (pH 7.4) for 24 hours, lyophilized and stored at 4°C . Prior to use, D-polyglutamate was dissolved in 5 mM Hepes/5% glucose buffer, (pH 7.4) at a final concentration of 270 mM. Fifty microliters of either albumin or D-polyglutamate solution was added to 450 μL of lipoplexes prepared as above. Each mixture was incubated at room temperature for 15 minutes. The diameter of the complexes was measured using dynamic light-scattering (Coulter Electronics, Hialeah, FL, USA) and the net surface potential determined with a Malvern Zetasizer IV (Malvern, UK).

3. Results

Effect of Polycation Pre-Injection on Lipoplex-mediated Transfection

It is known that the pre-formation of lipoplex is not necessary to achieve transfection *in vitro* (Felgner, 1989) and *in vivo* (Liu, 1998). Liu (1998) interpreted these observations to suggest that naked DNA itself was the active transfection agent. We address this assertion in chapter V of this dissertation. To confirm Liu's results *in vivo*, we IV-injected cationic liposomes 5 minutes prior to systemic administration of radiolabeled DNA or plasmid DNA encoding the luciferase reporter gene (Figures 1a and b). Pulmonary accumulation and reporter gene expression of the sequentially-administered DNA and of pre-formed lipoplex were virtually identical. This confirms Liu's (1998) surprising observation that the complexation of DNA with liposomes prior to injection is not necessary for efficient pulmonary gene delivery. In addition, these data demonstrate that interactions between cationic lipids and DNA occurs after the lipids have been in contact with serum for 5 minutes, implying that electrostatic binding may be a predominant factor governing pulmonary uptake and transfection following cationic lipoplex injection.

To determine if other polycations could mediate similar DNA retention and transfection in the lung, we injected various polycations followed 5 minutes later by radiolabeled DNA or luciferase reporter DNA. We analyzed lung DNA distribution 5 minutes after DNA injection and for lung reporter gene activity 24 hours after treatment (Figures 2a & b).

As shown in previous studies (Barron et al., 1998) pre-formed lipoplex delivers

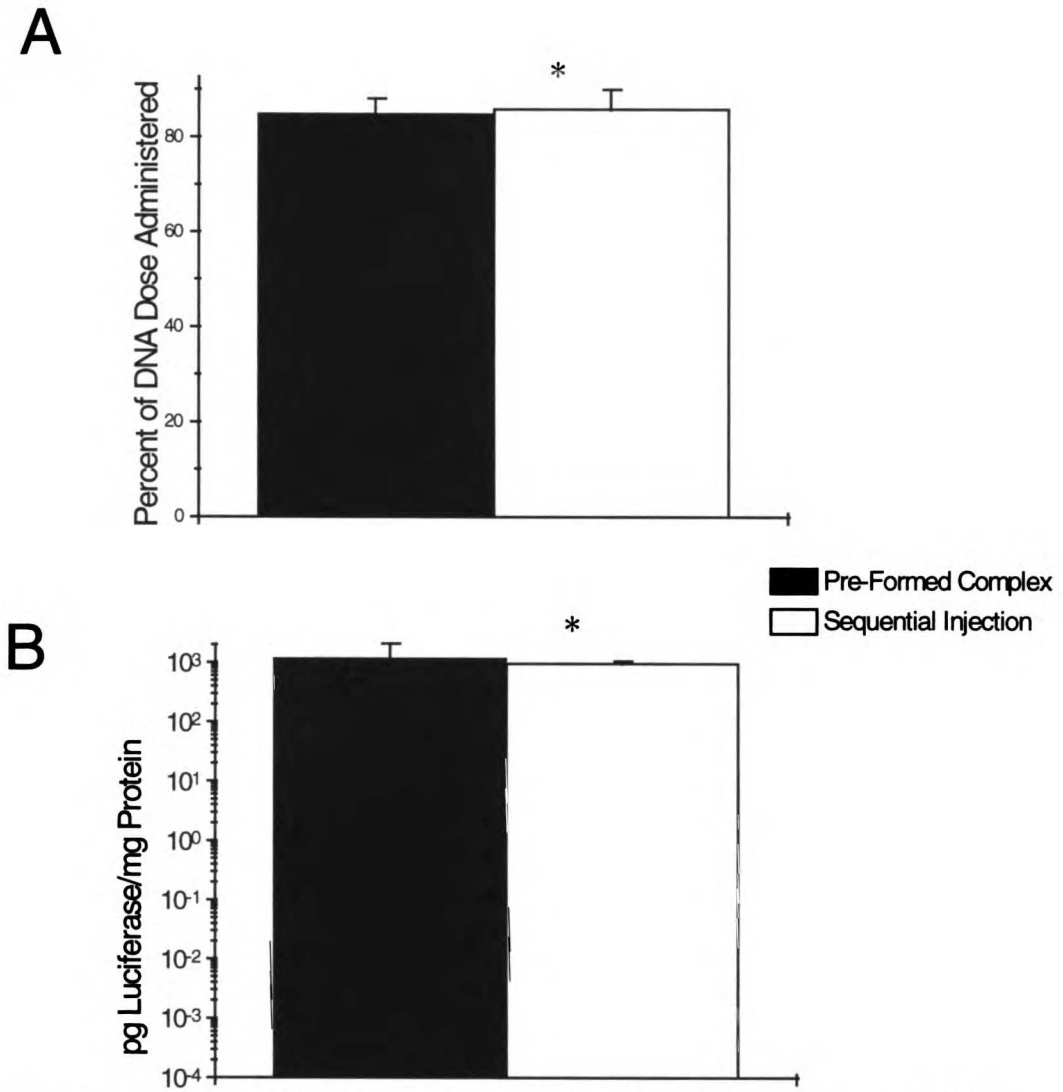


Figure 1A and B. Effect of sequential IV injection of cationic liposomes and luciferase reporter plasmid.

Control animals received a 100 uL bolus of cationic lipid-DNA complex prepared as in Materials and Methods. Animals in the sequential injection cohort were injected with a 100 uL IV bolus of DOTAP:CHOL liposomes prepared as in Materials and Methods.This was allowed to circulate for 5 minutes and then a sequential 100 uL bolus containing 30 ug of plasmid DNA was injected. In panel A, animals received radiolabeled DNA while in panel B, animals received a luciferase reporter gene. Animals were sacrificed and lungs assayed for luciferase activity 24 hours later. Results are expressed as the mean percent of dose administered ± S.E.M. for 6 animals. * No significant difference vs control group as determined by Student's t-test $p \leq 0.05$.

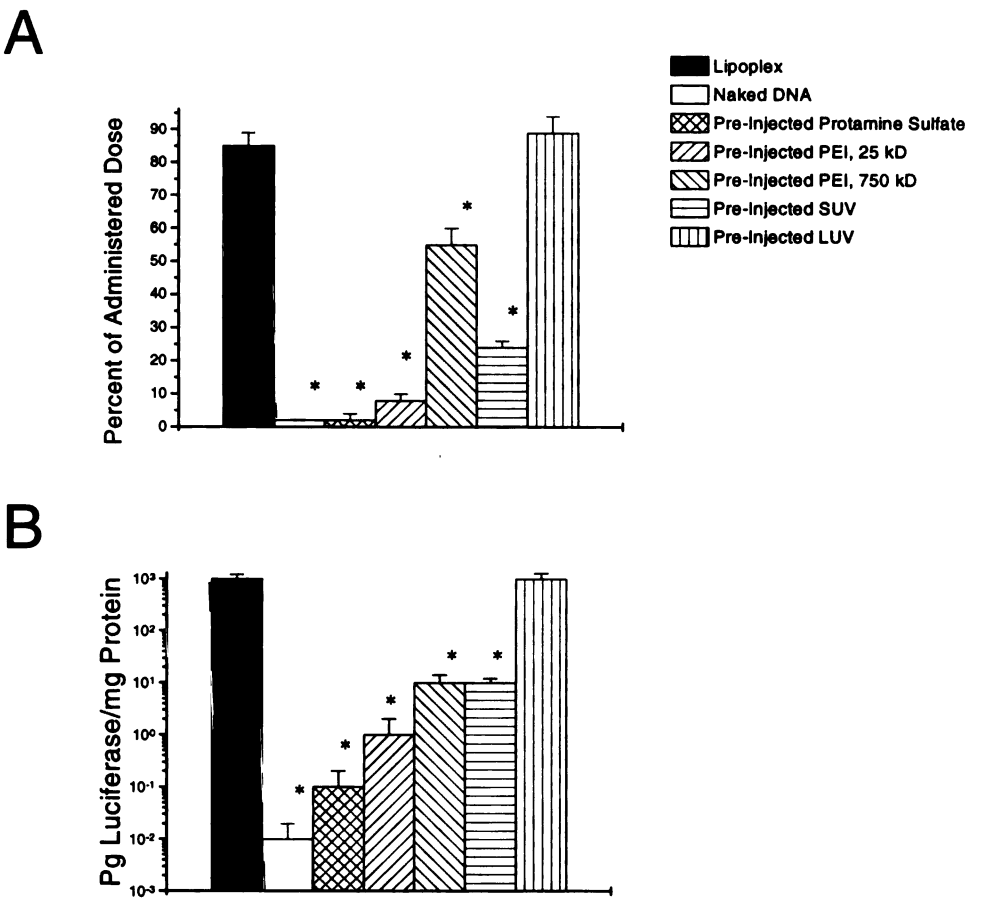


Figure 2A and B Effect of polycation injection on the distribution and transfection mediated by subsequently injected plasmid DNA.

Polycations were dissolved in 5mM Hepes/5% glucose buffer (pH 7.4) such that a 100 uL bolus of each constituted a nominal 450 nmol of positive charge. Liposomes (LUV and SUV) were prepared as outlined in Materials and Methods. Distribution of radiolabeled DNA was carried out 5 minutes following DNA injection. Luciferase activity was measured 24 hours post-injection. * Significant difference vs control group as determined by Student's t-test $p < 0.05$. (A, n = 6; B, n = 4).

85% of the administered DNA dose to the lung 5 minutes following IV administration. Naked DNA does not collect in the lung to any significant degree (Figure 2a). Although the amount of cationic charge delivered in the form of polymers was the same as that of the cationic LUVs, these polycations did not retain subsequently injected plasmid DNA to the same extent as the cationic LUV. Protamine sulfate retained only 2% of the injected DNA dose while, following 25,000 MW polyethylenimine (PEI) injection, 8% of the DNA dose distributed to the lung. The higher molecular weight polyethylenimine (750,000 MW) retained 55% of the injected DNA dose in the lung. The pre-injection of small unilamellar vesicles led to the retention of 24% of plasmid DNA injected 5 minutes hence. The pre-injection of large unilamellar vesicles (LUV) retained 89% of the administered DNA which is not significantly different than that of pre-formed complexes (Figure 2a).

The transfection mediated by the various polycations followed a similar pattern to that of distribution (Figures 2a & b). Naked reporter plasmid DNA did not yield significant transfection in the lungs. Protamine sulfate pre-injection yielded low transfection upon reporter administration but it was slightly more effective than DNA alone. The pre-injection of high molecular weight PEI yielded greater transfection when compared to low molecular weight PEI but gene expression was still lower than that of the LUV pre-treatment. Pre-injected SUVs mediated similar levels of transfection to that of high MW PEI even though they retained much less DNA in the lungs. Pulmonary transfection following sequential injection of LUVs and reporter DNA, as above, was the same as that of pre-formed lipoplex. These data suggest that pulmonary DNA retention and transfection is dependent on the size and structure of the pre-injected polycation

moiety after which it is injected.

Effect of Protamine Sulfate Pre-Injection on Lipoplex-mediated Transfection

Based on the assumption that cationic lipoplex binding in the lung is dependent on electrostatic interactions between the lipoplex and the endothelium, we reasoned that the pre-injection of a polycation such as protamine sulfate should attenuate transfection in the lung. The doses of protamine sulfate chosen- 90 μg and 900 μg per animal, constituted a nominal charge equivalent and a 10-fold charge excess, respectively, versus positive charge conferred by the cationic lipid component of the lipoplex. At stated times subsequent to protamine sulfate injection, lipoplex was injected IV and then 24 hours after administration, the lungs were assayed for luciferase activity (Figure 3). No gross toxicity was observed from this protocol following administration or upon sacrifice and dissection. When up to a 10-fold excess of protamine sulfate was delivered prior to lipoplex injection, lung transfection was not affected (Figure 3). These data suggest that either electrostatic interactions are not important in mediating transfection; or that protamine sulfate does not effectively compete with cationic lipoplex for binding sites on the pulmonary endothelium. The 10-fold lower gene expression overall in the experiments corresponding to data in figures 3 and 5 relative to that of figures 1b, 2b and 6 is a reflection of the different plasmid constructs employed in the two sets of experiments.

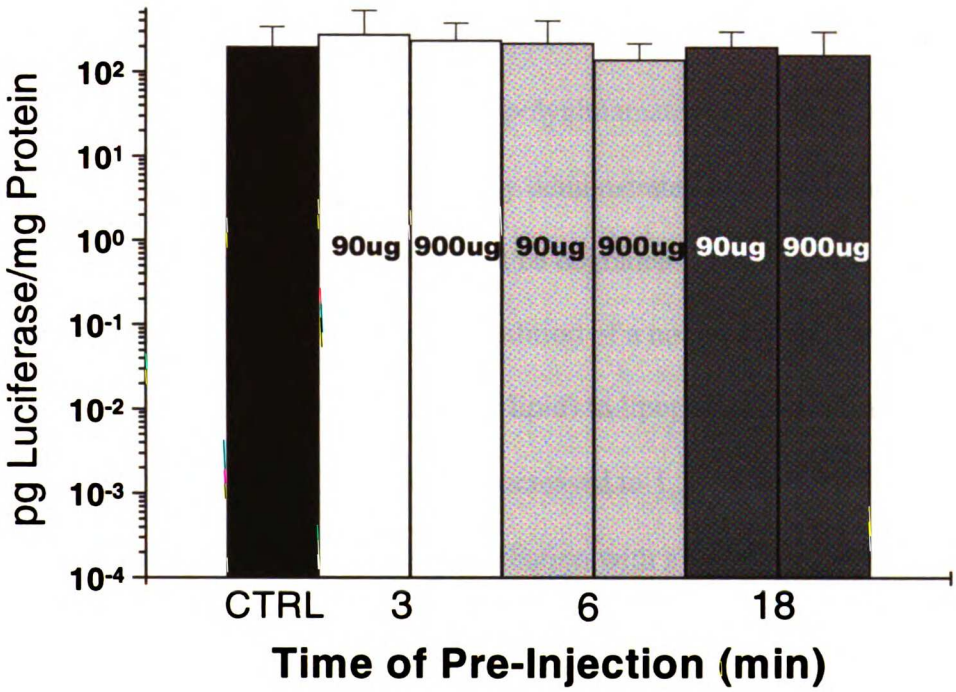


Figure 3. Effect of protamine sulphate pre-injection on cationic lipoplex mediated transfection in murine lungs.

A 100 uL bolus of either 90 or 900 ug of protamine sulphate was injected at the stated times prior to injection of DOTAP:CHOL-luciferase DNA complex (5:1, +/-). Tissues were assayed 24 hours later for luciferase activity as described in Materials and Methods(n = 6). No significant difference vs control group was detected in any treatment group as determined by Student's t-test $p \leq 0.05$.

Physicochemical Characterization of Lipoplex following Incubation with Polyanions

To ascertain whether the incubation of lipoplex with polyanions would neutralize the lipoplex and block transfection when subsequently injected IV, lipoplex prepared as above, was incubated in buffered polyglutamate or albumin solutions. Prior to incubation with polyglutamate, the lipoplex had a mean diameter of 450 nm and a net surface potential (ζ potential) of +36 mV (Table 1). D-polyglutamate was chosen as a lipoplex-coating molecule because it has been previously demonstrated to associate with lipoplex *in vitro* without displacing DNA and would not be degraded as rapidly *in vivo* as L-polyglutamate (Xu and Szoka, 1996). The addition of a nominal 6-fold charge excess of D-polyglutamate (versus that of the cationic lipid) to lipoplexes caused an increase in turbidity of the preparation. Particle diameter increased to 1.2 μm and the ζ potential decreased to –60 mV (Table 1). Following incubation with a 24 mg/mL albumin solution, a marked increase in the turbidity of the final mixture was observed; however, no precipitation or large aggregates were apparent. The diameter of the complex increased to 5.0 μm and the ζ potential was -20 mV (Table I).

In Vivo *Distribution of Coated Lipoplex*

To determine the effect of the lipoplex coating protocols on the distribution of the lipoplex, plasmid DNA containing a trace amount of radiolabeled DNA was complexed with the cationic lipid and injected IV. At 15 minutes and 24 hours post-injection, animals were sacrificed and tissues assayed for radioactivity. In agreement with previous work, the systemic injection of the lipoplex led to the accumulation of the vast majority of the DNA label in the lung, while less than 10% collected in the liver. In the lung and

Preparation	Diameter (μm) Mean ± S.D.	ζ Potential (mV) Mean ± S.D.
Lipoplex Alone	0.45 ± 0.12	+ 36 ± 5
Lipoplex + pGlu	1.20 ± 0.80	- 60 ± 5
Lipoplex + Albumin	5.00 ± 1.80	- 20 ± 5

Table 1. Particle diameter and zeta potential of cationic lipid-DNA complexes alone and following incubation with albumin or polyglutamate.

Lipoplexes were prepared as detailed in Materials and Methods. Albumin or D-polyglutamate was added to lipoplexes and each mixture was incubated at room temperature for 15 minutes. The diameter of the complexes was measured using dynamic light-scattering and the net surface potential determined as in Materials and Methods.

liver, the incubation of the cationic lipoplex with the albumin solution prior to administration did not affect the distribution of the radiolabeled DNA versus that of lipoplex that was not pre-incubated with polyanion (Figures 4a & b). The incubation of the lipoplex with the D-polyglutamate prior to injection led to the accumulation of 80% of the label in the liver and 11% in the lung 15 minutes post-administration (Figure 4a). After 24-hours, the discrepancy between the distribution of the various preparations tended to decrease, probably as a result of the degradation, redistribution and clearance of the DNA label (Figure 4b).

The interaction between lipoplexes and albumin did not cause a significant change in gene expression in the lung or liver versus lipoplex alone (Figure 5); however, the exposure of lipoplexes to a charge excess of D-polyglutamate led to a profound reduction in both pulmonary distribution and transfection (Figure 5). This observation indicates that polyglutamate coats the lipoplex more thoroughly than albumin thus preventing subsequent electrostatic binding in the lung. Although the incubation of lipoplex with D-polyglutamate effected an 8-fold increase in the accumulation of labeled DNA in the liver 15 minutes after injection (Figure 4a), no significant alteration in transgene expression was observed in that tissue (Figure 5).

