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Delivery of macromolecules by cationic liposomes

Hassan M. F. Farhood

To the Graduate Council:

I am submitting herewith a dissertation written by Hassan M. F. Farhood entitled "Delivery of macromolecules by cationic liposomes." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Leaf Huang, Major Professor

We have read this dissertation and recommend its acceptance:

John Koontz, Barry Rouse, Jeff Becker

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Associate Vice Chancellor
and Dean of The Graduate School

DELIVERY OF MACROMOLECULES BY CATIONIC LIPOSOMES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Hassan M. F. Farhood

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DEDICATION

This dissertation is dedicated to my mother Majida for her endless and dedicated support of my education.

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ABSTRACT

Delivery of macromolecules such as DNA and protein to mammalian cells is a critical step for gene expression studies and for the new field of gene therapy. Cationic liposomes are useful and simple vehicles for carrying macromolecules into cells efficiently and reproducibly. New formulations of cationic liposomes containing cationic cholesterol derivatives and the phospholipid dioleoyl phosphatidylethanolamine (DOPE) have been designed and tested for delivery of DNA to cells in culture in an effort to improve the efficiency and eliminate the toxic effects of cationic liposome formulations. Derivatives with a tertiary amine head group had efficient transfection activity, negligable cytotoxic activity, and no protein kinase C inhibition activity. In contrast, derivatives with a quaternary amine head group had no tranfection activity and showed higher toxicity in addition to protein kinase C-inhibition activity. The membrane stabilizing phospholipid, dioleoyl phosphatidylcholine (DOPC), did not replace the efficient role of DOPE (a membrane destabilizer) in transfection. The contribution of DOPE to superior transfection activity was studied in more detail which led to the development of a new DNA delivery assay that demonstrated the essential and quantitative role of DOPE in DNA delivery. DOPE was shown to be a major contributer to delivery of DNA into cells. Complete replacement of DOPE with DOPC abolished transfection while partial replacement had partial elimination of transfection activity. The lysosomotropic agent chloroquine also inhibited

transfection suggesting that endocytosis is the major route of DNA delivery by cationic liposomes.

Cationic liposomes were also tested in the delivery of a model protein to mammalian cells. The protein delivered was the human immunodeficiency virus-1 (HIV-1) trans-activator protein (tat), a transcription factor that induces the expression of genes under the control of HIV-1 promoter element. To improve the delivery of tat, known to undergo cell surface binding, endocytosis, and inefficient entry into the cytosolic compartment., different cationic liposome formulations were successfully tested allowing up to 150-fold enhancement in tat delivery. Tat delivery was facilitated by the inclusion of DOPE, but not DOPC, in the liposome formulations. Similar to DNA delivery, tat protein delivery was dependent on high DOPE content in the liposome formulation. Co-internalization of tat and cationic liposomes was required for maximal delivery activity implicating a common compartment from which tat was released into the cytosol. This was proposed to be the endosome compartment based on the known membrane disrupting function of DOPE (especially under low, endocytic pH) and the natural endocytic fate of tat. To test the possibility of DNA and protein co-delivery, tat protein was complexed with DNA, coding for a reporter gene under the control of HIV-1 promoter, and cationic liposomes. Transfection of cells with the ternary complex showed detectable reporter gene expression with tat protein concentrations as low as 10 ng/ml. Gene induction was shown to be

specific to tat-responsive promoter. The co-delivery system was applicable to different cell types using different cationic liposome formulations. This co-delivery strategy may be useful where transient and maximum gene expression may be necessary in certain applications such as cancer gene therapy.

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LIST OF ABBREVIATIONS

CAT	chloramphenicol acetyltransferase
CF	cystic fibrosis
CTL	cytotoxic T lymphocytes
DC-Chol	3β [N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol
DEAE	diethylaminoethyl
DOPC	dioleoyl phosphatidylcholine
DOPE	dioleoyl phosphatidylethanolamine
DOSG	1, 2-dioleoyl-sn-3-succinyl glycerol
DOTAP	*N* -[1-(2,3-dioleoyloxy)propyl]-*N,N,N* trimethylammonium methyl sulfate
DOTMA	N - [1-(2,3-dioleyloxy) propyl] - N,N,-trimethylammonium chloride
HIV	human immunodeficiency virus
HSV	herpes simplex virus
LTR	long terminal repeat
MHC	major histocompatibility
PE	phosphatidylethanolamine
PC	phosphatidylcholine
PCR	polymerase chain reaction
SUV	small unilamellar vesicles
tat	transcriptional activator protein