Effect of Heparinase I Pre-Injection on Lipoplex-mediated Transfection

The fact that cationic liposomes are capable of retaining plasmid DNA in the lung 5 minutes after their injection implies that cationic residues are available for binding to the subsequently injected anionic plasmid. In addition, this observation suggests that pulmonary transfection could result from binding between the cationic lipoplex and

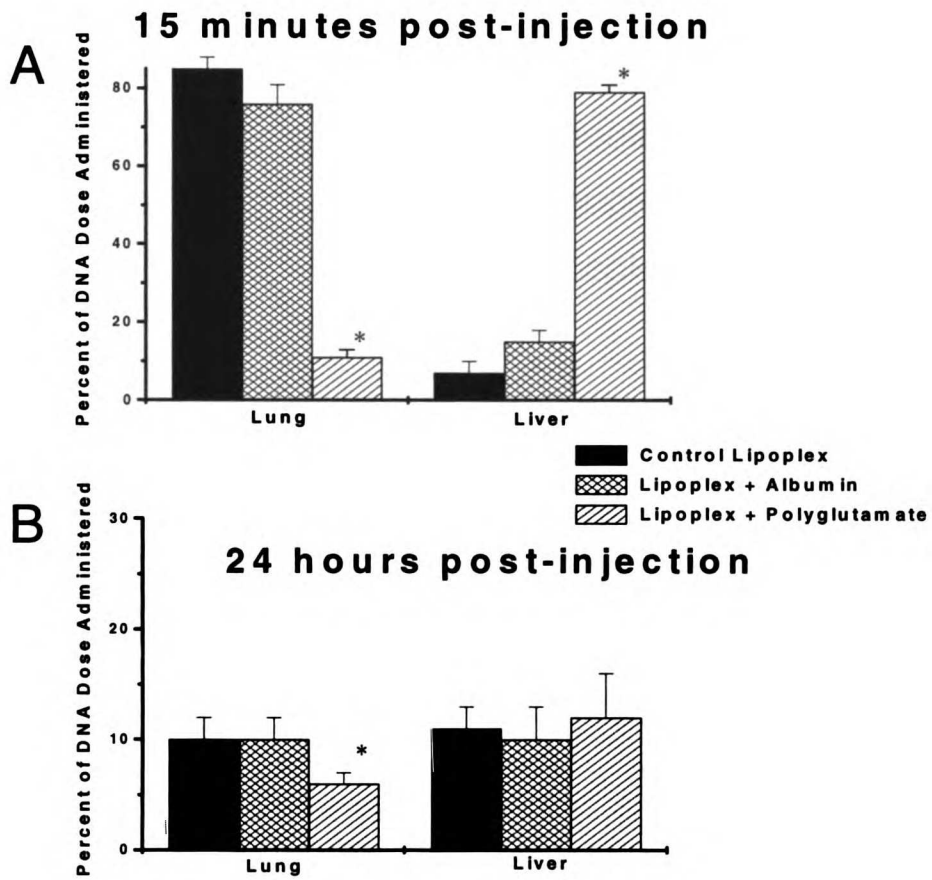


Figure 4A & B. Effect of lipoplex coating protocols on the distribution of the lipoplex.

Distribution of IV-administered DOTAP:CHOL-plasmid DNA complex (5:1, +/-) labeled with ^{125}I -dCTP plasmid DNA in mice at 15 minutes (A) and 24 hours post-administration (B). Results are expressed as the mean percent of dose administered ± S.E.M. for 4 animals. *Significant difference vs control group as determined by Student's t-test $p \leq 0.05$.

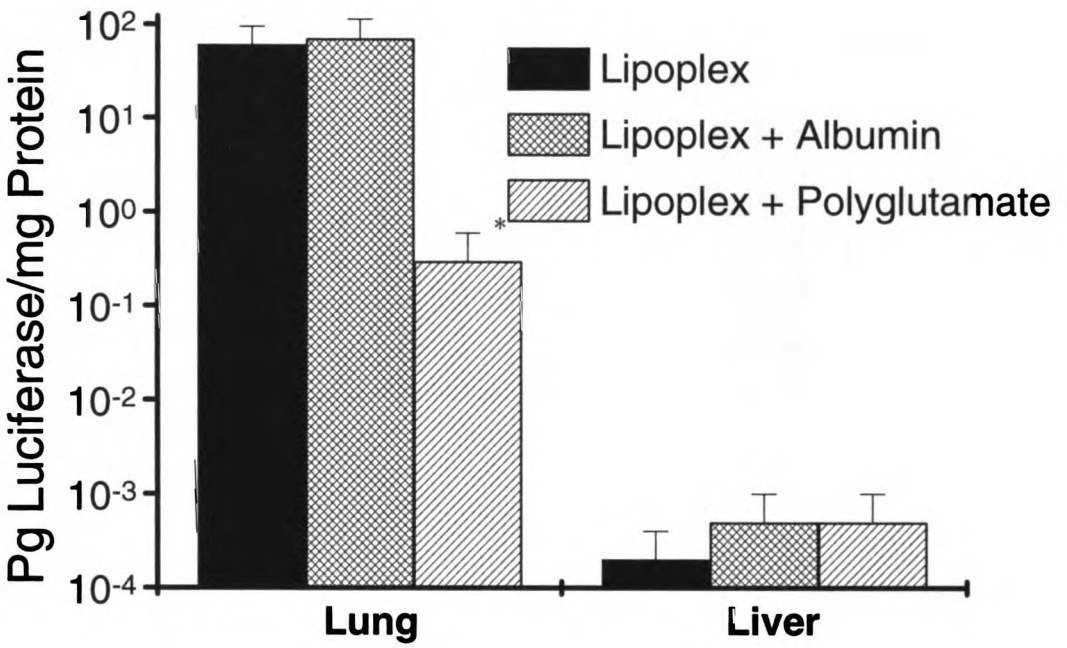


Figure 5. Effect of lipoplex coating protocols on lipoplex-mediated transfection in murine lungs.

DOTAP:CHOL-luciferase plasmid DNA complex (5:1, +/-) with or without coating protocol was administered IV. Twenty-four hours post-administration, the lungs were assayed for luciferase activity as outlined in Materials and Methods. Results are expressed as the mean ± S.E.M. for 6 animals. *Significant difference vs control group as determined by Student's t-test $p \leq 0.05$ ($n = 6$).

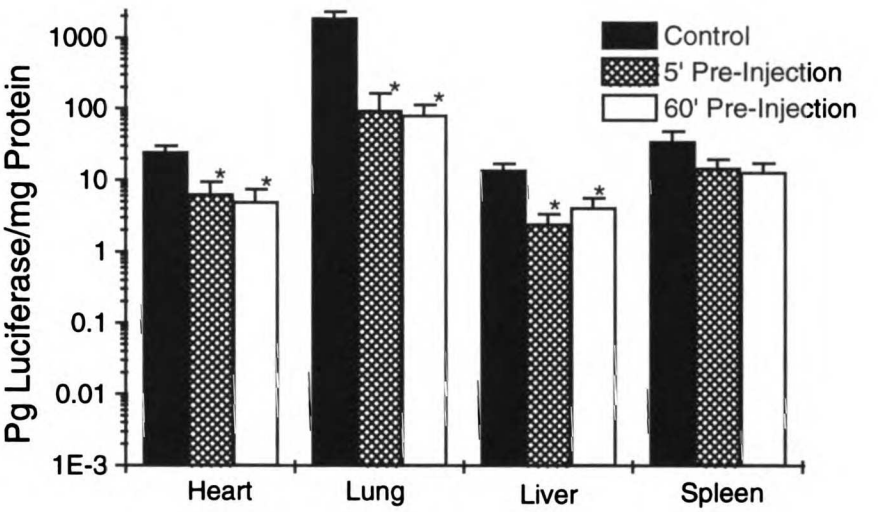


Figure 6 Luciferase expression in murine tissues following administration of heparinase I.

A 30U bolus of heparinase I was administered 5 or 60 minutes prior to lipoplex injection. Luciferase activity in tissues was assayed 24 hours later as outlined in Materials and Methods.

* Independent t-test significant difference at $P < 0.05$ versus control ($n = 6$).

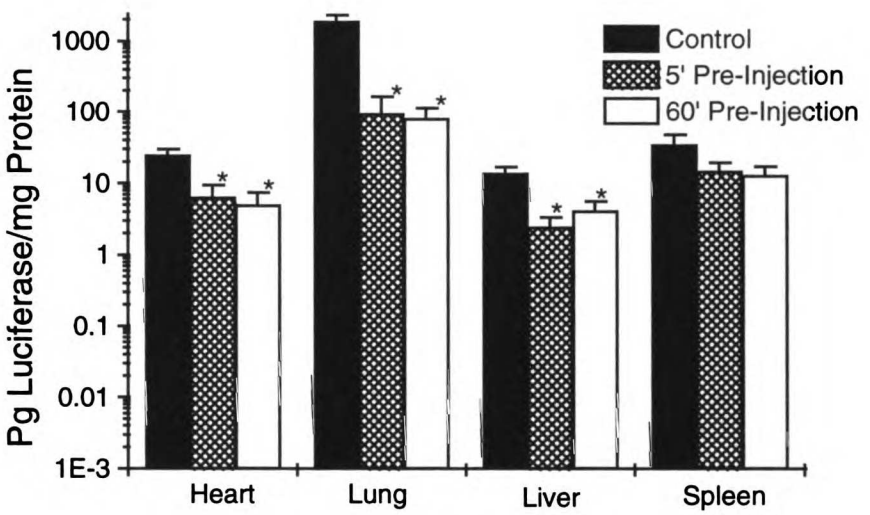


Figure 6 Luciferase expression in murine tissues following administration of heparinase I.

A 30U bolus of heparinase I was administered 5 or 60 minutes prior to lipoplex injection. Luciferase activity in tissues was assayed 24 hours later as outlined in Materials and Methods.

* Independent t-test significant difference at $P < 0.05$ versus control ($n = 6$).

anionic residues on the endothelium. A candidate for such anionic sites on the endothelium are glycosaminoglycans. The administration of heparinase 5 or 60 minutes prior to lipoplex injection resulted in a 20-fold reduction in lung transfection measured 24 hours after lipoplex administration, thus confirming the findings of Mounkes and colleagues that glycosaminoglycans may be important for lung transfection (Mounkes et al., 1998)(Figure 6). Significant reductions in transfection of the heart, liver and lung were also realized. These results can be interpreted in three fashions: 1) cell surface heparin and heparan sulfate are important in the transfection of the heart, lung, liver and spleen, 2) heparin released into circulation by heparinase treatment can interfere with cationic lipoplex transfection by binding to and neutralizing the complex as demonstrated above with D-polyglutamate, 3) released heparin may interact with the lipoplex and cause it to dissociate as demonstrated by Xu and Szoka *in vitro* (Xu and Szoka, 1996). Therefore, heparin appears to interact with the lipoplex following IV administration, but it is not clear if this interaction occurs at the cell surface and subsequently mediates lipoplex uptake in the lung or if the effect is caused by released heparin that interacts with circulating lipoplexes.

Lipoplex-mediated Transfection in the Analbuminemic Rat

The coating of the lipoplex with albumin did not alter its distribution or transfection *in vivo* upon systemic administration. This observation and the fact that albumin receptors have been demonstrated on the pulmonary endothelium (Galis et al., 1988; Ghitescu et al., 1986) provided the rationale for testing the hypothesis that albumin associated with lipoplex was responsible for the characteristic distribution and

transfection patterns in the lungs following IV injection of cationic lipoplexes. This was done by comparing transfection mediated by lipoplex in Sprague Dawley (S.D.) rats and a mutant form of this animal- the Nagase analbuminemic rat. Nagase rats typically possess plasma albumin levels that are 7000-fold lower than in wild-type S.D. rats (Nagase et al., 1979).

Although the injection of lipoplexes into the tail-vein of S.D. rats yielded pulmonary gene expression that was at least 2 orders-of-magnitude lower than that of the mouse, significant luciferase gene expression was observed in the heart, lung and liver of these animals (Figure 7). The systemic injection of lipoplexes into analbuminemic rats did not mediate gene expression that was significantly different from that of the S.D. rats. In addition, the incubation of the lipoplex with a purified albumin solution prepared as above did not affect transfection observed in these tissues in the Nagase animals. These data suggest that albumin coating of the lipoplex does not influence the distribution of nor reduce lipoplex-mediated transfection. Therefore, it appears that albumin it is not required for gene delivery to the lung.

4. Discussion

In analyzing the mechanism by which cationic lipoplexes mediate transfection in the lung following IV injection, we have hypothesized that electrostatic interactions between the lipoplex and anionic plasma proteins and/or cell surface components dictate the site and extent of cationic lipoplex binding and uptake. The experiments in this chapter were designed to study electrostatic interactions occurring at the cell surface and

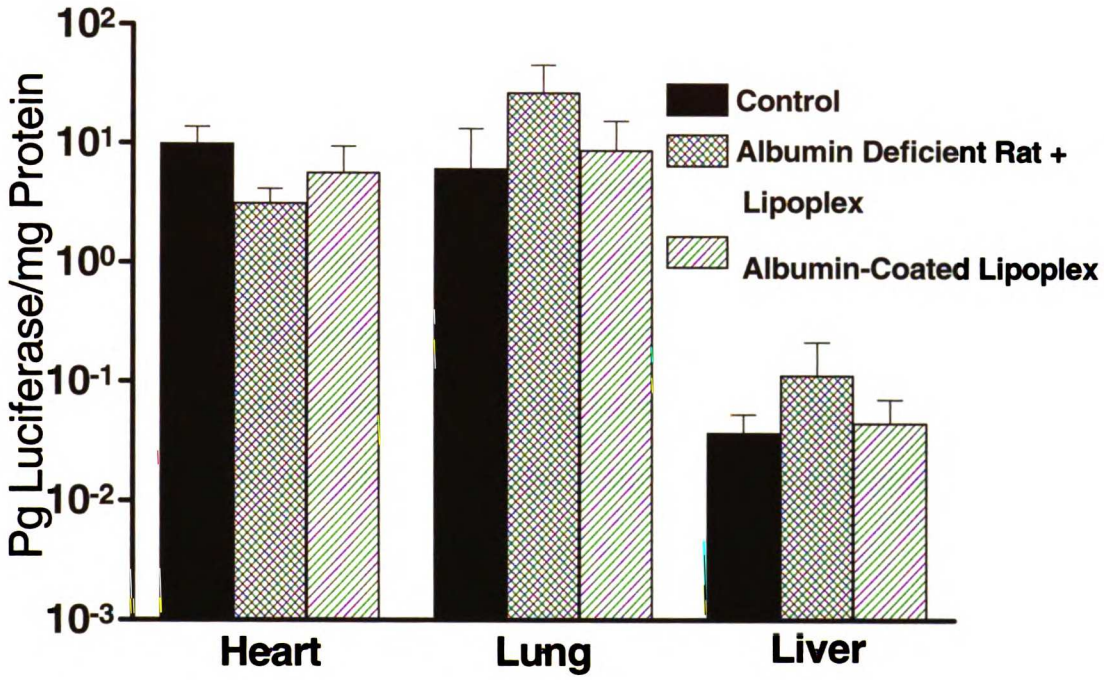


Figure 7. Comparison of transfection mediated by lipoplex in Sprague Dawley (S.D.) and Nagase analbuminemic rats.

DOTAP:CHOL-luciferase plasmid DNA complex (5:1, +/-) with or without coating protocol, was administered IV. Twenty-four hours post-administration, the lungs were assayed for luciferase activity as outlined in Materials and Methods. Results are expressed as the mean ± S.E.M. for 4 animals. No significant difference vs control group was detected in any treatment group as determined by Student's t-test $p \leq 0.05$.

interactions between the lipoplex and plasma proteins which subsequently target this ternary complex specifically to a plasma protein receptor following IV injection.

We confirmed the surprising observation of Liu and colleagues (Liu, 1998) that the distribution and transfection associated with an IV dose of plasmid DNA injected 5 minutes following IV liposome injection were the same as those of pre-formed lipoplex (Figures 1a and b). The fact that the cationic liposomes maintain the ability to bind DNA after being exposed to serum for 5 minutes implies that cationic residues still exist on the surface of the liposome after its exposure to anionic components in the blood. Thus, we speculated that electrostatic interactions between cationic lipoplex and components of the vascular compartment may influence lipoplex behavior following systemic administration.

To determine if the structure associated with a given polycation is important in mediating DNA binding and transfection, we IV injected liposomes and polymers of differing molecular weights (MW) followed by either radiolabeled DNA or reporter plasmid DNA. Generally, increasing the MW of the pre-injected liposome or polymer led to greater DNA retention and transfection in the lung (Figures 2a and b). Significant 3-dimensional structure or branching may be necessary to present a sufficient surface area to effectively interact with the endothelium and then, after exposure to circulation for several minutes, maintain the capacity to bind plasmid DNA. The low molecular weight of protamine sulfate may be the reason it was not particular effective for gene delivery nor was it able to inhibit transfection mediated by subsequently-injected cationic lipoplex (Figure 3).

Although pre-injected 750 kD PEI retained a similar amount of DNA in the lung

to that of pre-injected sonicated vesicles (Figure 2a), transfection following the SUV treatment was much higher (Figure 2b). It is not possible to determine the precise cause for this difference based on these data, but it may be related to events in the transfection process that occur subsequent to cell binding. For example, liposome-bound DNA may be more apt to be released from the endosome than DNA complexed to a polymer such as PEI.

We next asked the question whether coating of the lipoplex itself with an anionic polymer would block lung uptake. Incubating the lipoplex with D-polyglutamate led to a moderate increase in particle size and a profound decrease in the net surface charge suggesting that the cationic lipoplex was thoroughly coated by the polyanion. When injected IV, this complex did not distribute to the lung, rather it was cleared by the liver. Transfection was negligible in both organs. These data emphasize the necessity maintaining a positively-charged particle for lipoplex-mediated uptake and gene delivery in the lung.

When lipoplex was coated with albumin and injected IV, there was a marked increase in particle size and a decrease in ζ potential but lung uptake and transfection were unchanged relative to lipoplex controls. The fact that albumin incubation produced a particle with a less negative surface potential and a larger diameter implies that albumin was less effective at completely coating the lipoplex thus leaving exposed positive residues which facilitated particle cross-linking. The resulting gene delivery to the lung upon IV injection of this complex was interpreted to indicate that exposed positive regions on the lipoplex were binding negative components in the vasculature thus promoting pulmonary uptake and showing that thorough charge neutralization of the

lipoplex obviates pulmonary gene delivery.

Although it is possible to interpret the effect of albumin to be that of promoting the formation of aggregates which subsequently collect in the lung as suggested by Litzinger and colleagues (Litzinger et al., 1996), McLean and coworkers were unable to find any evidence of significant aggregate formation upon IV injection of lipoplexes and subsequent confocal microscopic analysis of the vasculature (McLean et al., 1997). These findings and the fact that we fail to see obvious respiratory distress or other gross toxicity upon IV injection of lipoplexes leads us to believe that significant aggregate formation does not promote lung transfection.

The ability of cationic lipoplexes to directly interact with negative residues on the endothelium is presented as an important process in the mechanism for lipoplex-mediated gene delivery *in vitro* and *in vivo* (Mislick and Baldeschwieler, 1996; Mounkes et al., 1998). We have confirmed this observation *in vivo* (Figure 6); however, the data do not conclusively define the nature of the interaction between cationic lipoplexes and proteoglycans following IV injection. Future experiments must address whether the decrease in systemic transfection following sequential IV administration of heparinase and then lipoplex is a reflection of reduced cell surface binding or simply neutralization or dissociation of the lipoplex by released heparin residues.