PART I

INTRODUCTION

1. History and Background

Introduction of genetic material (DNA) into cells has been attempted through numerous and diverse methods which were physical, chemical, and biological in nature (1). However, the ultimate application of gene transfer, i.e. gene therapy of human diseases, limits the applicable methodologies to only a few (1). Physical methods for DNA transfer include direct injection of DNA into muscles of live animals (2), with successful applications in DNA vaccination against disease (3), and microprojectile biolistics using a gene propelling gun mostly for gene therapy purposes (4). These physical delivery methods have not yet been tested on humans. Biological methods of gene delivery include viral (5) and retroviral vectors (6) both of which have been effectively used for gene therapy in animal models and humans (7-9). However, certain concerns such as safety, toxicity, and immunogenecity are still unresolved for some of these gene vectors (10). Chemical methods for DNA delivery are also among the promising available technology for gene tranfer to combat disease. Targeted cationic polymers (11) and liposomes (12-14), cationic (15,16) and non-cationic (17), are the only chemical delivery techniques potentially availabLe for gene therapy. In practice, however, only cationic liposomes have reached the realm of human clinical trial (18-20).

Liposomes are lipid vesicles constituted of a lipid bilayer membrane entrapping a volume of aqueous solution inside the vesicle. The lipid bilayer is hydrophobic in the interior and hydrophilic on the surface. The vesicle can be multilamellar, composed of multiple concentric lipid bilayers, or unilamellar, composed of a single lipid bilayer, depending on the method of liposome preparation (21). Unilamellar vesicles can entrap larger volume of aqueous solution per unit lipid mass than the multilamellar vesicles of the same vesicle diameter. Also depending on the preparation method, the size of liposomes can vary widely between 30-50 nm up to several microns in diameter (21). Liposomes can carry and protect molecules such as conventional drugs or macromolecules (DNA, RNA, or protein) which can be entrapped in the aqueous volume inside the liposome. Once the carrier liposome fuse with or enter the target cell a portion of the entrapped molecules is released into the cytoplasm (22). Hydrophobic molecules such as water-insoluble drugs can be made soluble by entrapment of the drug in the hydrophobic interior of the lipid bilayer with relatively high entrapment efficiency (21). An alternative method for carrying molecules by liposomes is through adsorption of charged molecules to the surface of liposomes with an overall opposite charge from that of the carried molecules. Negatively charged macromolecules such as DNA can adsorb to, and complex with, cationic liposomes without entrapment of DNA inside the liposome (14).

Seven years ago, the first formulation of a cationic liposome, Lipofectin™, was introduced by Felgner *et al* (23). Lipofectin is composed of a sonicated preparation of equal amounts (by weight) of a synthetic cationic lipid, *N* - [1- (2, 3, dioleoyloxy) propyl]-*N, N, N* - trimethylammonium chloride (DOTMA) and a membrane destabilizing phospholipid dioleoyl phosphatidylethanolamine (DOPE) (Figure 1). Sonication of the dried and hydrated lipids, with buffer or water, produces small unilamellar vesicles with an approximate size range of 50-300 nm in diameter. The net positive charge of the liposome allows it to complex with the negatively charged DNA by electrostatic interaction, without the cumbersome and inefficient encapsulation of DNA inside the liposomes. The result of the electrostatic binding of DNA to cationic liposomes is a complex with a net positive charge, at an optimal ratio of liposome to DNA, which enables the complex to adsorb to the negatively charged cell surface. Following the adsorption, cellular uptake of the complexed DNA facilitates intracellular DNA delivery and subsequent gene expression (23).

The composition of Lipofectin™ was the prototype for most of the subsequent cationic liposome formulations prepared in different laboratories (14-16). All cationic liposomes used for successful gene transfer are made of an amphipathic cationic lipid with or without DOPE. In contrast to the non-variable phospholipid component (DOPE) of cationic liposomes, the cationic lipid of the different available liposome formulations differs markedly and can be a cationic cholesterol (24-26),

cationic detergent (27,28), single or multiple tertiary and quaternary amine-diacyl conjugates (23,24,29-32), lipospermine (33), and lipopolylysine (34,35). These cationic amphiphiles have only two components in common: the net cationic charge on the hydrophilic headgroup of the amphiphile and the hydrophobic tail that anchors the molecule to the liposome lipid bilayer (14). The chemical structure of the cationic lipids is quite diverse and each molecule can have a single or multiple cationic charges. In addition, and in contrast to the natural phospholipid component of cationic liposomes, the cationic lipid component is synthetic and is not found naturally among the lipids in biological membranes. This and the nondegradable bond that connect the cationic headgroup to the hydrophobic tail, found in some liposome formulations, may be the main reason behind the toxic effects seen with high concentrations of virtually all cationic liposome formulations. Fortunately, the cationic liposome concentration used for successful gene transfer is below the toxic level in the majority of applications.

2. Lipid Composition of Cationic Liposomes

DNA delivery by cationic liposomes to target cells is dependent on the quantitative content of both the cationic lipid and DOPE in the liposome. Three exceptions to this general rule have been reported (29,33,34). These three different liposome formulations lack DOPE and are composed of 100% cationic lipid. The mechanism of DNA delivery

using these formulations is unknown. One cationic lipid, i.e. lipopoly-L-lysine, was found to deliver DNA only after scraping the transfected cells in culture (34). However, scraping was unnecessary if DOPE was added to the cationic lipid to form liposomes (35). However, the other two cationic lipid formulations, i.e. lipospermine (33) and the cationic detergent O,O'-didodecyl-N-[p-(2-trimethylammonioethyloxy)be-nzoyl]-(L)-glutamate bromide (29), do not require any mechanical assisstance for transfection, confirming a role for DNA delivery by the cationic lipid independent of DOPE.

The role of the cationic lipid in DNA delivery:

As a result of the large number of phosphodiester anionic charges on DNA the majority of the cationic lipid in cationic liposomes is required for DNA complexing, and hypothetically for DNA condensation. A small amount of excess cationic lipid is required for binding the liposome to the cell surface. These two requirements probably create a critical quantitative ratio of DNA to cationic liposomes. The critical ratio must be reached for successfull gene transfer. This ratio is optimal when the net ionic charge of the DNA/liposomes complex is positive. The negative charges exposed on the DNA molecule must be completely neutralized by the addition of sufficient amount of cationic liposomes. There is a highly reproducible distinctive sharp increase in transfection activity when this critical ratio is reached (25,26,33,34). Any subsequent addition of cationic lipids to the

complex does not enhance DNA delivery and only increases the toxicity to the treated cells (25).

In addition to the optimal transfection activity achieved with a critical ratio of DNA to cationic liposomes, the formation of aggregates is prominent with several formulations of cationic liposome. Aggregation occurs by bridging between DNA molecules mediated by the complexed cationic liposomes. The aggregate size varies and can be several microns in diameter. These large aggregates are not expected to enter cells by endocytosis because the size of endosome is limited to a few hundred nanometers in diameter (36). Smaller DNA/liposome complexes may enter cells by endocytosis as was shown by electron microscopy (35). A model implicating endocytosis in the uptake of lipospermine-complexed DNA was proposed by Barthel et al (33). The model sees the complex as a globule with a net cationic charge that undergo a zipper-like mechanism of uptake by endocytosis (Figure 2). However, no direct data was provided to support the model. On the other hand, the model proposed by Zhou et al (35) provided direct electron microscopy evidence for endocytosis of lipopolylysine-complexed DNA (Figure 3). Lipospermine and lipopolylysine are cationic lipids with similar overall structures and components and both are capable of complexing and efficiently condensing the DNA. Whether the model is applicable with other cationic liposome formulations is currently unclear.

The role of the phospholipid in DNA delivery:

The role of DOPE in cationic liposome formulations is dual. Some cationic lipids, such as cationic cholesterols, can not form vesicles without the phospholipid component (25,26,31). This particular role for vesicle formation and stabilization can be performed by many types of lipid and is not unique to DOPE (35). However, the second proposed role of the phospholipid is dependent on DOPE and in general can not be substituted by other lipids (26,35). DOPE has a strong tendency to form inverted hexagonal (H_{II}) phase under physiological conditions (37). The primary amine group of DOPE hydrogen bonds with neighboring phosphate group resulting in a poor hydration of the ethanolamine head group (38). The bulky oleoyl chains of the molecule further favor an aggregation state of inverted micelle (38). In the presence of charged amphiphiles, such as a cationic lipid, the level of interfacial hydration is sufficiently increased such that bilayer liposomes (L_α phase) are stabilized. When the charge of the amphiphile is neutralized by binding with an opposite charge, such as DNA, it is highly likely that DOPE will revert to the inverted micellar structure (Figure 4). If sufficient amount of DOPE is added to a biological membrane, it is also conceivable that the membrane would be destabilized by forming localized non-bilayer structures. This property of DOPE is important for the penetration of DNA/liposome complex across a biological membrane for transfection. Substitution of DOPE with the membrane stabilizing phospholipid known as dioleoyl phosphatidylcholine

(DOPC) (Figure 1) impaired the DNA delivery function of lipopolylysine liposomes which again became dependent on scraping (35). DOPC is an $L\alpha$ phase forming lipid which stabilizes lipid bilayer as a result of the readily hydratable choline head group (38).