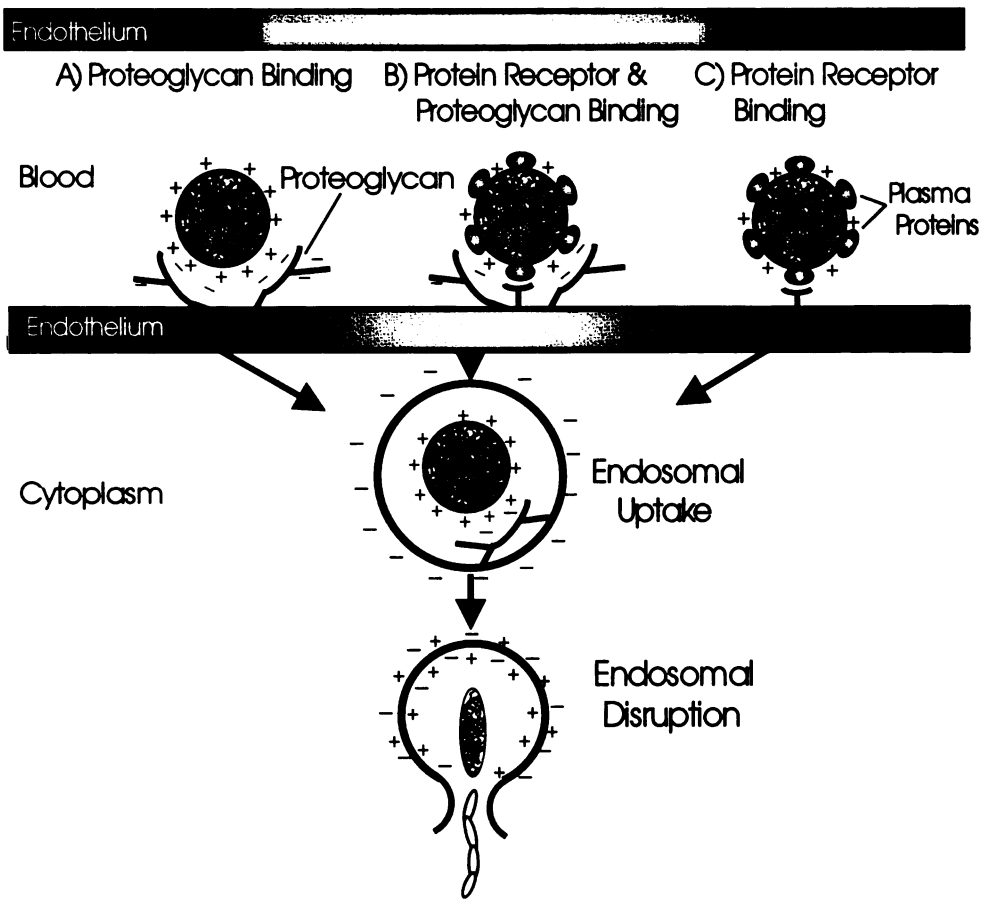
We hypothesized that a critical electrostatic interaction occurs between the lipoplex and albumin and that the lipoplex-associated albumin then targets the entire complex to albumin receptors on the pulmonary endothelium. This conjecture was supported by the fact that coating the lipoplex with albumin did not affect gene delivery to the lung after IV injection. We tested this hypothesis by injecting the lipoplex into the

analbuminemic Nagase rat model and found that transfection was unaffected relative to wild-type rats thus indicating that albumin was not necessary for successful gene delivery to the lung.

These experiments show that, after IV injection, lipoplexes maintain the capacity to participate in electrostatic interactions and these binding phenomena are likely to be important factors in lipoplex-mediated gene delivery to the lung. The exact nature of this binding and the components with which the lipoplex interacts have not been completely elucidated. The potential for lipoplex binding to occur at the level of the glycocalyx (Figure 8a) has been demonstrated but in order to explain the differential uptake of lipoplex in various regions of the body, (Barron et al., 1998; McLean et al., 1997; Song et al., 1997) there must exist qualitative or quantitative differences in proteoglycan distribution in the various regions of the endothelium such that preferential uptake occurs. Systematic analyses of endothelial proteoglycan content in various tissues are generally lacking; however, it is interesting to note that the ovaries, sites of significantly increased endothelial glycoconjugate expression (Augustin et al., 1995) have been identified by McLean and colleagues as having enhanced IV-injected lipoplex binding capacity (McLean et al., 1997). Alternatively, cationic lipoplex-mediated lung transfection may be facilitated through electrostatic interactions between the lipoplex and specific proteins for which receptors exist on the pulmonary endothelium (Figure 8c). Although we showed that albumin was not involved in facilitating transfection in this manner, it is possible that other proteins such as low-density lipoproteins may promote pulmonary transfection upon binding to the lipoplex. Finally, non-specific binding of cationic lipoplexes to anionic endothelial proteoglycans may combine with more specific

Figure 8

Lipoplex Internalization in the Lung



protein-mediated interactions in a given endothelium to yield the characteristic gene expression profile of cationic lipoplexes (Figure 8b).

The goal of the experiments described in this section was to determine the nature of lipoplex binding to endothelial cells in the lung. Toward that end, they serve to emphasize the importance of electrostatic interactions on pulmonary gene delivery following systemic administration of cationic lipoplexes. The positive charge of the lipoplex renders them inherently reactive with many components of the vascular compartment; these can be divided into two general categories:1) negative residues on the cell surface such as membrane gangliosides, glycoproteins and proteoglycans or 2) circulating plasma proteins. Future mechanistic studies will better define the precise role played by proteoglycans and glycoproteins in mediating systemic gene delivery. We have speculated that the distinctive distribution and transfection associated with IV administered cationic lipoplexes is dictated by electrostatic binding to a particular plasma protein which facilitates targeting to a discrete endothelial region. Chapter III of this dissertation addresses the role of lipoplex interactions with complement proteins in systemic distribution and transfection. Chapter IV defines the time window within which binding and uptake occur and finally, chapter V demonstrates that the role of the cationic lipid component of the lipoplex goes beyond that of acting as a binding agent for DNA retention and delivery in the lung.

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Chapter III: Effects of Complement Depletion on the Pharmacokinetics and Gene Delivery Mediated by Cationic Lipid-DNA Complexes

1. Introduction

The work in chapter II focussed on the role of electrostatic interactions in cationic lipoplex-mediated transfection following systemic injection. In this chapter, we present studies conducted to characterize the nature of cationic lipoplex interactions with complement proteins. This work was carried out with 2 principal objectives: First, we sought to determine if the complement system was activated by cationic lipoplexes; such interactions are important in the context of determining the immunogenic potential of these systems and our second aim was to resolve whether complement activation influenced the distribution and transfection of IV injected cationic lipoplex.

There is a paucity of studies addressing the interactions between cationic lipid-DNA complexes and blood proteins. Senior and colleagues have shown that upon incubating cationic lipids in serum, a dose-dependent increase in turbidity was observed, ultimately leading to the formation of clot-like masses (Senior et al., 1991). Litzinger and co-workers (Litzinger et al., 1996) have shown that complexes formed from DC-Chol:DOPE, (3 β[N-(N',N' dimethylaminoethane) carbamoyl] cholesterol:dioleoylphosphatidylethanolamine) liposomes and oligonucleotides at a

charge ratio of 4:1 (+/-) were preferentially distributed to the lung following tail vein injection. Less than 10% of the dose was found in the liver. The authors suggested that the accumulation of complex in the lung may result from interactions with blood components leading to an increase in the size of the complex, thereby facilitating lodging in the pulmonary capillary bed (Litzinger et al., 1996).

The ability of cationic liposomes alone, or when complexed with plasmid DNA, to interact with plasma complement has been demonstrated *in vitro* (Devine et al., 1994; Plank et al., 1996). Activation of complement *in vivo* could lead to the coating of complex with complement proteins thereby targeting it to complement receptors present on pulmonary endothelium (Zhang et al., 1986)(Varsano et al., 1995). Indeed, Loughrey and colleagues (Loughrey et al., 1990) have shown that the platelet-dependent localisation of liposomes in the lung and liver of rabbits (Doerschuk et al., 1989) is mediated by complement. In addition, several investigators have suggested that complement proteins mediate the clearance of cationic lipid-based moieties *in vivo*, via the reticuloendothelial system, thus limiting the efficacy of cationic liposomes as systemic drug or gene delivery vehicles (Chonn et al., 1991; Devine et al., 1994; Plank et al., 1996). Nevertheless, complement-mediated clearance of cationic liposomes has not been demonstrated *in vivo* nor has complex-dependent complement activation. We have hypothesized that if cationic lipid/DNA complexes activate complement, the resulting interaction could influence systemic gene delivery. Our goal was to determine if preventing opsonization of lipid-DNA complex by complement would alter the extent of gene expression in the lung or influence the tissue distribution of cationic lipid-DNA complexes upon IV injection.

2. Materials and Methods

Animals

Experiments were conducted on 4-6 week-old female ICR mice purchased from Simonsen (Gilroy, CA). All animals were handled in accordance with protocols established by the National Institute for Health Guidelines for the Care and Use of Laboratory Animals, and with the approval of the Committee for Animal Research at the University of California, San Francisco. Animals were sacrificed at stated times with a sodium pentobarbital overdose.

Chemicals and Reagents

The cationic lipid 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids (Alabaster AL, USA). Cholesterol and Cobra Venom Factor (CVF) were purchased from Sigma (St. Louis, MO, USA). Goat anti- mouse C_3 antibodies were purchased from Cappel (Durham, NC).

Plasmid DNA

pCMVLuc plasmid DNA encoding the firefly *Photinus pyralis* luciferase gene was the generous gift of Dr. Keelung Hong (Hong et al., 1997; gene expression studies) and GeneMedicine Inc., (The Woodlands, TX, USA) (distribution experiments). Plasmid DNA was amplified in DH5α sub-cloning efficiency *E. coli* (Gibco BRL, Gaithersburg,

MD, USA), extracted by alkaline lysis and purified by centrifugation through two cesium chloride/ethidium bromide gradients according to standard laboratory procedures (Sambrook, 1989). Plasmid DNA was then dialyzed against 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and ethanol precipitated. The purity and identity of the plasmid DNA preparation was confirmed by absorbence measurements at 260 and 280 nm and through gel electrophoresis of plasmid restriction digest products. Endotoxin was reduced to less than 20 EU/mg plasmid DNA by passing the DNA through a polymyxin B column (Bio-Rad, Hercules, CA. USA). Endotoxin levels were measured with a limulus amebocyte lysate assay kit (Kit QCL-1000, Biowhittaker, Walkersville, MD, USA).

Cationic Lipid-DNA Lipoplex Preparation and Injection

DOTAP and cholesterol were dissolved in chloroform at a 1:0.9 molar ratio then evaporated to dryness on a rotary evaporator and placed under vacuum overnight to remove the remaining solvent. The dried lipid film was hydrated in 10% glucose/10mM Hepes buffer (pH 7.4) over a a period of 30 minutes during which time the suspension was agitated on a vortex mixer for 30 second intervals about 10 times. The hydrated lipid suspension was extruded through a 0.2 μm polycarbonate membrane. Liposome-DNA complexes were formed at room temperature by mixing equal volumes of plasmid DNA diluted in dH_2O with the liposome preparation to yield a complex with a 5:1 charge ratio (+/-) and were allowed to stand for 15 minutes prior to injection. The diameter of the complex was measured using dynamic light-scattering (Coulter Electronics, Hialeah, FL,

USA). Each mouse received a 100 μL dose administered via tail-vein injection containing 900 nmol total lipid and 90 nmol (30 μg) plasmid DNA except where stated otherwise.

Luciferase Assay

Twenty-four hours following administration of cationic lipid/DNA complexes, animals were anesthetized with an intraperitoneal (IP) injection of pentobarbital (75 mg/kg). Approximately 1 mL of blood was removed via intracardiac puncture. One milliliter of phosphate buffered saline (PBS) was perfused through the right cardiac ventricle. The heart, lung and a 300 mg portion of the liver were removed and reporter gene expression measured with the Luciferase Assay System (Promega, Madison, WI, USA). Tissues were placed in 2 mL polypropylene tubes containing 1.25 mL of lysis buffer (Promega, Madison, WI, USA) and homogenized utilizing a bead-mill (Mini-Beadbeater; Biospec Products, Bartlesville, OK, USA). Samples were then spun at 12,000 RPM in a microcentrifuge (7 minutes at 12,000x *g*) and 10 μL of the supernatant analyzed for luciferase activity as previously described (Meyer et al., 1995). Reporter gene activity was expressed as picograms of luciferase activity per mg of tissue protein.

Complement Depletion Procedure

Cochrane and colleagues (Cochrane et al., 1970) have demonstrated that a glycoprotein in the venom of *Naja naja* termed cobra venom factor (CVF), has significant anti-complementary activity. CVF was administered in 3 IP injections 12 hours apart beginning 48 hours prior to administration of cationic lipid-DNA complex. The total dose

of CVF (250 U/kg) was dissolved in PBS and administered in 3 equal doses. To ensure complete complement depletion, antibodies against complement factor C3 were administered intraperitoneally 2 hours prior to cationic lipid-DNA complex injection.

Complement Assay

Complement activity was measured utilizing an hemolytic assay adapted in our lab (Plank et al., 1996) to a 96-well plate format from methods described by Whaley (Whaley, 1985). Briefly, sheep red blood cells obtained from the UCSF Animal Care Facility were sensitized with anti-sheep red blood cell stroma according to Whaley (Whaley, 1985) and diluted to a concentration of 2 x 10^9 cells/mL in GVB^+ (145 mM sodium chloride, 25 mM sodium barbital, 1mM magnesium chloride, 0.15 mM calcium chloride, and 1% gelatin, pH 7.4)

Serum was collected according to established laboratory procedures from mice following anesthetization with pentobarbital as outlined above. In order to accurately determine CH50 values for serum from animals without complement depletion, it was necessary to initially execute 3-fold serum dilutions. Subsequent 1.2-fold serial dilutions of serum were performed in round-bottomed 96-well plates with GVB^+. To the same wells, sensitized erythrocytes were added to a final concentration of $1x10^9$ cells/mL. Samples were incubated at 37 °C for 1 hour with vigorous shaking. Unlysed erythrocytes were pelleted by centrifugation at 2,000 x *g* and 100 µL of the supernatant was transferred to flat-bottomed plates and released hemoglobin measured at 410 nm with an ELISA plate reader. CH50 values were then calculated according to published procedures

(Whaley, 1985). The CH50 value describes the serum dilution necessary to lyse 50% of the erythrocytes in a given assay. The degree of complement depletion in treated mice was expressed by comparing their CH50 value with that of untreated mice (Plank, 1996).

Complement C3a Measurement

Human blood was drawn and allowed to stand at room temperature for 30 minutes then serum was prepared by standard lab procedures. Five hundred microliters of serum was incubated for 30 minutes with a quantity of complex reflecting that of the dose injected IV into mice in *in vivo* experiments (450 nmol total lipid and 45 nmol (15 μg DNA). Alternatively, to establish the level of C3a in serum upon complete complement activation, serum was incubated with 4.5 U of cobra venom factor for 30 minutes. Complex was added to the latter treatment following incubation, but prior to C3a measurement, to control for possible complex-based interference with the assay. Samples were frozen in liquid nitrogen and shipped on dry-ice for ELISA analysis by the National Jewish Center for Immunology and Respiratory Care (Denver, CO).

Radiolabeling of Plasmid and Lipid Components of Lipoplex

To study the distribution of the cationic lipid DNA complexes *in vivo,* radio-labeled DNA and lipid were combined, injected into the tail-veins of mice and then tissue levels were measured at various time points.

Plasmid DNA was labeled according to the protocol of Meyer and colleagues (Meyer et al., 1995). Plasmid DNA was nick-translated with ^{125}I-dCTP (NEN Research

Products, Boston, MA, USA), DNase I (GIBCO-BRL), and DNA Polymerase (GIBCO-BRL). The nick translation procedure was optimized to yield intact plasmid DNA with a specific activity of 0.32 μCi/μg DNA. p-hydroxybenzamidine phosphatidylethanolamine (BPE) was synthesized and radio labeled with $Na^{131}I$ as previously described (Abra et al., 1982). Labeled lipid, in chloroform was added to the DOTAP:CHOL preparation to yield a specific activity of 75 mCi/mole total lipid (assuming 2 $x10^{12}$ dpm/Ci). Liposomes were then prepared as outlined above.

3. Results

In Vivo *Complex-mediated Complement Depletion*

Complement depletion by cationic liposomes or cationic lipid-DNA complexes has been demonstrated *in vitro* (Chonn, et al., 1991; Devine et al., 1994; Plank et al., 1996) but to date, similar experiments have not been performed *in vivo*. We measured serum complement levels in mice 24 hours after tail-vein injection of DOTAP:CHOL-plasmid DNA complex (900 nmol total lipid and 90 nmol (30 μg) plasmid DNA) prepared at a 5:1 charge ratio (+/-). Figure 1 shows that serum complement was depleted

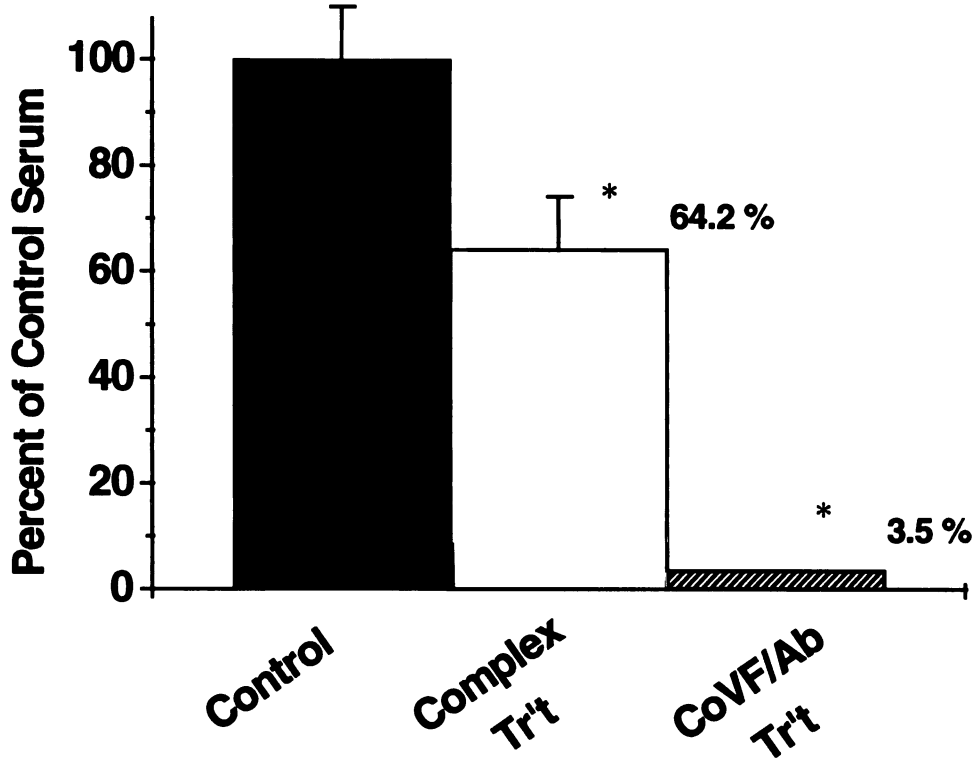

Figure 1. Complement Depletion in Mice Following Administration of Lipoplex or CVF and C3 Antibodies

Complement depletion in murine serum after injection of DOTAP:CHOL-plasmid DNA complex or CVF/C3 Ab treatment. The assays were carried out 24 hours after lipoplex injection as outlined in Materials and Methods. CH50 values obtained upon injection of complex or CVF/C3 Ab were expressed as a percentage of control serum obtained from untreated animals ± S.E.M. Each group consisted of 6 animals. * Significant difference vs control group as determined by Student's t-test $p \leq 0.05$.

by 36 ± 4% when compared to saline-injected control mice measured 24 hours post-injection.

To demonstrate that the depletion of complement-mediated hemolytic activity was a reflection of complement activation and not adsorption of complement proteins to the cationic complex, we measured the plasma concentration of C3a, a circulating product of C3 activation (Table 1). C3a levels were quantified upon incubation of 500 μL of human serum with complex for 30 minutes at 37°C. The quantity of complex added (450 nmol total lipid and 45 nmol (15 μg) DNA) reflected that of the dose injected IV into mice in *in vivo* experiments. We found that C3a levels in serum were elevated considerably relative to those of untreated serum. Quantitation of complement activation is possible based on the assumption that the amount of CVF added to serum prior to incubation yields complete activation of serum complement (Cochrane, 1970; Pepys 1975). According to the calculation below, C3a levels are elevated to 33 % of maximal following incubation with complex.

$$\text{C3a Activation} = \frac{([\text{C3a}]\text{ in serum incubated with complex}) - ([\text{C3a}]\text{ in control serum})}{([\text{C3a}]\text{in serum treated with CVF and complex}) - ([\text{C3a}]\text{ in control serum})}$$

This indicates that the decrease in hemolytic activity observed in mice following complex injection (Figure 1) is the product of complex-mediated complement activation rather than a non-specific complex-complement interaction.

Serum Treatment	Serum C3a Concentration
	μg/mL
Control Serum	7.8 (5.6)[a]
Serum Incubated with Complex	45.5 (1.3)[a]
Serum Incubated with CVF	167.1 (2.4)[a]
Serum Incubated with CVF + Added Complex[c]	123.4 (3.3)[a]

[a] Values represent means of two samples and the range in ng C3a per mL serum.
[b] Serum blank is untreated human serum.
[c] Samples were incubated with CVF for 30 minutes at 37°C prior to addition of complex.

Table 1. ELISA analysis of complement component C3a generated in untreated human serum and after incubation with complex or CVF.

Generation of complement component C3a in human serum as measured by ELISA. Five hundred microliters of human serum was incubated for 30 minutes at 37°C with DOTAP:CHOL-plasmid DNA complex prepared at a 5:1 charge ratio (+/-) (900nmol total lipid and 90nmol (30μg) plasmid DNA), 4.5U CVF alone or with CVF followed by the addition of complex.

In Vivo Complement Elimination

Mice were treated with CVF and C3 antibodies as outlined in materials and methods. Twenty-two hours after the final IP CVF administration, goat anti-mouse C3 antibodies were injected IP. Animals were sacrificed 2 hours later and serum complement activity was measured in treated mice using an hemolytic assay. Plasma complement depletion was greater than 95% when compared to saline-injected control animals as shown in Figure 1. This confirms that the CVF and C3 antibody protocol employed was an effective means of eliminating complement activity in mice.

Effect of Complement Elimination on Systemic Gene Transfer

To determine if complement influences intravenous cationic lipid-mediated gene transfer, complement-depleted mice were injected via the tail-vein with increasing doses of DOTAP:CHOL-pCMVLuc plasmid DNA complex prepared at a 5:1 charge ratio (+/-). The heart, lung, liver and spleen were assayed for luciferase activity 24 hours later. The dose-response curve for luciferase expression in all animals was linear between 10 μg and 60 μg plasmid DNA per animal (Figure 2). In this dose-range, expression was highest in the lung, followed by the heart, liver and spleen . At doses ranging from 3-60 μg plasmid DNA and a corresponding increase in cationic lipid, no significant alteration in transgene expression was observed in tissues of complement depleted mice when compared to complement intact animals. These data establish that serum complement proteins are not modifying the ability of systemically administered cationic lipid-based gene delivery vectors to transfect tissues.

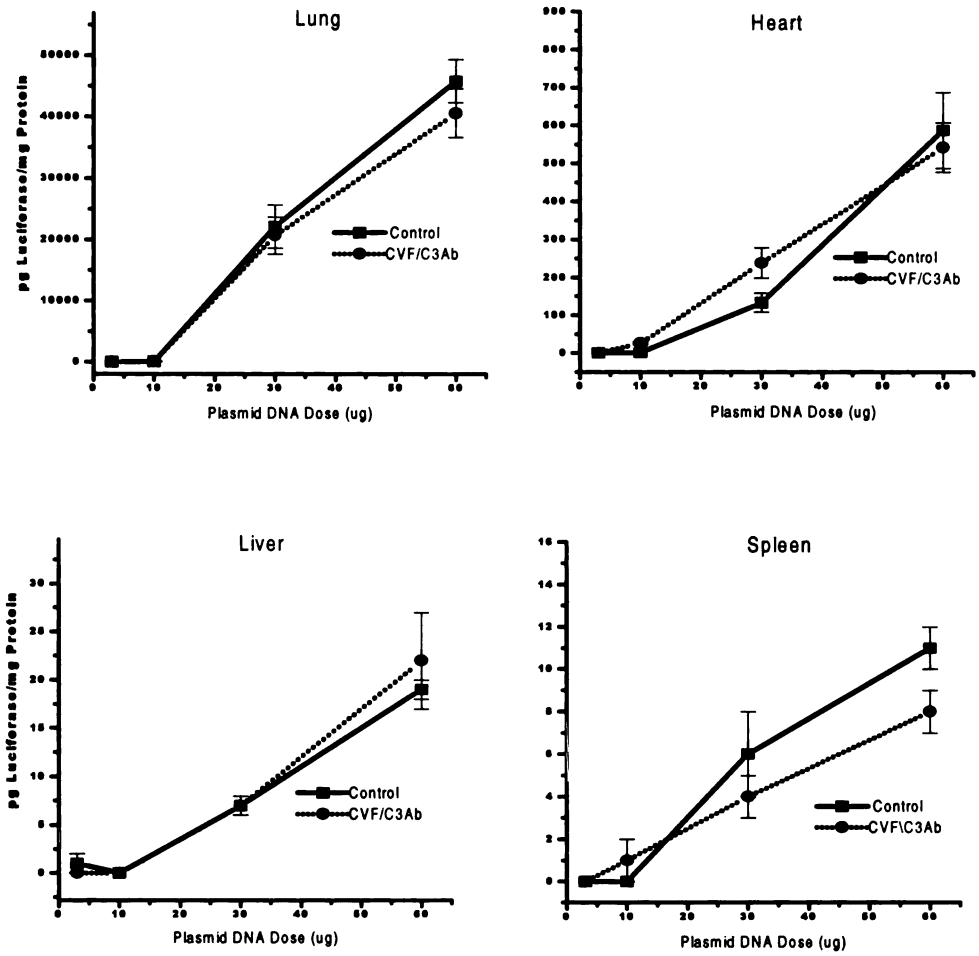

Figure 2. The Effect of Complement Depletion on Luciferase Gene Dose Response in Transfected Murine Tissues

Luciferase reporter gene expression after intravenous complex injection in complement intact and complement depleted female ICR mice. Individual mice were either untreated or complement depleted as described in Materials and Methods. Animals then received the indicated dose of pCMVLuc plasmid DNA complexed with a corresponding DOTAP:CHOL (1:1 mol:mol) liposome preparation to yield complex with a 5:1 charge ratio (+/-). Twenty-four hours later, animals were sacrificed and tissues analyzed for luciferase activity as described in Materials and Methods. Results are expressed as the mean of six samples ± S.E.M.

Effect of Complement Elimination on the Distribution and Pharmacokinetics of Cationic Lipid-DNA Complex

We have investigated the effect of complement depletion on the distribution of IV-administered DOTAP:CHOL-plasmid DNA complex (5:1, +/-) by administering radio labeled complex to complement-depleted mice and measuring the radioactivity associated with tissues at various times post-injection (Figures 3a and 3b). We found that the systemic disposition of the complex was not dependent on the presence of serum complement proteins.

In both treatment groups, blood clearance of complex was very rapid, leaving less than 1% of the injected dose in circulation 5 minutes after injection (data not shown). Labeled plasmid DNA and lipid accumulation in the lung at 5 minutes and 15 minutes post-injection exceeded 70% of the dose administered in both treatment groups (Figures 3a and 3b). Forty-five minutes post-injection, at least 60% of the administered dose of complex was present in the lung. After 135 minutes, 40% of both the plasmid DNA and lipid label is detected in the lung. Both labels decline to approximately 5% at 1215 minutes post-injection (Figures 3a and 3b). Initial distribution of complex to the liver was lower but tended to increase slowly with time. In the lung, heart, liver and spleen, the amount of accumulated complex did not vary significantly between complement depleted and complement intact animals at any of the times assayed (Figures 3a and 3b; heart and spleen not shown), demonstrating that cationic lipid-DNA complex biodistribution was not complement dependent.

To investigate the relative amount of each complex component distributed post-

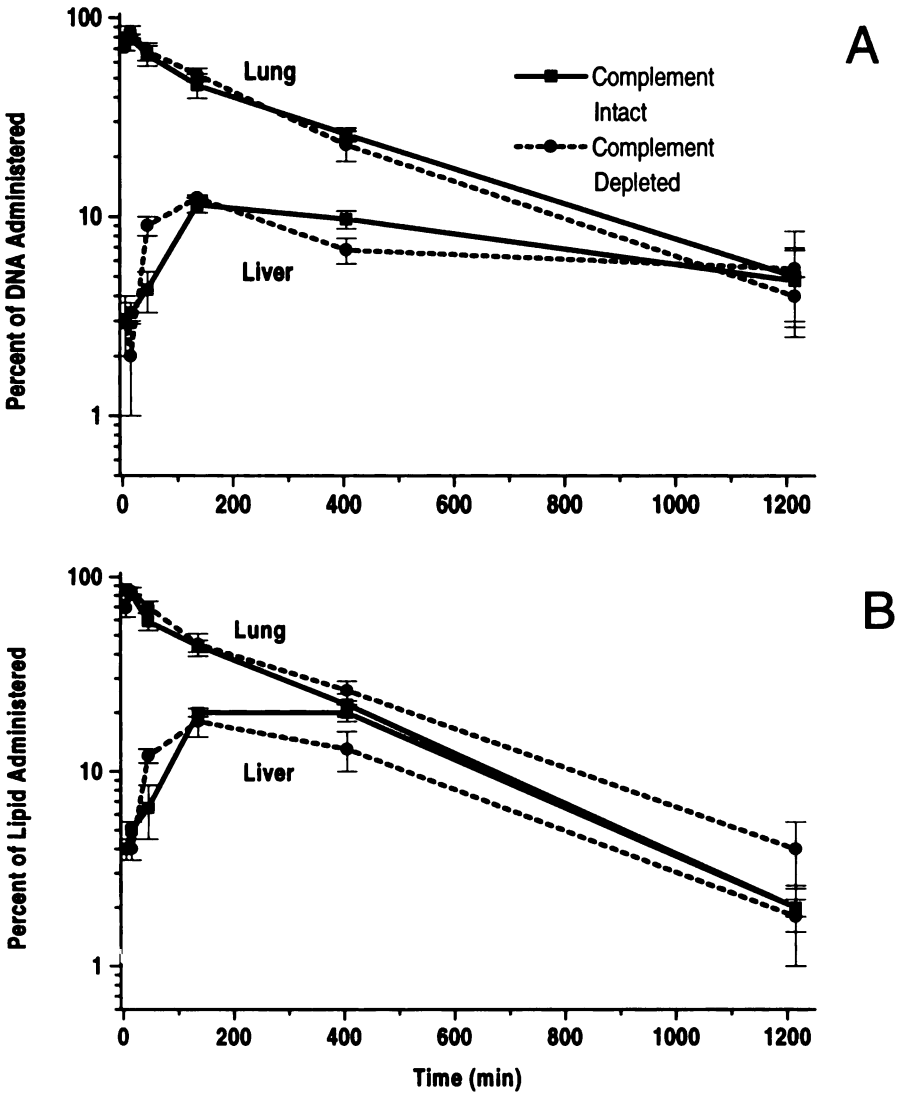

Figure 3a and 3b. Lipoplex Component Distribution In Murine Tissues Following I.V. Injection

Distribution of I.V.-administered DOTAP:CHOL-plasmid DNA complex (5:1, +/-) labeled with ^{125}I -dCTP (plasmid DNA) and ^{131}I-BPE (liposomes) in mice as a function of time. Complex was injected into the tail-vein of female ICR mice and radioactivity measured in various tissues at time points given. Results are expressed as the mean percent of dose administered ± S.E.M. for 4 animals.

injection, we expressed the percent of the dose of DNA and lipid recovered in each tissue as a ratio of ^{131}I (lipid) to ^{125}I (DNA) over time (Figure 4). The starting ratio of labeled DNA to labeled lipid was 1.5. Although label ratios varied from tissue-to-tissue, the ratio of lipid to DNA did not differ between the complement-depleted and intact animals. Five minutes subsequent to injection, the ratio of DNA to lipid in the lung had decreased to approximately 1.0 (Figure 4). For a given tissue, the relative proportions of the two labels remained constant over all but the last time point. After 1215 minutes, the ratios calculated for the lung and liver (and other tissues measured but not shown) decreases, indicating a relative loss of ^{131}I. After approximately 2 hours, the proportion of lipid label versus DNA label begins to decrease in the thyroid and stomach (data not shown). This is consistent with the degradation of the ^{125}I DNA label causing the release of free iodide which then is taken up by the thyroid and stomach. This observation is corroborated by previous work done in this group in which the degradation of the DNA label was monitored with Southern blot assays (Levy, 1996). We found that the time point at which radioactivity began accumulating in thyroid and stomach coincided with a loss of DNA integrity as evidenced with Southern blots generated with DNA extracted at the same time point. The ^{131}I label on BPE is less labile in serum and is primarily secreted in the urine (Abra et al., 1982).

4. Discussion

Although a number of groups have demonstrated that the efficiency of nucleic

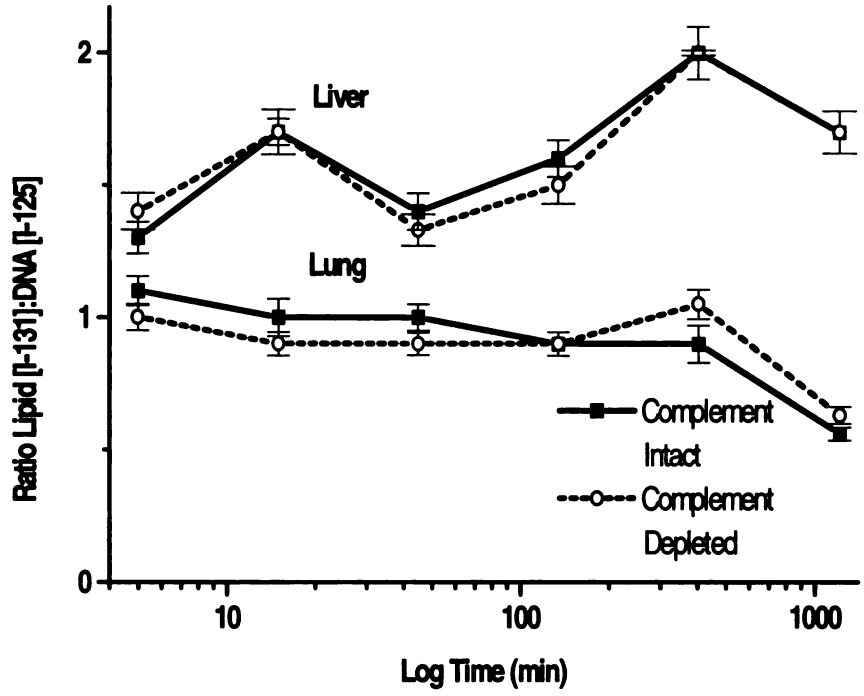

Figure 4. Ratio of ^{131}I-labeled Cationic Liposome to ^{125}I-labeled Plasmid DNA in Murine Tissues as a Function of Time.

Following I.V.-administration of DOTAP:CHOL-plasmid DNA complex (5:1, +/-) labeled with ^{131}I-BPE (liposomes) and ^{125}I -dCTP (plasmid DNA), tissues were assayed for associated radioactivity and the measurements were expressed as a ratio of the percent of dose administered of ^{131}I to ^{125}I at indicated time points. Dotted lines represent complement depleted animals. Each ratio is the mean of 4 animals ± S.E.M.

acid delivery is reduced *in vitro* in the presence of serum, (Felgner et al., 1987; Senior et al., 1991; Zelphati et al., 1996) systemic gene transfer has been achieved in mice after IV administration of cationic lipid-DNA complexes (Brigham et al., 1989; Brigham et al., 1993; Canonico et al., 1994; Solodin et al., 1995; Liu et al., 1997a; Liu et al., 1997b). Thus, the significance of the role that blood components play in modulating gene delivery via cationic lipids is not clear. It is conceivable that plasma proteins interact with cationic lipid-nucleic acid complexes and attenuate their activity upon IV injection through dissociation of the complex components, by targeting the complex for phagocytosis via opsonisation or simply by charge neutralizing the complex upon coating by serum proteins. Conversely, gene delivery to some tissues may be facilitated by interactions between positively charged cationic lipids and negatively charged plasma proteins. We have demonstrated that increasing the charge ratio associated with cationic lipid-DNA complexes can increase the transfection mediated by these complexes in the heart and lung (unpublished results). It is possible that the increase in transfection is a reflection of increased electrostatic interactions between cationic lipids and anionic serum proteins leading to deposition of complex in tissues through the formation of complex-based aggregates that lodge in capillary beds. Alternatively, plasma proteins that associate with the complex might target the complex to cell surface receptors that interact with these plasma proteins. Litzinger and colleagues (Litzinger et al., 1996) have speculated that increased delivery of cationic lipid oligonucleotide complex to the lung upon IV injection may be facilitated by the interaction between blood components and complex.

We have shown, for the first time, that cationic-lipid DNA complex-mediated

depletion of complement occurs *in vivo* such that complement-mediated hemolytic activity is reduced by 35 % in mice (Figure 1). Moreover, we assayed human serum for a product of C3 activation, complement C3a, and found its levels increased following incubation with cationic-lipid DNA complex (Table 1). This indicates that the depletion of complement activity *in vivo* is the manifestation of complement consumption rather than a simple electrostatic interaction between complex and complement proteins.

Although DNA is capable of binding to (Uwatokao et al., 1990) and activating complement (Jiang et al., 1992), Plank and colleagues (Plank et al., 1996) found that DNA, at concentrations similar to those present in liposome-DNA complexes, did not activate complement to the same degree as the complex itself. Cationic lipid alone caused greater complement activation than equal amounts of lipid complexed with DNA at a high charge ratio (6:1, +/-). As Plank and coworkers state, the implication is that complement activation, in this context, is dependent on the availability of cationic groups for interaction with complement proteins. A fraction of these cationic groups would be neutralized by anionic phosphates present on DNA molecules thus attenuating the complement activation properties of complex versus cationic lipid alone.

To study the effect of complement activation on systemic complex-mediated transgene expression, we applied a protocol developed for the elimination of serum complement *in vivo*. Cochrane and colleagues (Cochrane et al., 1970) and later Pepys (Pepys, 1975) demonstrated that the IP injection of cobra venom factor (CVF) is an effective means of complement elimination in mice. This protocol, when combined with IP administration of anti-mouse C3 antibodies, reduced serum complement by 97%

(Figure 1). This treatment facilitated a comparison of cationic lipid mediated gene delivery between complement intact animals and otherwise identical, complement depleted animals.

We found no detectable difference in gene expression between the complement intact and complement depleted animals in the tissues tested, indicating that complement proteins do not influence gene delivery by cationic lipid-based complexes (Figure 2). Several groups, however, have demonstrated that neutral and negatively charged liposomes interact with serum complement proteins (Chonn et al., 1991; Chonn et al., 1992; Harashima et al., 1994; Liu et al., 1995a; Liu et al., 1995b). Cationic liposomes also deplete serum complement proteins and this depletion is apparently a reflection of serum complement recognition of the charged lipid as foreign, resulting in opsonization *in vitro* (Chonn et al., 1991; Devine et al., 1994; Plank et al., 1996). Although we have found that complement is activated to a measurable degree upon injection of DOTAP:CHOL-plasmid DNA complex, it appears that this interaction neither interferes with nor increases transfection mediated by the complex.

Plank and colleagues (Plank et al., 1996) have speculated that complement receptors present on the pulmonary endothelium (Zhang et al., 1986; Varsano et al., 1995) may act to target opsonized complex to the lung and may explain the considerable transfection noted in this organ upon IV injection (Plank et al., 1996) however, our results demonstrate that complement is not promoting the transgene expression we observe in the lung and heart following IV injection of complex (Figure 2).