The content of DOPE in the liposomes varies from 0-50 mole% depending on the preparation. Approximately 30-50 mole% of DOPE is need for DNA delivery. Most attempts to replace DOPE with other lipids have failed to produce an efficient DNA carrier (26,35). Only a few cationic liposomes can deliver DNA without DOPE (mentioned earlier). The work described in chapters 3 and 4 of this dissertation demonstrate a direct relationship between macromolecule (DNA and protein) delivery and % DOPE content. Optimal delivery was shown at 80 % DOPE. However, available cationic liposome formulations do not exceed 50 % DOPE content because of the need for high cationic lipid content (50-70 %) to complex with and neutralize the large quantity of negative charges of DNA.

In general, entry of macromolecules into cells (largely studied with DNA) mediated by cationic liposomes has been shown to take place essentially by adsorptive endocytosis with minor delivery activity directly through the plasma membrane (17,35). The available models of macromolecules entry into cells predict the disruption of endosomal membrane (facilitated by DOPE) by cationic liposomes, an event that

releases the macromolecules into the cytosol of transfected cells. The electron microscopy observations (35) showing release of DNA/lipopolylysine complexes into the cytosol of mammalian cells through a disrupted endosomal membrane lend strong support to the endocytosis model. Additonal support for this model comes from micrographs showing uptake of DNA complexes by coated pits at the plasma membrane surface of cells (35). The mechanism by which DNA molecules enter into the nucleus from the cytosol is completely unknown.

3. Delivery of Macromolecules by Cationic Liposomes

DNA delivery was the motivation for the development of cationic liposomes (23). The choice of DNA is justified by the stability of DNA in comparison to other macromolecules including RNA and protein. Moreover, DNA, being the information source of both RNA and protein, can provide a constant and long-lasting supply of both macromolecules. Additionaly, gene therapy by DNA transfer can potentially cure a genetic disease by permanent replacement of the defective gene with a normal gene. Permanent therapy of genetic diseases is not possible without DNA delivery. In practice, however, this ideal form of gene therapy is not currently feasible and repeated doses of the gene (DNA), RNA, or protein can provide a temporary relief for the patients.

The large size of DNA and the difficulties associated with its delivery has lent support to attempts for delivery of macromolecule other than DNA using cationic liposomes. In addition, the need for transient expression of a gene is best fulfilled by delivery of unstable gene products such as RNA and protein.

RNA delivery by cationic liposomes:

The first attempt to deliver RNA by cationic liposomes was reported by Ballas et al (27). Tobacco mosaic virus RNA was successfully delivered into tobacco and petunia plant protoplasts using cationic liposomes and was assayed using a viral capsid protein quantitation assay. The cationic liposome composition used was atypical and contained cholesterol, phosphatidylcholine and a quaternary ammonium detergent. The liposomes were complexed with RNA by simple mixing similarly to DNA complexing. Removal of the cationic detergent abolished the majority of RNA delivery activity. This is an expected result since the cationic detergent is used for electrostatic interaction with RNA and cells. An interesting comparison was made between the delivery of RNA complexed to the cationic liposomes and the delivery of the same RNA encapsulated in the same cationic liposomes (27). Complexed RNA showed higher delivery efficiency than the encapsulated RNA, was simpler and more reproducible, and showed no toxic effects.

Another delivery study of RNA complexed to cationic liposomes was reported a year later (39). This study used Lipofectin™ to deliver luciferase RNA, synthesized *in vitro*, into a variety of mammalian cells as well as amphibian and insect cells. The study was aimed toward a comparison between the role of capped vs uncapped RNA and the role of β-globin 5' and 3' untranslated sequences on translation in a reporter gene expression assay. The study showed a 1,000-fold enhancement in gene expression when capped RNA coding for the untranslated sequences was used. The kinetics of RNA delivery was rapid with easily detectable reporter protein activity in less than 1 hour after the delivery was initiated. Within 1 hour of RNA delivery, 71 % of the delivered RNA was shown to adhere to cells, 32 % of which were RNase-resistant which shows the protecting effect of cationic liposome complexing. The delivery using cationic liposomes was simple, efficient, and reproducible. Delivery was also quite versatile and successful with respect to the variety of target cells including those normally refractive to transfection.