To determine if the biodistribution of complex was altered in the presence of

serum complement proteins, we administered radio labeled cationic lipid-DNA complex IV to mice and monitored its distribution in animals with either intact or depleted complement systems. We found that complement depletion did not significantly affect complex distribution in any of the tissues assayed. The lack of influence that complement depletion had on the distribution of complex in the liver and spleen was particularly surprising in light of the role complement typically plays in opsonization and targeting of foreign moieties to the reticuloendothelial system. Our distribution data was similar to that of previous studies (Mahato et al., 1995; Litzinger et al., 1996; Liu et al., 1997a) which showed cationic-lipid nucleic-acid complexes prepared with an excess positive charge were rapidly cleared from the blood, and initially accumulated in the lung following tail-vein injection. Initial accumulation in the liver was approximately 10%.

There is a clear correlation between the distribution of complex to the lung and the expression detected therein. When prepared at a 5:1 charge ratio, the complex is primarily taken up by the lung (Figure 3a and 3b), and it is in this tissue where the greatest level of gene expression was detected (Figure 2). The considerably lower transfection level detected in the liver is a function of the small amount of complex delivered to this tissue. It is also likely that much of the complex delivered to the liver is taken up by and degraded in Kupffer cells, obviating subsequent gene delivery. Taken together with the expression data, (Figure 2), the biodistribution patterns suggest that complement proteins do not promote the localization of complex in the lung via complement receptors present on the lung endothelium.

Although the initial ratio of lipid label to DNA label in the complex is 1.5, the

measured ratio of the dose percent of lipid recovered to that of DNA in the lung is approximately unity at 5 minutes post-injection (Figure 4). This is likely a reflection of the high charge ratio at which the complex is formulated: Since the cationic lipid component is formulated to be in excess, some liposomes may remain uncomplexed and of smaller size than the lipoplex and therefore they are not extracted from circulation by the pulmonary microvasculature. Litzinger and co-workers (Litzinger et al., 1996) have shown that liposomes injected alone do not localize in the lung, rather they accumulate in the liver following IV injection. However, if cationic liposomes were complexed with oligonucleotides prior to injection, they found that complex is delivered preferentially to the lung by a size-dependent mechanism, possibly involving the formation of emboli in the lung microvasculature. In any event, we have found that the ratio of the 2 labels in the lung remains approximately one until the last time point of the experiment (Figure 4). This suggests that the complex, after establishing an equilibrium between the proportion of lipid and DNA component localized at the lung, may remain relatively intact for a considerable amount of time. The elimination half-life in the lung for both the lipid and the DNA is approximately 6 hours.

The ratio of labels taken up in the liver (Figure 4) remains close that of the originally injected complex -1.5. Considering that the lung appears to be preferentially extracting complex from circulation, one would expect the liver uptake of circulating uncomplexed lipid to be reflected in a higher ratio than 1.5, yet, at 5 minutes post-injection, it does not. This phenomenon may indicate that the liver, like the lung, is initially taking up only complex, however, by 15 minutes post-administration,

accumulation of uncomplexed lipid in the liver is reflected in the ratio increase at this time point. In the spleen, a significant amount of label does not accumulate initially, due to its small size.

The marked increase in the ratio of ^{131}I to ^{125}I after 1215 minutes in the lung and liver, suggests that at this point, the ^{131}I bound to BPE or its metabolites were being slowly excreted in the urine as described by Abra and colleagues (Abra et al., 1982) while the ^{125}I accumulates in the thyroid and stomach (data not shown). The greater accumulation of ^{131}I versus ^{125}I in the bladder at this time point supports this explanation (data not shown).

In summary, in spite of the fact that cationic lipid-DNA complexes activate the complement system to a considerable degree, there was no significant difference in reporter gene expression levels nor complex distribution in various tissues upon depletion of complement proteins. This implies that the interaction between complex and complement does not alter the properties of the injected complex to the extent that gene delivery is altered. Given the clear role in immunosurveillance that the complement system maintains and the plethora of experimental work supporting the hypothesis that complement plays an important role in liposome distribution and clearance, this result is surprising. The simple interpretation of this phenomenon is that, in spite of significant opsonization, the majority of the complex is trapped in the pulmonary microvasculature where it is taken up or degraded such that it can no longer transfect other tissues. In effect, the lung capillary beds act as a very efficient filter.

It is also conceivable that other proteins coat the positively charged complex so

thoroughly upon injection that significant activation of complement is prevented. Indeed, in chapter II we have shown that the net surface charge on complex upon incubation with serum albumin is negative suggesting that significant interactions between and lipoplex and plasma proteins is possible.

Blood components which could potentially influence cationic lipid-DNA mediated gene delivery are manifold. The positive charge of these moieties makes them intrinsically reactive in the blood, the protein and cellular components of which are almost exclusively negatively-charged. Complement proteins and albumin have been eliminated as significant candidate proteins for governing lipoplex-mediated systemic transfection, but future work will involve analysis of other plasma proteins that may function to promote or inhibit the cationic lipid-DNA mediated transfection we observe following IV injection.

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Chapter IV: Lipoplex Mediated Gene Delivery to the Pulmonary Endothelium Occurs within 60 Minutes of Systemic Administration

1. Introduction

In chapters II and III, we studied the nature of lipoplex binding interactions in the vascular compartment and discussed how such couplings lead to endothelial uptake following IV injection. In this chapter, we investigate the time period following administration within which lipoplex internalization occurs.

When positively charged lipoplexes are administered systemically, the lipid and DNA components are cleared from circulation within minutes of injection and can then be detected in the lungs for several hours post-injection. (McLean et al., 1997; Liu et al., 1997a; Liu et al., 1997b and Barron et al., 1998). Pulmonary accumulation peaks within 5 minutes following IV injection and declines to approximately 10% of the administered dose by 24 hours post-injection (Barron et al., 1998). The early accumulation of cationic lipoplexes in the lung coincides with that tissue yielding the highest level of gene expression after IV lipoplex administration. Liu and colleagues detected pulmonary luciferase reporter activity 2 hours subsequent to injection of lipoplex containing the luciferase gene (Liu et al., 1997a). This activity increased to a maximum at 10-24 hours post-administration and then declined to less than 10% of peak levels in 72 hours.

We were interested in learning if there is a critical time period within which pulmonary binding and uptake occurs after systemic administration of lipoplexes. We

reasoned that disruption of the lipoplex should block lung transfection. To dissociate the lipoplex we used anionic liposomes which have previously been shown to effectively displace DNA from the cationic lipid -DNA complex *in vitro* (Xu and Szoka, 1996). We injected anionic liposomes at defined time points post-lipoplex injection to disrupt the lipoplex following its distribution into the pulmonary microvasculature and then measured the level of gene expression 24 hours later. We show that there is a critical interval of 60 minutes after the injection of the lipoplex when transfection of the lung can be dramatically reduced by this protocol.

2. Materials and Methods

Animals

Experiments were conducted on 3-6 week-old female CD-1 mice purchased from Simonsen (Gilroy, CA). All animals were handled in accordance with protocols established by the National Institute for Health Guidelines for the Care and Use of Laboratory Animals, and with the approval of the Committee for Animal Research at the University of California, San Francisco. Animals were sacrificed at stated times by exsanguination following anesthetization with an intraperitoneal (IP) injection of ketamine (200mg/kg), xylazine (10mg/kg) and acepromazine (2mg/kg).

Chemicals and Reagents

One-palmitoyl-2-oleoyl-*sn*-glycero-3-(phospho-*rac*-(1-glycerol)) (POPG), 1,2 dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids

(Alabaster AL, USA). Cholesterol was purchased from Sigma (St. Louis, MO, USA).

Plasmid DNA

pCMVLuc plasmid DNA encoding the firefly *Photinus pyralis* luciferase gene was the generous gift of GeneMedicine Inc., (The Woodlands, TX, USA). Plasmid DNA was amplified in DH5α sub-cloning efficiency *E. coli* (Gibco BRL, Gaithersburg, MD, USA), extracted by alkaline lysis and purified by centrifugation through two cesium chloride/ethidium bromide gradients according to standard laboratory procedures (Sambrook et al., 1989). Plasmid DNA was then dialyzed against 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and ethanol precipitated. The purity and identity of the plasmid DNA preparation was confirmed by absorbance measurements at 260 and 280 nm and through gel electrophoresis of plasmid restriction digest products. Endotoxin was reduced to less than 20 EU/mg plasmid DNA by passing the DNA through a polymyxin B column (Bio-Rad, Hercules, CA. USA). Endotoxin levels were measured with a limulus amebocyte lysate assay kit (Kit QCL-1000, Biowhittaker, Walkersville, MD, USA).

Cationic Lipid-DNA Complex Preparation

DOTAP and cholesterol were dissolved in chloroform at a 1:0.9 molar ratio then evaporated to dryness on a rotary evaporator and placed under vacuum overnight to remove residual solvent. The dried lipid film was hydrated in 10% glucose/10mM Hepes buffer (pH 7.4) at 25°C over a period of 30 minutes during which time the suspension was agitated on a vortex mixer for 30 second intervals about 10 times. The final

concentration of DOTAP in the lipoplex preparation was 4.5 mM while that of cholesterol was 4.1mM. The hydrated lipid suspension was extruded through a 0.2 μm polycarbonate membrane. Liposome-DNA complexes were formed at room temperature by mixing equal volumes of plasmid DNA diluted in distilled water with the liposome preparation to yield a complex with a 5:1 charge ratio (+/-) and were allowed to stand for 15 minutes at room temperature prior to injection. The diameter of the complex was measured using dynamic light-scattering (Malvern Instruments Model 1000, Malvern, UK). Utilizing a multimodal data analysis package, the ζ average diameter of the lipoplex was determined to be 350 nm.

Small Unilamellar Vesicle Preparation

POPG was combined with either DOPE or cholesterol and dissolved in chloroform at a 1.9:1 molar ratio then evaporated to dryness on a rotary evaporator and placed under vacuum overnight to remove the remaining solvent. The dried lipid film was hydrated in 5% glucose/5 mM Hepes buffer (pH 7.4) over a period of approximately 15 minutes during which time the suspension was agitated on a vortex mixer for 5 second intervals about 5 times. The suspension was sonicated for 15-20 minutes in a bath sonicator (Laboratory Supplies Company Inc., Hicksville, NY) at 10°C until a translucent lipid dispersion was obtained. This preparation was formulated to yield a final POPG concentration of 22.5 or 67.5 mM which corresponded to a 5 or 15-fold charge excess, respectively, relative to that of the cationic lipid which was prepared at a final concentration of 4.5 mM. The diameter of the liposomes was measured using dynamic light-scattering scattering (Malvern Instruments Model 1000, Malvern, UK). Utilizing a

multimodal data analysis package, the ζ average diameter of the lower concentration anionic liposomes was 48 nm while that of the higher concentration was 98nm.

In Vitro *Lipoplex Dissociation and DNase I Susceptibility*

Cationic lipid-DNA complexes containing 15 μg of plasmid DNA were mixed with a 5-fold charge excess of anionic liposomes to a final volume of 150 μL and then incubated for 5 minutes at 37°C in 5 mM Hepes/5% glucose, pH 7.4. Serum incubations were carried out in the presence of 33% mouse serum. Ten units of DNase I were then added in 850 μL of digestion buffer (200 mM Tris, pH 8.4, 20 mM $MgCl_2$, 500 mM KCl) and the incubation was continued for a further 10 minutes. A 750 μL aliquot was removed and incubated in DNA extraction buffer (100 mM NaCl, 10 mM Tris, 25 mM EDTA (pH 7.4), 0.5% SDS, and 0.1 mg/mL Protease K,) in a final volume of 1.5 mL at 50 °C for 2 hours. A 200 μL aliquot was extracted twice in 200 μL of phenol:chloroform:isoamyl alcohol (25:24:1 vol/vol) and then precipitated in 2 volumes of ethanol and 1/10 volume 7.5 M ammonium acetate. The pellet was resuspended in 30 μL of distilled water, loaded onto a 1% agarose gel and electrophoresed at 110 V for approximately 1.5 hours and then the gel was stained with ethidium bromide.

Radiolabeling of Plasmid DNA and Lipid

To study the distribution of the cationic lipid DNA complexes *in vivo,* lipoplexes containing radio-labeled DNA or lipid were injected into the tail-veins of mice and tissue isotope levels were measured at various time points.

Plasmid DNA was labeled with the Gibco BRL nick translation kit (Kit # 18160-010, Gibco BRL, Gaithersburg, MD, USA) and ^{125}I-dCTP (NEN Research Products, Boston, MA, USA). The nick translation procedure yielded high molecular weight DNA with a specific activity of 0.32 μCi/μg DNA.

p-Hydroxybenzamidine phosphatidylethanolamine (BPE) was synthesized and radio-labeled with Na^{125}I as previously described (Abra et al., 1982). Labeled lipid, in chloroform was added to the DOTAP:CHOL preparation to yield a specific activity of 75 mCi/mole total lipid. Liposomes were then prepared as outlined above and the ensuing lipoplex preparation yielded a radiolabel dose of 200,000 cpm per injection.

In Vivo *Luciferase Assay*

Mice were injected intravenously with a 100 μL bolus of the lipoplex preparation described above containing 900 nmol total lipid and 90 nmol (30 μg) luciferase plasmid DNA. Twenty-four hours later animals were anesthetized with an intraperitoneal (IP) injection of ketamine (200 mg/kg), xylazine (10 mg/kg) and acepromazine (2 mg/kg) and sacrificed by exasanguination via intracardiac puncture. One milliliter of phosphate buffered saline (PBS) was perfused through the right cardiac ventricle. The heart, lung and a 300 mg portion of the liver were removed and reporter gene expression measured with the Luciferase Assay System (Promega, Madison, WI, USA). Tissues were placed in 2 mL polypropylene tubes containing 1.25 mL of lysis buffer (Promega, Madison, WI, USA) and homogenized utilizing a bead-mill (Mini-Beadbeater; Biospec Products, Bartlesville, OK, USA). Samples were then spun at 12,000 RPM in a microcentrifuge (7 minutes at 12,000x g) and 10 μL of the supernatant analyzed for luciferase activity as

previously described (Meyer et al., 1995). Reporter gene activity was expressed as picograms of luciferase activity per mg of tissue protein. A standard curve describing the correlation between relative light units and picograms of luciferase activity was constructed using luciferase enzyme obtained from Analytical Bioluminescence Laboratory (Product #2400, San Diego CA, USA).

In Vivo *Lipoplex Perturbation*

Reporter gene assays subsequent to anionic liposome injection were conducted as follows: At stated times following IV injection of a 100 μL lipoplex bolus, a 100 μL bolus of anionic liposomes was injected IV. Twenty-four hours post-lipoplex injection, animals were anesthetized as above, sacrificed by exsanguination and tissues assayed as outlined for luciferase activity.

DNA and lipid distribution assays following lipoplex dissociation were conducted as follows: Animals were IV injected with lipoplex containing radiolabeled DNA or lipids followed by an anionic liposome injection at stated times. Five minutes after the anionic liposome dose, animals were anesthetized as above and various tissues were removed for measurement of radiolabel at stated times.

3. Results

In Vitro *Lipoplex Dissociation*

To determine whether anionic liposomes could be employed to disrupt lipoplexes *in vivo*, we first assessed their ability to dissociate lipoplex in the presence of serum in an *in vitro* DNase I digestion assay (Figure 1). This assay is based on the ability of the

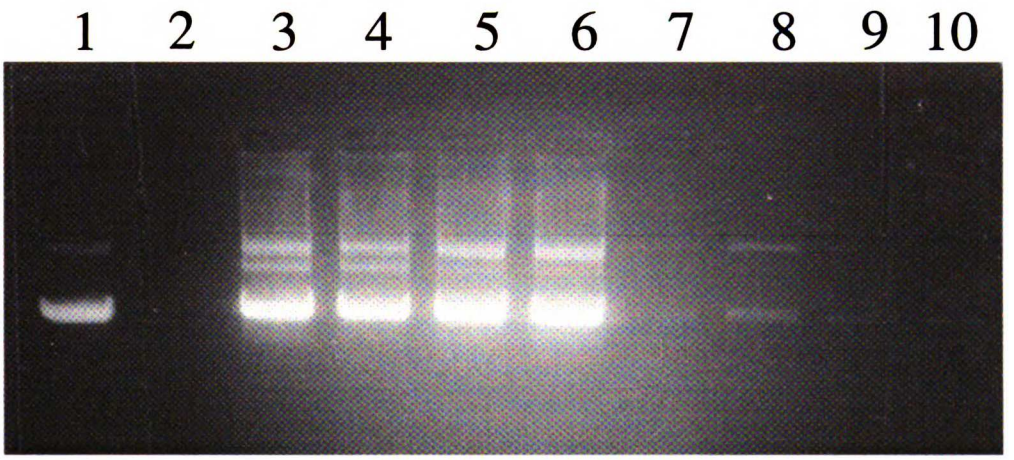

Figure 1. DNase I digestion of plasmid DNA upon incubation of complexes under various conditions

Cationic lipid-DNA complexes were prepared at a 5:1 charge ratio (+/-) as described in Materials and Methods section and then mixed with the indicated agents. The resulting preparations were then subjected to DNase I digestion for 10 minutes at 37°C. DNA was extracted and electrophoresed through a 1% agarose gel. Lane 1, Plasmid DNA (1.8 μg; lane 2, 6 μg plasmid DNA digested with DNase I; lane 3, lipoplex; lane 4, lipoplex digested with DNase I; lane 5, lipoplex incubated with serum; lane 6, lipoplex incubated with serum and then digested with DNase I; lane 7, lipoplex incubated with a 5-fold charge-excess of PG:CHOL-based (1.9:1, mol:mol) liposomes and then digested with DNase I; lane 8, lipoplex incubated with serum, a 5x charge-excess of PG:CHOL-based liposomes, and then digested with DNase I; lane 9, lipoplex incubated with a 5x charge-excess of PG:DOPE-based (1.9:1, mol:mol) liposomes and then digested with DNase I; lane 10, lipoplex incubated with serum, a 5x charge-excess of PG:DOPE-based liposomes and then digested with DNase I.

lipoplex to protect DNA from DNase I digestion, thus, DNA released from the lipoplex is degraded. As shown in lane 2 of figure 1, complete DNase I digestion of 2 μg of plasmid DNA occurs within 10 minutes; however, in the presence of cationic lipids, degradation is virtually eliminated (lane 4). Incubation of the lipoplex with 33% serum did not significantly affect the integrity of complexed DNA in the presence or absence of DNase I (lanes 5 and 6). The mixing of a 5-fold charge excess of anionic liposomes with the lipoplex yielded extensive release of plasmid DNA from the lipoplex as evidenced by the marked decrease in high molecular weight DNA present in lanes 7-10. A small amount of DNA is apparent in lane 8 suggesting that PG:CHOL liposomes were not as efficient at releasing DNA in the presence of serum as PG:DOPE liposomes (lane 10). These data demonstrate that PG-based liposomes are capable of releasing DNA from the lipoplex in the presence of serum.

Lipoplex-Mediated Luciferase Expression In Vivo Following Anionic Liposome Administration

Anionic liposome-mediated dissociation of the lipoplex was effected *in vivo* to determine the time period post-lipoplex injection within which lipoplex integrity is critical for transfection. Animals were injected with lipoplexes consisting of DOTAP:CHOL liposomes complexed with a luciferase reporter gene and then, at defined intervals post-lipoplex injection, a single bolus of anionic liposomes comprised of POPG:DOPE (1.9:1, mol:mol) or POPG:CHOL (1.9:1, mol:mol) was delivered IV. The anionic liposome dose was calculated to yield a nominal 5-fold charge excess versus that of the cationic lipid. No toxicity was apparent following either injection. The animals

were sacrificed 24 hours later and assayed for luciferase expression in the heart, lungs and liver (Figure 2). Pulmonary gene expression was significantly decreased in animals that received either anionic liposome preparation 5 minutes subsequent to lipoplex injection. Luciferase expression in the lungs was decreased 70-fold in animals injected with POPG:CHOL-based liposomes while those receiving liposomes comprised of POPG:DOPE exhibited a 200-fold reduction in this tissue. The larger decrease in gene expression is consistent with the notion that the DOPE-based anionic liposomes are more efficient in dissociating the lipoplex as demonstrated by the *in vitro* experiments above (Figure 1). When the negatively-charged liposomes were administered 60 minutes following lipoplex administration, there was no significant reduction in pulmonary gene expression. Luciferase activity in the heart and liver, although uniformly lower than in the lungs, followed a similar expression pattern in that both anionic liposome treatments depressed gene expression when administered at 5 minutes post-lipoplex injection. When DOPE was utilized as a helper lipid, greater reductions in gene expression were observed in the heart and liver than when CHOL was used. Although expression was higher in the heart when a 60 minute interval between lipoplex and negative liposomes was maintained, a significant reduction versus animals not treated with anionic liposomes still existed. IV injections of the 5mM Hepes/5% glucose and neutrally-charged POPC:DOPE liposomes were performed 5 minutes following lipoplex injection (Figure 3). Neither of these treatments significantly influenced transfection, indicating that the attenuation of gene expression following the injection of anionic liposomes may be attributed to the lipoplex-dissociative capacity of the negatively-charged liposomes and not to a non-specific lipid effect or due to buffer flushing lipoplexes from the lungs.

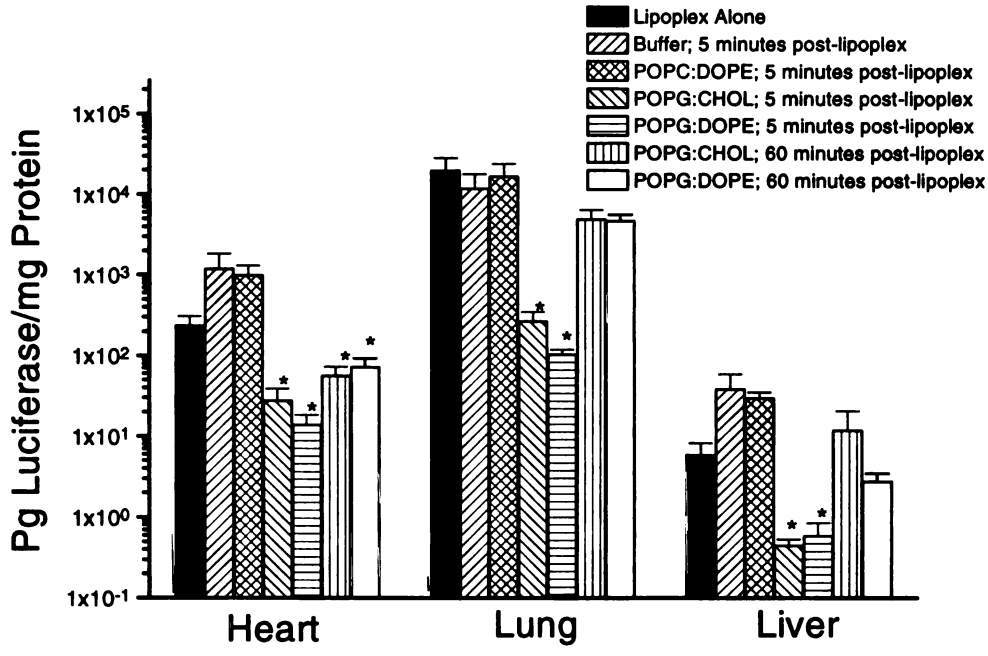

Figure 2. Lipoplex-mediated pulmonary luciferase expression in murine tissues following injection of a 5-fold charge excess of POPG:CHOL or POPG:DOPE liposomes.

Lipoplexes comprised of DOTAP:CHOL and a luciferase reporter gene were injected into the tail-vein of female CD-1 mice followed at stated time intervals by an IV bolus of anionic liposomes. Animals were sacrificed 24 hours later and tissues were assayed for luciferase activity as described in Materials and Methods. Results are expressed as the mean of 6 samples ± S.E.M. * Independent t-test significant difference at $P < 0.05$ versus lipoplex alone.

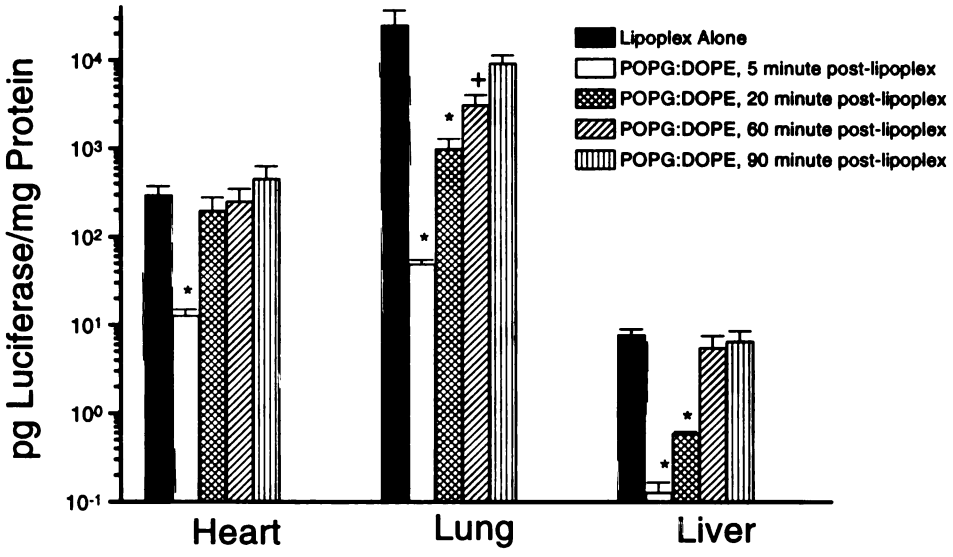

Figure 3. Lipoplex-mediated pulmonary luciferase expression in murine tissues following injection of a 15x charge excess of POPG:DOPE anionic liposomes.

Female CD-1mice were injected with lipoplex comprised of DOTAP:CHOL complexed with a luciferase reporter gene alone or lipoplex injection was followed 5 minutes later with 5mM Hepes/5% Glucose or neutral liposomes (POPC:DOPE). Anionic liposome-treated animals received lipoplex IV followed at stated time intervals by an IV bolus of anionic liposomes. Animals were sacrificed 24 hours later and tissues were assayed for luciferase activity as described in Materials and Methods. Results are expressed as the mean of 6 samples ± S.E.M. $^{+}$ Independent t-test significant difference at $P < 0.10$ versus lipoplex alone. * Independent t-test significant difference at $P < 0.05$ versus lipoplex alone.

Since the POPG:DOPE-based liposomes appeared to have a greater capacity to dissociate the lipoplex, this formulation was employed in subsequent experiments. To further define the time-window within which lipoplex dissociation affects gene expression, animals were dosed with POPG:DOPE liposomes at 5, 20, 60 and 90 minutes post-lipoplex injection. This experiment was conducted with negative liposomes constituting a nominal 15-fold charge excess versus the cationic lipid to determine if a greater excess of anionic liposomes would further decrease expression in the tissues assayed. Generally, the higher anionic liposome dose only moderately decreased expression observed in the lungs and liver versus that elicited by the 5-fold excess of negatively-charged lipids. In the heart, there was little change versus that in animals dosed with a 5-fold excess of anionic liposomes. When this anionic liposome preparation was injected 5 minutes post-lipoplex administration, pulmonary gene expression was reduced 400-fold versus animals injected with lipoplex only. Gene expression in the lungs was statistically significantly lower when negative liposome administration was carried out at 20 minutes post-lipoplex injection; however, it was 50-fold higher than that of animals injected with anionic liposomes at 5 minutes post-lipoplex administration. When anionic liposomes were administered at 60 minutes post-lipoplex injection the difference in pulmonary expression versus animals injected with lipoplex alone was significant at $p \leq 0.10$ while no significant difference was detected in animals treated with anionic liposomes at 90 minutes subsequent to lipoplex injection. In the liver, there was an 80-fold reduction in gene expression when anionic liposomes were injected 5 minutes subsequent to lipoplex administration. As in the lung, this difference was markedly

reduced when the negative liposomes were injected 20 minutes after the lipoplex. Although the administration of anionic liposomes 5 minutes after lipoplex injection produced a significant ($p \leq 0.05$) reduction in gene expression in the heart, negative lipid administration 20 or more minutes post-lipoplex injection did not attenuate gene expression.

These data indicate that the ability of anionic liposomes to attenuate lipoplex-mediated gene expression is inversely related to the time interval separating the injection of the lipoplex from that of the anionic liposomes.

Plasmid DNA and Lipid Displacement In Vivo

To confirm that the observed decrease in pulmonary gene expression was indeed a manifestation of lipoplex dissociation, radiolabeled plasmid DNA was complexed with the cationic lipid and injected IV (Tables 1a and b). In control mice, that is, animals injected with lipoplex only, at 10 minutes post-lipoplex injection, 2% of the administered dose remained in the blood while over 70% of the administered dose had accumulated in the lungs. The liver contained 6% of the labeled DNA dose at this time point. The amount of labeled DNA in the lung gradually decreased in the control animals to approximately 40% of the dose administered by 60 minutes post-lipoplex injection. There was a concomitant rise in radioactivity in the liver, suggesting that the DNA label was released from the lung and thereafter accumulated in the liver; however; the accretion of DNA in the liver did not result in increased transfection of that organ (Figures 2 and 3).

In animals receiving the sequential anionic liposome IV bolus 5 minutes post-lipoplex injection, the amount of labeled DNA remaining in the lungs was only 14% of

A DNA Distribution Following Anionic Liposome Displacement Protocol

	Lung Mean ± S.E.M.		Blood Mean ± S.E.M.		Liver Mean ± S.E.M.	
Time Post-Lipoplex Injection*	Lipoplex Alone	Lipoplex + Anionic Liposomes	Lipoplex Alone	Lipoplex + Anionic Liposomes	Lipoplex Alone	Lipoplex + Anionic Liposomes
10	73 ± 2	14 ± 6	2 ± 0.5	19 ± 1	6 ± 3	18 ± 3
25	57 ± 1	15 ± 1	3 ± 0.1	18 ± 1	4 ± 0.5	19 ± 1
65	38 ± 8	25 ± 2	1 ± 0.5	2 ± 1	9 ± 2	12 ± 2
95	35 ± 4	30 ± 3	1 ± 0.5	3 ± 1	6 ± 1	15 ± 3

B Lipid Distribution Following Anionic Liposome Displacement Protocol

	Lung Mean ± S.E.M.		Blood Mean ± S.E.M.		Liver Mean ± S.E.M.	
Time Post-Lipoplex Injection*	Lipoplex Alone	Lipoplex + Anionic Liposomes	Lipoplex Alone	Lipoplex + Anionic Liposomes	Lipoplex Alone	Lipoplex + Anionic Liposomes
10	82 ± 2	16 ± 3	1 ± 0.2	25 ± 6	5 ± 3	50 ± 3
25	79 ± 3	18 ± 4	1 ± 0.3	9 ± 4	8 ± 2	49 ± 7
65	60 ± 12	28 ± 3	1 ± 0.5	7 ± 1	19 ± 3	41 ± 6
95	40 ± 3	26 ± 9	1 ± 0.5	3 ± 2	35 ± 7	35 ± 6

* In animals receiving anionic liposomes, this time point corresponds to 5 minutes post-anionic liposome injection.

Table 1A and B. Distribution of DOTAP:CHOL-plasmid DNA complex labeled either with ^{125}I-labeled dCTP (plasmid DNA; A) or ^{125}I-labeled BPE (liposomes; B) following IV-administration.

Control animals were injected IV with lipoplex alone and tissues assayed for radioactivity at stated time-points. Animals treated with the anionic lipid displacement protocol were injected IV with a 15-fold charge excess (relative to cationic lipid) of POPG:DOPE liposomes 5, 20, 60 or 90 minutes after lipoplex injection and five minutes prior to measurement of radioactivity.

the administered dose 5 minutes after anionic liposome injection while that in the blood and liver was 19% and 18%, respectively. These data demonstrate that, when administered 5 minutes after lipoplex injection, the anionic liposomes caused the release of DNA label from the lungs into the blood. The labeled material is then cleared from circulation by the liver as would be expected of blood-borne free DNA (Kawabata et al., 1995). As the time interval between the administration of the lipoplex and the anionic liposomes increases, the difference in pulmonary DNA-label distribution between control mice and mice receiving the subsequent bolus of anionic liposomes decreases. This is a reflection of the time-dependent clearance of label from the lungs of the control mice and the fact that, at later time points, more of the lipoplex has probably been internalized.

These data are consistent with the assertion that the observed decreases in pulmonary gene expression following anionic liposome injection are a consequence of DNA displacement from lung tissue. At all time points, greater than 90% of the administered dose was recovered.

The distribution of the lipid component of the lipoplex was also analyzed following anionic liposome-mediated displacement and compared to animals which did not receive a sequential anionic liposome bolus. As in the DNA distribution experiments, lipid label was cleared from the lung upon administration of anionic liposomes at 5 and 20 minutes post-lipoplex injection; however, the lipid label accumulated to a greater extent in the liver than in the lung. The greater accumulation of the label in the liver is likely a reflection of rapid plasma clearance of the lipid marker. A similar increase in the spleen was noted (data not shown). At all time points, greater than 90% of the administered dose was recovered.

To determine the long-term effects of the displacement protocol, on the DNA content in the lung, we assayed for tissue radioactivity at 24 hours post-lipoplex injection in 3 different groups of animals. One cohort was injected with lipoplex only, while two others received a bolus of anionic liposomes at 5 minutes or 60 minutes post-lipoplex injection (Figure 4). The amount of DNA label remaining in the heart 24 hours after lipoplex injection was 1.2% of the originally administered dose. When the displacement protocol was executed 5 minutes after lipoplex administration, the amount of recovered label was significantly reduced ($p \leq 0.05$) to 0.1% of the dose. In the lung, there was also a significant reduction ($p \leq 0.05$) in residual DNA label in the lung following the 5 minute displacement protocol. In addition, the percent of dose remaining in the lung following the 5 minute protocol was significantly lower ($p \leq 0.05$) than when the displacement protocol was performed 60 minutes after lipoplex injection. Although a similar pattern was noted in the liver, no significant differences existed between the treatment groups. The mean total dose recovered 24 hours post-administration for the lipoplex-only procedure was 58%, for the 5 and 60 minute displacement procedures, recoveries were 48 and 46%, respectively.

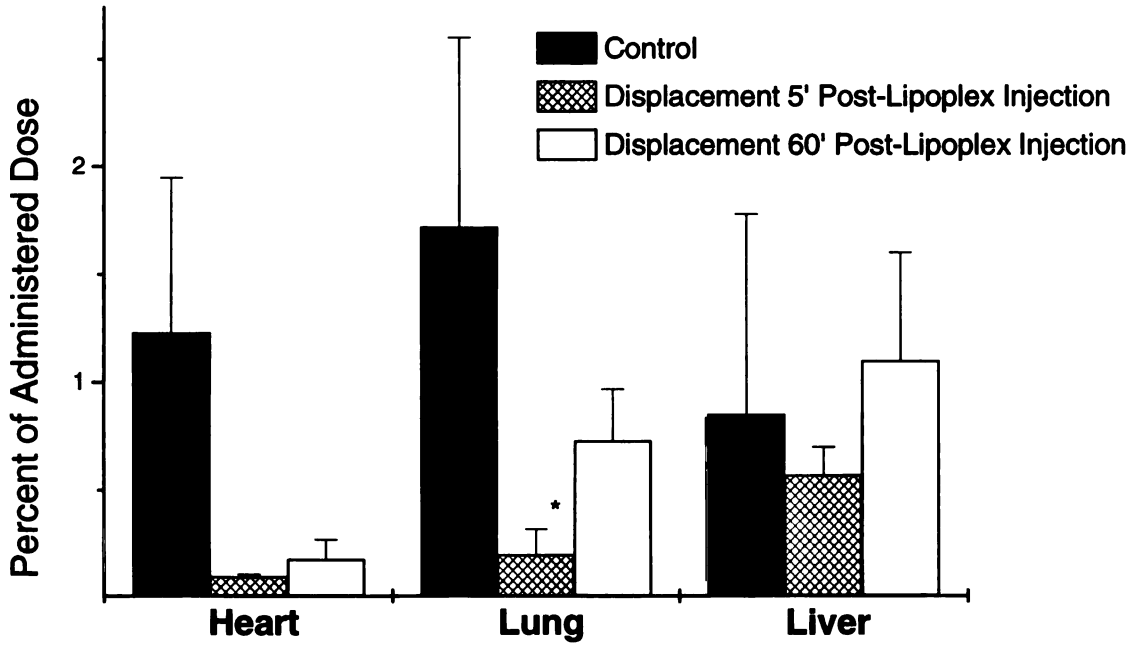

Figure 4. Long-term DNA Distribution Following Anionic Liposome Displacement Protocol

Distribution of DOTAP:CHOL-plasmid DNA complex labeled with ^{125}I-labeled dCTP plasmid DNA following IV-administration. Control animals were injected IV with lipoplex alone and tissues assayed for radioactivity at stated time-points. Animals treated with the anionic lipid displacement protocol were injected IV with a 15-fold charge excess (relative to cationic lipid) of POPG:DOPE liposomes 5, or 60 minutes after lipoplex injection. Measurement of radioactivity in tissues was carried out 24 hours later.

* Independent t-test significant difference at $P < 0.05$ versus control ($n = 4$).

In Vitro Lipoplex Dissociation

Following anionic liposome administration 60 minutes post-lipoplex injection, approximately 30% of the lipid and DNA dose administered is present in the lung. To determine if a 60 minute exposure of lipoplex to serum would render the lipoplex impervious to dissociation by anionic liposomes, a DNase digestion assay was performed. The lack of high molecular weight DNA in Lane 2 of figure 5 demonstrates, as shown in figure 1 above, that in the absence of cationic lipid, DNA is digested by DNase. The complexation of DNA with cationic lipids protects the plasmid DNA from degradation; however, after a 60 minute incubation of the lipoplexes in serum, anionic liposomes are still potent agents for displacing DNA from the lipoplex.

The anionic liposome injection protocol may manipulate the lipoplex in two fashions: 1) The lipoplex could, as a whole, be cleared from the lung relatively intact, or 2) The lipoplex is dissociated into its component parts. The notion that dissociation of the lipoplex occurs *in vivo* is supported by previous work (Xu and Szoka, 1996) and the *in vitro* data presented in figure 1. The latter scenario is also supported by the fact that, upon clearance form the lung, the DNA and the lipid label are distributed separately with the DNA initially found in the blood and the lipid accumulating primarily in the liver.

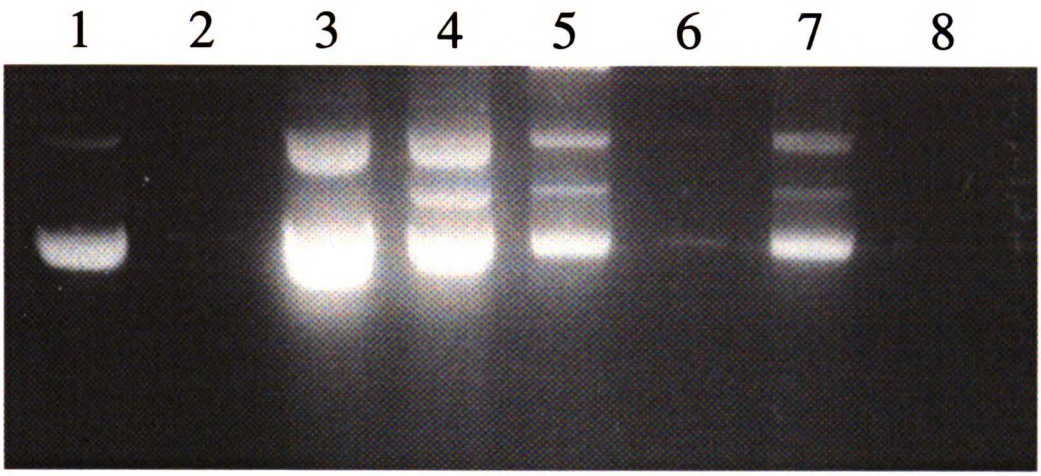

Figure 5: DNase I digestion of plasmid DNA upon incubation of complexes under various conditions.

Lipoplexes were subjected to DNase I digestion for 10 minutes at 37°C (except samples in lanes 1 and 3). DNA was extracted and electrophoresed through a 1% agarose gel. Lane 1, Plasmid DNA 1.8μg; lane 2, plasmid DNA (6μg) digested with DNase I; lane 3, plasmid DNA from lipoplex prepared as in Materials and Methods; lane 4, plasmid DNA from lipoplex digested with DNase I; lane 5, plasmid DNA from lipoplex incubated with serum for 5 minutes and then digested with DNase I; lane 6, plasmid DNA from lipoplex incubated with serum for 5 minutes, POPG:DOPE liposomes for 5 minutes and then digested with DNase I; lane 7, plasmid DNA from lipoplex incubated with serum for 60 minutes and then digested with DNase I; lane 8, plasmid DNA from lipoplex incubated with serum for 60 minutes, POPG:DOPE liposomes for 5 minutes and then digested with DNase I.

4. Discussion

Although it has been demonstrated that IV-injected cationic lipid-DNA complexes initially distribute into and ultimately transfect the lung (Liu et al., 1997a; Liu et al., 1997b; McLean et al., 1997; Barron et al., 1998), the initial fate of the lipoplex upon localizing in the pulmonary microvasculature (McLean et al., 1997) is not known. In previous chapters of this work, we have suggested that electrostatic interactions between cationic lipoplexes and anionic residues on the pulmonary endothelium or on plasma proteins (for which there are receptors on the endothelium) govern the site of lipoplex uptake and transfection. In this chapter we have defined the time-interval subsequent to lipoplex injection within which binding and uptake occurs.

Xu and Szoka have shown that negatively-charged liposomes, when combined with cationic lipid-DNA complexes cause the release of plasmid DNA from the lipoplex (Xu and Szoka, 1996). Exposure of the lipoplex to an equal molar charge ratio of anionic to cationic lipids renders previously bound DNA accessible to small intercalating agents such as ethidium bromide and macromolecules such as DNase I (Xu and Szoka, 1996). Upon centrifugation through sucrose gradients, it was shown that almost 50% of the previously bound DNA is entirely released from the lipoplex. This phenomenon is thought to be the result of a combination of electrostatic and hydrophobic interactions resulting in the diffusion of anionic lipids into the lipoplex where they form charge neutral ion pairs with cationic lipids therein, leading to the release of DNA from the admixture (Xu and Szoka, 1996). The goal of this study was to use anionic liposome-mediated lipoplex dissociation *in vivo* to determine the necessary time interval within which the IV-injected lipoplex must maintain its integrity to yield significant transfection.

Zelphati and colleagues (Zelphati et al., 1998) have shown that pre-incubation of lipoplex with serum or BSA prevented dissociation of the lipoplex by heparin and oleic acid. We wanted to determine if the serum components, particularly proteins, could coat the lipoplex and render it impervious to the dissociating effects of subsequently-added anionic liposomes. The DNase I digest experiment depicted in figure 1 demonstrated that in the presence of 33% mouse serum, POPG:DOPE and POPG:CHOL anionic liposomes, are capable of releasing significant quantities of DNA from the lipoplex. This was demonstrated by the paucity of high molecular-weight DNA present in these samples following DNase I digestion. Although it is difficult to make quantitative conclusions based on this experiment, it appears that DOPE-based liposomes displaced a greater quantity of the DNA from the lipoplex than CHOL-based liposomes.

To ascertain whether the IV-injection of anionic liposomes following IV lipoplex administration could be utilized to interrupt transfection in various murine tissues, lipoplexes were injected systemically, and permitted to distribute for at least 5 minutes prior to the injection of a 5-fold charge excess of negative liposomes (relative to nominal charge conferred by cationic lipid). Previous experiments have shown that greater than 98% of injected lipoplex is cleared from circulation by 5 minutes post-injection (Barron et al., 1998). A minimum 5-minute delay was adopted to facilitate complete lipoplex distribution prior to perturbation by anionic liposome injection. Failure to allow the complete plasma clearance of the lipoplex prior to administration of the anionic agent could result in altered distribution of the lipoplex due to interactions with the administered anionic component while in circulation.

Experiments with POPG:DOPE and POPG:CHOL liposomes showed that substantial decreases in systemic gene transfer could be effected by administration of the negative liposomes at a 5-fold charge excess 5 minutes post-lipoplex injection (Figure 2). The administration of the POPG:DOPE liposomes yielded a greater decrease in pulmonary luciferase expression than injection of a like amount of POPG:CHOL. If the decline in luciferase activity *in vivo* may be interpreted as an indication of lipoplex displacement from the lungs or lipoplex disruption, the anionic liposomes formulated with DOPE as the helper lipid were more effective in this capacity than cholesterol-based liposomes. This is consistent with the ability of DOPE to destabilize lipid bilayers (Litzinger and Huang, '92). Such destabilization would facilitate more intimate interactions between anionic lipids and the cationic lipid component of the lipoplex leading to ion pairing, charge neutralization and ultimately to DNA release from the lipoplex (Szoka et al., 1996; Zelphati and Szoka, 1997).

The fact that anionic liposomes only decreased transfection when given at less than 60 minutes post-lipoplex injection indicates that cationic lipid-DNA uptake occurs within this 60 minute time-window thus rendering the lipoplex invulnerable to anionic liposome-based disruption. Hepes/glucose buffer vehicle or neutrally-charged liposomes (POPC:DOPE) did not alter gene expression in any of the tissues assayed suggesting that the influence of the anionic liposome injections is a function of the liposome's negative charge and not simply the injection of a buffer or lipid bolus (Figure 2).

To more precisely define the time window within which the integrity of the lipoplex is necessary for transfection, a 15-fold charge excess of POPG:DOPE liposomes was injected at 5, 20, 60 and 90 minutes post-lipoplex injection. When tissues were

assayed for luciferase activity 24 hours later, once again, a pattern of greatly reduced gene expression was observed when the negative lipids were injected at early time points while, as the interval between lipoplex and anionic liposome injection was increased, the reduction in gene delivery decreased (Figure 3). A 3-fold greater dose of anionic lipids was injected in this experiment versus that referred to in figure 2; however, the reduction in pulmonary gene expression was still of the same order-of-magnitude; that is, 400-fold lower than when lipoplex alone was injected. Similar, although less pronounced, trends were noted in the heart and liver.

To verify that the reduction in luciferase gene expression following anionic liposome injection was a result of lipoplex dissociation and DNA release, experiments were conducted to analyze the distribution of the DNA and lipid components of the lipoplex upon negative liposome injection. Both the DNA and lipid distribution data show that injection of anionic liposomes a short time after lipoplex injection yields a large decrease in pulmonary lipoplex retention. This phenomenon supports the contention that the decrease in gene expression attributable to anionic liposome injection after lipoplex injection is a manifestation of lipoplex dissociation and clearance from the lungs. Dissociation of the lipoplex 5 minutes after its injection initially leads to a considerable accumulation of labeled lipid in the liver while the DNA at early time points tends to collect in the blood suggesting that the lipoplex is not simply released from the pulmonary vasculature as a whole, but is dissociated into its component parts allowing for discrete redistribution of the lipoplex components.

The reduction in reporter expression when negative liposomes are administered at early time points is observed in the liver and the heart as well as the lung. The fact that

the most profound effect is seen in the lung is likely a reflection of the high uptake and expression of lipoplex in this tissue.

As the time interval between lipoplex and negative liposome administration increases, the ability of the anionic liposomes to disrupt the lipoplex and thereby attenuate resulting gene transfer, decreases. This indicates that lipoplex properties are altered as the time post-lipoplex injection elapses. The ability of the negative liposome bolus to displace DNA and lipid from the lung demonstrates that the lipoplex must initially be exposed in the vascular compartment to the circulating anionic liposomes. At 1 hour post-lipoplex injection, the amount of labile DNA in the pulmonary vasculature is reduced considerably. This suggests that the DNA remaining in the lung at this time has been taken up by cells of the pulmonary vasculature. The possibility that the residual pulmonary DNA and lipids are contained in a lipoplex structure that, over time, becomes invulnerable to the disrupting effects of the injected anionic liposomes is refuted by the data depicted in figure 5 which show that even after incubation in serum for 60 minutes, the lipoplex is still vulnerable to disruption by anionic liposomes.

Twenty-four hours after lipoplex administration, there is significantly less residual DNA label present in the lung (Figure 4) following the 5 minute interval post lipoplex-injection rather than with the 60-minute interval. This supports the idea that more DNA is internalized prior to lipoplex disruption in animals subjected to the latter protocol. Ultimately, the higher transfection observed in this group is likely a manifestation of greater transfectionally-active DNA uptake in the 60 minutes following lipoplex injection.

We have proposed 3 models of lung lipoplex uptake in chapter II (Figure 8). Briefly, we hypothesized that the positively charged lipoplex binds with anionic residues either on the endothelium or on plasma proteins or possibly with both and that these interactions lead to lipoplex uptake in the pulmonary endothelium. The objective of this work was to determine whether lipoplex uptake occurs within a discrete time interval in the lungs following IV injection. By defining the time window within which critical events in the course of transfection occur, we have established a temporal framework within which future mechanistic studies may be conducted. We have shown that transfectionally active lipoplex is taken up from the microvasculature in the first 60 minutes following systemic administration; therefore, studies designed to characterize cationic lipid-DNA uptake ought to be focussed within this time interval.

The implications as to mechanism of *in vivo* transfection notwithstanding, our results should be considered in the context of strategies to ameliorate toxicity and immunogenicity of IV-injected lipoplexes. The systemic administration of lipoplex into mice is fatal when the lipid dose exceeds 1μmol of cationic lipid per animal (Song et al., 1997). The cause of this toxicity is not known although a number of biological systems are affected by lipoplex administration. Bottega and Epand have shown that cationic lipids are potent inhibitors of protein kinase C activity and may, thereby affect downstream cell-signaling mechanisms (Bottega and Epand, 1992). Administration of lipoplexes into the airways caused a marked increase in cytokines and an influx of macrophages and neutrophils in the lung (Freimark et al., 1998; Scheule et al., 1997). Intravenous administration of lipoplexes consisting of 450 nmol of cationic lipid

complexed with 30 μg of plasmid DNA into mice activated the complement system (Barron et al., 1998).

To counteract these effects, it may be possible to administer anionic liposomes subsequent to systemic lipoplex injection. Although the time frame within which the adverse effects of cationic lipoplex administration occur is not clear, the disruption of residual lipoplex from the pulmonary vasculature after 1 hour may attenuate toxicity through minimizing the opportunity for endothelial membrane damage by the un-internalized lipoplex.

In addition to the displacing properties of the polyanions, the dissociative capacity of the anionic liposomes may also diminish the immune reactivity to residual lipoplex inasmuch as Freimark and coworkers (1998) have observed that the ability of the cationic lipid and DNA components of the lipoplex to induce the production of inflammatory mediators is greater when they are complexed; moreover, the release of plasmid DNA from the lipoplex renders the DNA susceptible to degradation, thus rapidly reducing its potential to stimulate cytokine release and inflammation.

The foregoing findings are important in that they address 2 important issues in the province of cationic lipoplex-mediated gene delivery. The first is the relatively low gene delivery efficiency of these vectors. Further progress toward improving cationic lipids as gene delivery vehicles requires a greater understanding of the mechanism by which the current transfection levels are achieved and how they can be enhanced. We have now defined a clear time-frame within which *in vivo* uptake studies may be focussed. The second contribution lies in the potential of polyanionic disruption to decrease the toxicity associated with cationic lipids by releasing the lipoplex back into circulation where it can

be cleared and degraded. This may also permit the administration of higher lipoplex doses initially, thus increasing efficacy if not efficiency.

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Chapter V: Cationic Lipids are Essential for Gene Delivery Mediated by Intravenous Administration of Lipoplexes

1. Introduction

Recently, Liu injected cationic liposomes intravenously (IV) in mice 5 minutes prior to an IV injection of a reporter gene (Liu, 1998). Surprisingly, the level of transgene expression in the lung, as measured 24-hours later, was the same as that obtained following injection of the preformed lipoplex. Based on these data, Liu suggested that extracellular DNA released from the lipoplex is the transcriptionally active agent, and that the cationic lipid component serves only to increase pulmonary retention of DNA following systemic administration (Liu, 1998). Given that naked plasmid DNA is capable of transfecting striated muscle, (Wolff et al., 1990; Buttrick et al., 1992) the thyroid gland (Sikes et al., 1994), the liver (Hickman et al., 1994), the lung (Meyer et al., 1995), solid tumors (Yang and Huang, 1996), and synovial tissue (Nita et al., 1996), we found Liu's observations intriguing and believed the hypothesis to be an important conjecture. If the proposal was correct, then any sustained release system for DNA should mediate transfection in the lung. Potentially, one could obtain gene expression without the adverse effects associated with the cationic lipid.

We repeated Liu's experiments and confirmed the observations. The pre-injection of cationic-liposomes, followed 5 minutes later by a luciferase reporter gene, yielded plasmid DNA distribution and transfection in the lung that was not significantly different than for DNA delivered as a component of the preformed lipoplex (Chapter II, Figure 1).

We hypothesized that if free DNA is the active transfection agent in pulmonary gene delivery following IV lipoplex injection, then establishing a relatively high steady-state plasma concentration of transcriptionally active DNA would yield transfection comparable to or greater than that of IV-injected lipoplexes.

To test our hypothesis, we defined the time period within which lung transfection occurs upon systemic injection of cationic lipoplex by the subsequent IV injection of anionic liposomes. This strategy causes lipoplex dissociation *in vitro* (Xu and Szoka, 1996). In chapter IV of this document, we determined that lipoplex integrity is critical for lung transfection only in the first 60 minutes after its injection (Chapter IV; Barron and Szoka, submitted). Following this time period, clearance of lipoplex from the lung and other tissues by anionic liposome injection did not significantly influence transfection. This suggests that pulmonary uptake of the transcriptionally active species occurs substantially within the first 60 minutes post-lipoplex injection.

The objective of this body of work was to determine if the role of the cationic lipid component of the lipoplex goes beyond that of acting as a passive release matrix for plasmid DNA in the pulmonary vasculature. Our strategy for addressing this question was to infuse reporter DNA up to steady-state plasma concentrations comparable to that delivered by lipoplex bolus. The studies in chapter IV provided us with a time window within which plasmid DNA infusion was to be carried out; therefore, we maintained a steady state DNA concentration for 60 minutes and then assayed for luciferase activity 24 hours later. In addition, we infused high levels of non-coding plasmid DNA, a potential competitor for reporter DNA uptake, for 60 minutes after administration of lipoplex, and determined whether this decreased reporter gene expression.

2. Materials and Methods

Animals

Experiments were conducted on 3-6 week-old female CD-1 mice purchased from Simonsen (Gilroy, CA). All animals were handled in accordance with protocols established by the National Institute for Health Guidelines for the Care and Use of Laboratory Animals, and with the approval of the Committee for Animal Research at the University of California, San Francisco. Animals were sacrificed at stated times by exsanguination following anesthetization with an intraperitoneal (IP) injection of ketamine (200mg/kg), xylazine (10mg/kg) and acepromazine (2mg/kg).

Extraction of Plasmid DNA and Gel Electrophoresis

Plasmid DNA encoding the chloramphenicol acetyl transferase (CAT) gene, (pCT0129.142, a generous gift of GeneMedicine Inc.) was dissolved in 5 mM HEPES/5% glucose buffer at pH 7.4 at a final concentration of 4 mg/mL. Animals were anesthetized by intraperitoneal injection of ketamine (200 mg/kg), xylazine (10 mg/kg) and acepromazine (2.0 mg/kg) dissolved in phosphate buffered saline at pH 7.4. A 27 gauge butterfly infusion set was inserted into the tail vein and the infusion was carried out at a rate of 20 μL/min for 10 minutes with a Bioanalytical Systems infusion pump (West Lafayette, IN). Animals were then sacrificed and blood was drawn via cardiac puncture. Plasma was harvested by centrifugation of blood at 500xg and incubated in lysis buffer (100 mM NaCl, 10 mM Tris, 25 mM EDTA, 0.5% SDS and 0.1 mg/ml

protease K, pH 8.0) for 2 hours at 50° C. DNA was then extracted according to standard procedures (Sambrook et al., 1989) and quantified at A_{260}.

Two micrograms of DNA were either loaded directly onto a 1% agarose gel or subject to *Sac* I restriction prior to electrophoresis at 120 V for 1 hour.

In Vitro Transfection Activity of Extracted DNA

Luciferase plasmid DNA (pLC0888.143, a generous gift of GeneMedicine, Inc.), native and recovered after infusion into animals (numbered 1 to 4), was transfected into CV-1 cells using heat activated poly-amidoamine dendrimer. Cells were seeded at 20,000 per well (96-well plate) and grown overnight (37°C, 5% CO_2) in 10% serum supplemented medium (DME-H21, with 100 U/mL penicillin, 100 U/mL streptomycin, 10 mM HEPES, and non-essential amino acids). Complex was made by adding DNA to dendrimer at a 1:4 mass ratio (Tang et al., 1996) and added in a 100 µL aliquot to cells in 1mL of serum free medium. Incubation continued for 3 hours, followed by two salt washes (1 M NaCl, 10 mM HEPES, pH 7.4), one PBS wash, and replacement with serum supplemented medium. DNA recovered from luciferase plasmid infused animals was quantified by A_{260} and dosed at 3.0 µg per well. The standard curve was generated by transfection with native luciferase plasmid at the dose indicated on the ordinate axis. Dendrimer quantity and polyplex charge ratio were kept constant by adding a complement, to 3.0 µg total DNA, of non-luciferase coding plasmid DNA, which also had been infused and recovered (Table 1). The dilution protocol for the standard curve was carried out to control for any effects that degraded DNA or other residues isolated from mouse plasma could have on *in vitro* gene expression.

IV Lipoplex Bolus and Infused Plasmid DNA Administration in Mice

Cationic liposomes were prepared as follows: DOTAP and cholesterol were dissolved in chloroform at a 1:0.9 molar ratio then evaporated to dryness on a rotary evaporator and placed under vacuum overnight to remove the remaining solvent. The dried lipid film was hydrated in 10% glucose/10 mM HEPES buffer (pH 7.4) over a period of 30 minutes during which time the suspension was agitated on a vortex mixer for 30 second intervals about 10 times. The hydrated lipid suspension was extruded through a 0.2 μm polycarbonate membrane. Liposome-DNA complexes were formed at room temperature by mixing equal volumes of plasmid DNA diluted in dH_2O with the liposome preparation to yield a complex with a 5:1 charge ratio (+/-) and were allowed to stand for 15 minutes prior to injection. The diameter of the complex was measured using dynamic light-scattering (Coulter Electronics, Hialeah, FL, USA). Each mouse received a 100 μL dose administered via tail-vein injection containing 900 nmol total lipid and 90 nmol (30 μg) plasmid DNA. Animals to be infused were anesthetized as above. The infusion protocol was the same as that outlined above except that the plasmid used (pLC0888.143, A generous gift of GeneMedicine Inc.) contained the luciferase reporter gene and was infused for 60 minutes. Infused animals were allowed to recover from anesthesia and all animals were sacrificed 24 hours post-treatment. Luciferase activity was measured as detailed previously (Barron et al., 1998).

Infusion of Non-Coding DNA Following an Intravenous Bolus of Lipoplex in Mice

Each mouse received a 100 μL dose administered via tail-vein injection containing 900 nmol total lipid and 90 nmol (30 μg) luciferase plasmid DNA as for control animals in figure 3. Five minutes after lipoplex injection, treatment animals were anesthetized and infused with a non-coding CAT plasmid, (pCT0129.142) in a 5 mM HEPES/5% glucose buffer at pH 7.4. Plasmid concentration and infusion rate were identical to infusion protocol outlined above. The infusion was maintained for 60 minutes. Twenty-four hours post-administration, all animals were sacrificed and luciferase activity measured according to established protocols (Barron et al., 1998).

3. Results

Infused DNA Extraction and Gel Electrophoresis

Our first objective was to infuse DNA to a steady-state plasma DNA concentration (C_{ss}) that was similar to the 30 μg DNA dose administered through our standard lipoplex-based transfection protocol. Employing the published elimination rate for plasmid DNA of 1.4 mL/minute (Kawabata et al., 1995), the necessary infusion rate to achieve a C_{ss} of 60 μg DNA per mL plasma is 84 μg/minute (equation 1).

$$R_0 = C_{ss} * Cl \qquad \text{Equation 1}$$

Where R_0 is the infusion rate in μL/minute, C_{ss} is the steady-state plasma concentration in μg/μL and Cl is the clearance rate in μL/minute.

We infused 4 mice with plasmid DNA via the tail vein as outlined in table 1 and

determined the plasma concentration and integrity of the infused plasmid DNA at its C_{ss}. The 10 minute infusion caused no apparent toxicity in the animals. The DNA was extracted from plasma and quantified via UV spectroscopy (Table1). The mean concentration of plasmid DNA in plasma was determined by optical density to be 496 μg/mL or 8-fold greater than that of the value calculated through equation 1 and had a high A_{260}/A_{280} ratio, indicating that the extracted DNA was free of significant protein contamination. As a control, the extraction protocol was applied to plasma from animals into which we had not infused plasmid DNA. As expected, virtually no DNA was detected by absorbance (260nm) (Table 1). Thus, high plasma DNA concentrations can be achieved using this infusion protocol.

To determine the size and integrity of the circulating plasmid DNA at C_{ss}, the extracted DNA was subjected to agarose gel electrophoresis (Figure 1). Figure 1 is a reproduction of an agarose gel through which a typical DNA sample obtained from the infused mice was electrophoresed. To confirm the identity of the DNA observed on the gel, we included native and restricted plasmid controls (lanes 1 & 2). Lane 3 contains DNA extracted from the plasma of an untreated animal. There was no detectable DNA in this lane since nucleated cells are removed upon harvesting plasma (Table 1). Lane 4 contains DNA extracted from mouse plasma following the 10 minute infusion procedure. When compared to the native plasmid DNA in lane 1, it is apparent that the sample in lane 4 has been degraded to some degree. There is a prevalence of 'relaxed' versus that of supercoiled plasmid as indicated by the significant quantity of slower migrating DNA in lane 4. Relaxed plasmid DNA represents the effect of endonuclease activity to which the

Animal #	A_{260}/A_{280}	DNA Plasma Concentration μg/ml
1	1.85	454
2	1.81	467
3	1.71	578
4	1.72	484
Mean	1.77	496
Control[a]	N/A	<0.1

[a] DNA extracted from mouse plasma not exposed to plasmid DNA

Table 1. Characterization of Plasmid DNA Extracted from Mouse Plasma Following IV Infusion

Plasmid DNA was dissolved in 5 mM HEPES/5% glucose buffer at pH 7.4 at a final concentration of 4 mg/mL. Animals were anesthetized and a 27 gauge butterfly infusion set was inserted into the tail vein and the infusion was carried out at a rate of 20 μL/min for 10 minutes. Animals were then sacrificed and blood was drawn via cardiac puncture. Plasma was harvested and DNA extracted as outlined in Materials and Methods.

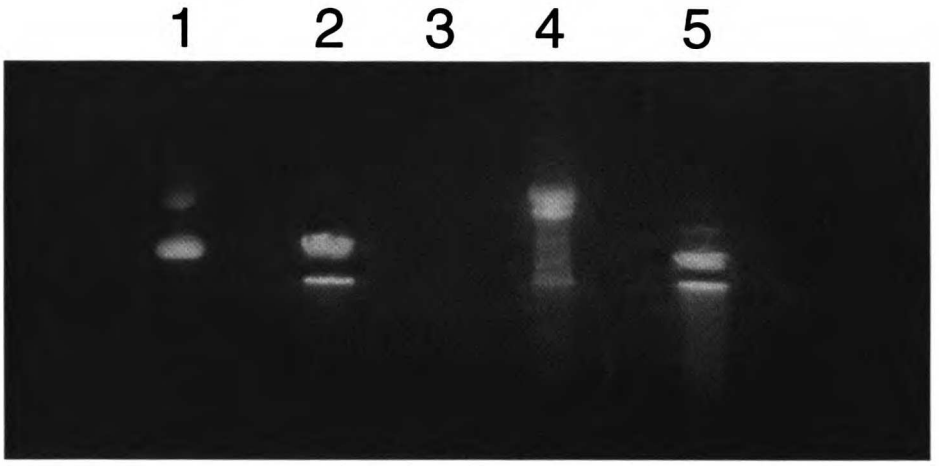

Figure 1. Gel Electrophoresis of DNA Extracted from Mouse Plasma

Two micrograms of DNA, either native, or extracted as detailed in Materials and Methods, were loaded directly onto a 1% agarose gel or subject to *Sac* I restriction prior to electrophoresis at 120 V for 1 hour. Lane 1: 1 μg of native plasmid DNA; lane 2: 1 μg *Sac* I restricted plasmid DNA; lane 3: DNA extracted from plasma of untreated animal; lane 4:1 μg DNA extracted from infused mouse; lane 5: 1 μg *Sac* I restricted DNA extracted from infused mouse.

DNA has been exposed in plasma. In addition, lower molecular weight DNA, is depicted as a more rapidly migrating 'smear' on the gel. To confirm the identity of the DNA sample in lane 4, we restricted a second aliquot of extracted DNA prior to electrophoresis (Lane 5). The restriction pattern of DNA in lane 5 was similar to that of lane 2 therefore, the DNA sample extracted from plasma is identical to the infused plasmid.
Although plasmid DNA degradation occurred, there was a significant proportion of high molecular weight DNA present in the extracted sample. The amount of ethanol precipitable DNA that remained in plasma as quantitated by absorption was 496 μg or 62% of the 800 μg originally infused. Upon extraction of DNA from an animal infused for 60 minutes, we found a similar gel electrophoresis profile with even higher plasma DNA concentration (810 μg/mL).

In Vitro *Transfection Activity of DNA Extracted from Plasma*

To determine what fraction of the extracted DNA was transcriptionally active, a standard curve was established in an *in vitro* transfection assay that facilitated the measurement of luciferase activity in the extracted DNA. Non-coding DNA that had been previously infused into mice was extracted from plasma and combined with varying amounts of intact luciferase plasmid to yield 3 μg of total DNA. The DNA was then complexed with fractured polyamidoamine dendrimer (Tang et al., 1996). The resulting polyplex was used to transfect CV-1 fibroblasts in a 96-well culture plate. A 3 μg aliquot of luciferase plasmid harvested from the plasma of infused animals was then used to transfect CV-1 cells in a similar fashion. Luciferase activity was assayed 48-hours following transfection (Figure 2). Extrapolation of the reporter gene activity to the

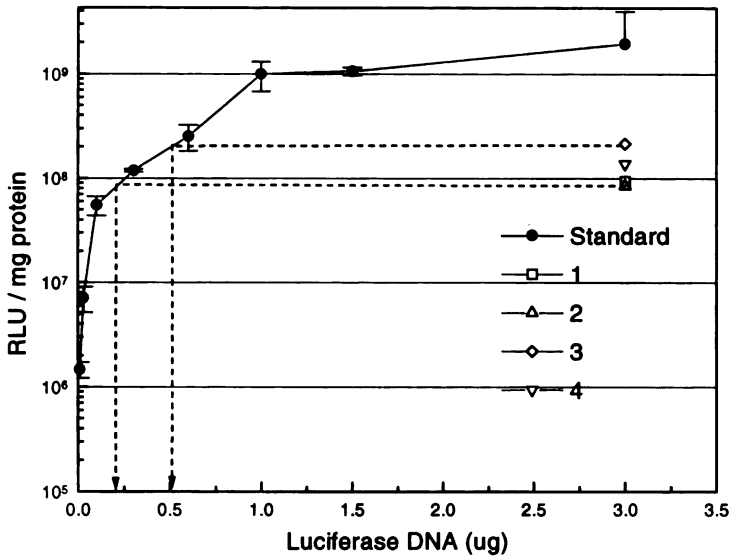

Figure 2. ***In Vitro*** **Transfection Activity of DNA Extracted from Mouse Plasma**

Luciferase plasmid DNA, native and recovered after infusion into animals (numbered 1 to 4), was transfected into CV-1 cells using heat activated poly-amidoamine dendrimer as outlined in Materials and Methods. The standard curve was generated by transfection with native luciferase plasmid at the dose indicated on the ordinate axis. Dendrimer quantity and polyplex charge ratio were kept constant by adding a complement, to 3.0 μg total DNA, of non-luciferase coding plasmid DNA, which also had been infused and recovered (Table 1). Values represent the mean ± S.D. n = 3.

standard curve indicated that between 5 and 15% of the extracted DNA is transcriptionally active. Given the circa 800 μg/ml concentration of high molecular weight DNA in plasma after the 60 minute infusion, this corresponds to 40-120 μg of transcriptionally-active DNA present in the blood at steady-state during the infusion procedure.

Transfection mediated by Free Plasmid DNA Infusion

Based on the data presented in table 1 and figures 1 and 2, it is clear that the infusion procedure outlined in figure 3 establishes a significant plasma concentration of high molecular weight, transcriptionally active DNA. The transfection activity mediated by this IV infusion protocol was then compared with that of lipoplex-mediated systemic transfection. Mice were either infused via the tail vein with luciferase plasmid DNA for 60 minutes, corresponding to the established critical time window of lipoplex transfection, or received a bolus injection of lipoplex containing the luciferase plasmid. Luciferase gene expression in the heart, lung, liver and spleen was measured 24 hours post-administration (Figure 3). Compared to transfection of tissues from animals that had received a tail-vein bolus injection of cationic lipid-DNA complexes, reporter gene activity in the infused animals was scarcely above background activity in each organ.

Since the infusion procedure involved the introduction of 1.2 mL of buffer into the circulation of the mouse, there was a potential for the buffer itself to have significant

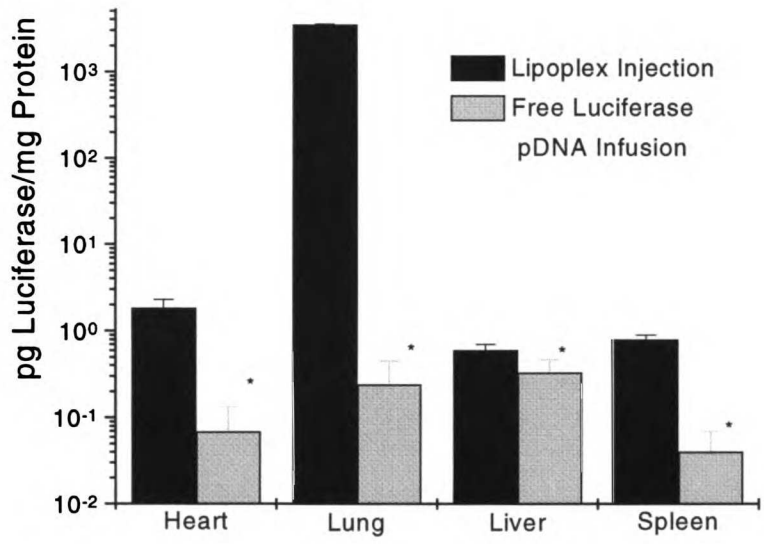

Figure 3. Comparison of Transfection in Various Organs Mediated by a Lipoplex Bolus or Intravenously Infused Plasmid DNA in Mice.

Lipoplexes were prepared as described in Materials and Methods. Lipoplex-treated animals received a 100 μL bolus administered via tail-vein injection containing 900 nmol total lipid and 90 nmol (30 μg) plasmid DNA. Animals to be infused were anesthetized and infused with 4.8 mg plasmid DNA over a 60 minute interval. Infused animals were allowed to recover from anesthesia and all animals were sacrificed 24 hours post-treatment. * Significant difference vs control group as determined by Student's t-test $p \leq 0.05$ ($n = 4$).

effects in the blood. To ensure that artifacts due to electrolyte dilution or excessive glucose load were not observed, we repeated the infusion procedure with DNA dissolved in phosphate buffered saline (PBS). DNA dissolved in PBS yielded similar results to that of the glucose-based buffer when infused IV, therefore we conclude that the buffer in which the DNA is dissolved had no significant effect on subsequent gene expression. These data demonstrate that free DNA is not capable of transfection in the tissues assayed, even when it is present in the blood in significant transcriptionally-active quantities.

Effect of Non-Coding DNA Infusion on gene Expression Mediated by subsequently-Injected Lipoplex

We further reasoned that if the release of free plasmid DNA from the lipoplex mediates transfection, then a high plasma concentration of non-coding plasmid DNA should compete with the coding plasmid for cell uptake and transfection should decrease. Lipoplex was administered as outlined in figure 3. We have previously observed that approximately 90% of the lipoplex is cleared from circulation 5 minutes post-injection (Barron et al., 1998). In order to avoid influencing lipoplex distribution, the infusion of non-coding DNA was initiated 5 minutes after lipoplex injection and maintained for 55 minutes thereafter. Animals were sacrificed 24 hours later for analysis of transgene expression in the heart, lung, liver and spleen (Figure 4). The infusion of the non-coding DNA molecule did not decrease transfection in any organ assayed.

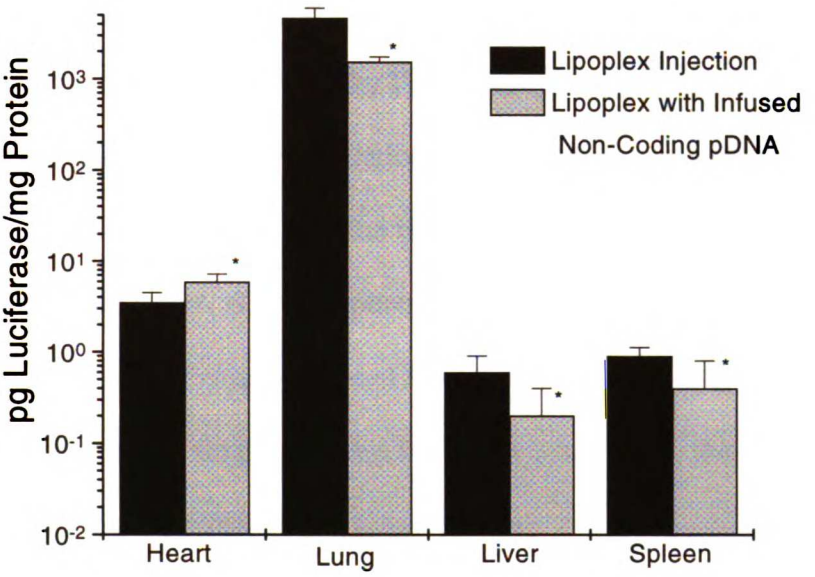

Figure 4. Effect of Non-coding DNA Infusion on Gene Expression Mediated by an Intravenous Bolus of Lipoplex in Mice

Each mouse received a 100 μL dose administered via tail-vein injection containing 900 nmol total lipid and 90 nmol (30 μg) luciferase plasmid DNA. Five minutes after lipoplex injection, animals to be infused were anesthetized and received 4.8 mg plasmid DNA over a 60 minute interval. Twenty-four hours post-administration, all animals were sacrificed and assayed for luciferase activity. Results are expressed as the mean luciferase activity ± S.E.M. for 6 animals. * No significant difference vs control group as determined by Student's t-test $p \leq 0.05$.

4. Discussion

We have previously shown that a 30 μg dose of DNA complexed with cationic lipids yielded significant transfection activity in the lung upon IV injection into the mouse (Barron et al., 1998). Our first objective then, was to establish a comparable steady-state plasma concentration (C_{ss}) by IV infusion of naked plasmid DNA. The fact that the measured plasma DNA concentration was ultimately much higher than that of the calculated value after a 60 minute infusion is presumably a reflection of the saturation of DNA clearance mechanisms. A similar phenomenon was observed by Emlen and Mannik upon IV bolus injections of single-stranded DNA (Emlen and Mannik, 1978). These investigators observed that single-stranded DNA was removed by the liver in a manner that could be described by Michaelis-Menten kinetics.

Although gel electrophoresis of the extracted DNA (Figure 1) demonstrated that the plasmid DNA in the blood was principally of high molecular weight, it was apparent that much of the DNA was in the relaxed form; that is, it had been subject to limited degradation. To determine if this damage rendered the plasmid DNA transcriptionally inactive, we employed the extracted DNA in an *in vitro* transfection assay and found that up to 15% of the extracted DNA was transcriptionally active. Given the 800 μg plasmid DNA circulating after infusion for 60 minutes, this translates to as much as 120 μg of transcriptionally-active plasmid DNA per mL of plasma and yet no significant transfection was observed in the heart, lung, liver or spleen.

The inability of the non-coding DNA to reduce transfection implies that there is no opportunity for it to compete with the initially complexed DNA for uptake; therefore, we conclude that the complexed DNA has been compartmentalized following injection either as a component of the lipoplex or through cellular uptake.

We have shown that the maintenance of significant plasma levels of high molecular weight, transcriptionally-active DNA does not yield measurable transgene delivery in the heart, lung, liver or spleen. In addition, the infusion of non-coding plasmid DNA, a potential competitor for reporter DNA uptake, did not cause a decrease in transfection. These data do not support the hypothesis that DNA released from the lipoplex in the extracellular compartment is the transcriptionally active agent observed in murine tissues upon IV administration of cationic lipid-DNA complex.

These data imply that the cationic lipid component of the lipoplex is an active agent in the delivery of the gene and the lipoplex constitutes more than just a sustained release mechanism for plasmid DNA. As outlined above, we believe the role of cationic lipids in transfection following IV administration of lipoplexes involves its ability to electrostatically bind anionic components in the vascular compartment. This may involve direct binding to the endothelium through anionic gangliosides, glycoproteins and proteoglycans. Alternatively, lipoplexes may couple with plasma proteins which indirectly target the lipoplex to the lung endothelium by binding their cognate receptors in the pulmonary microvasculature. Finally, a combination of the 2 phenomena may occur. The exact internalization mechanism is not yet clear; however, this work demonstrates that the the cationic lipid's role in lung transfection involves more than simple DNA retention at the endothelium and then its timely extracellular release, indeed,

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Publications resulting from this work:

Chapter 1
Barron, L.G. & F.C. Szoka, Jr. The Perplexing Delivery Mechanism of Lipoplexes, Academic Press, Submitted

Chapter 3
Barron, L. G., Meyer, K. B., and Szoka, F. C., Jr. (1998). Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid-DNA complexes. Human Gene Therapy *9*, 315-23.

Chapter 4
Barron, L. G., Lucie Gagne, and Szoka, F. C., Jr. (1999). Lipoplex mediated gene delivery to the pulmonary endothelium occurs within 60 minutes of systemic administration. Human Gene Therapy, Submitted

Chapter 5
Barron, L. G. L. S. Uyechi and F C. Szoka, Jr.(1999) Cationic lipids are essential for gene delivery mediated by intravenous administration of lipoplexes. Gene Therapy, In press.

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