Viral delivery by cationic liposomes:

Lipofectin™ was also tested for the delivery of murine leukemia virus and packaged retroviral vectors to cells normally uninfectable by these retroviral particles due to the absence of specific receptors on the cell surface (40). Simple addition of cationic liposomes to the virus incubated with target cells allowed efficient delivery of the viral genome into the

cells. In contrast to cationic liposomes, the presence of polybrene, a cationic polymer used for DNA transfection, did not allow the infection of cells. In addition to high infection efficiency, the simplicity and reproducibility of the cationic liposome-mediated viral infection of nonpermissive cell makes this delivery strategy an available remedy to overcome resistance of a variety of cells to infection.

Protein delivery by cationic liposomes:

Transient delivery of macromolecules such as proteins can be a challenge and/or toxic or injurous when the common delivery protocols are employed including electroporation (41), scrape-loading (42), and liposome encapsulation (22). The introduction of cationic liposome-mediated delivery of proteins by Debs *et al* in 1990 has simplified and popularized protein delivery (43). The report described the delivery of a purified recombinant glucocorticoid receptor fragment, a mammalian transcriptional regulator protein, to mammalian cells using Lipofectin™ (43). The protein was delivered with or without a *cis* -acting DNA element that codes for a reporter gene under the control of the element responsive to induction by the glucocorticoid receptor protein. High levels of gene expression was induced in this system. An unexpected result was the high efficiency of protein delivery when the cationic lipid component of Lipofectin (i.e. DOTMA) was used alone without DOPE (43).

Recently, protein delivery by cationic liposomes had application in the field of molecular immunology through three different studies all of which used cationic liposomes for delivery of antigens. The first was by Walker et al who delivered the herpes simplex virus (HSV) glycoprotein B antigen (44). The recombinant protein was delivered (44) to cells by mixing the protein with a cationic liposome formulation containing *N* -[1-(2,3-dioleoyloxy)propyl]-*N,N,N* -trimethylammonium methyl sulfate (DOTAP) and DOPE (31). The delivery resulted in the presentation of a peptide fragment of the protein by the class I major histocompatibility (MHC) complex receptor and sensitized the cells to lysis by class I MHC-restricted, HSV-specific cytotoxic T lymphocytes (CTL). In addition, a memory CTL response was evoked specific for the HSV protein delivered by the liposomes. This memory response was stimulated in spleen cells from HSV-infected mice after the mice were injected subcutaneously with a mixture of DOTAP liposomes and the HSV protein. A similar injection strategy with complete Freund's adjuvant did not produce any memory immune response (44). This novel strategy of immunization should help facilitate the induction of CTL response i*n vivo* without introduction of the whole virus *in vivo*. Additionally, this simple delivery system may help in dissecting the class I MHC antigen presentation pathway.

The second study used lipopolylysine liposomes to deliver the anionic and antigenic protein ovalbumin which could readily form complexes with the cationic liposomes (45). A mouse cell line was the target cells for

delivery of the antigen. Successful uptake of the complexed antigen caused sensitization of the mouse cells to killing by an ovalbumin-specific CTL. Optimum sensitization to killing ocurred at an ovalbumin concentration of 20 μg/ml. The delivery was more potent if the target cells were treated with phorbol myristate acetate which may activate endocytosis leading to enhanced uptake of the complex (45). Lipopolylysine delivery of antigen compared favorably to the osmotic loading method (45). Studying the antigen presentation pathway can be simplified using lipopolylysine mediated atigen delivery.

The third study reported using DOTAP for the delivery of ovalbumin protein with subsequent presentation by the class I MHC pathway (46). In this study, electroporation, osmotic loading, and cationic liposomes (DOTAP) were tested for ovalbumin delivery. Electroporation and liposomes were highly efficient delivery vehicles in comparison with osmotic loading. However, the toxicity associated with electroporation and the simplicity of cationic liposomes delivery make liposomes a prefered delivery carrier.

Enzyme delivery by complexing to cationic liposomes (Lipofectin™) was reported by Lin *et al* (47). The enzyme delivered was a purified prostatic acid phosphatase protein and human prostate carcinoma cells were used as targets for the delivery and enzymatic activity was demonstrated by a tartrate-sensitive acid phosphatase assay. Retention of enzymatic activity

after delivery was maintained for at least 48 hours. Immunofluorescence staining localized the delivered enzyme to the cytoplasm of cells. Delivery kinetics were as fast as RNA delivery as 70 % of the enzyme presented was incorporated into cells within one hour of incubation. This report took advantage of the acidic nature of the enzyme which is negatively charged at physiological pH allowing efficient complexing with cationic liposomes. The data from this work demonstrated the feasibility of enzyme delivery by cationic liposomes which may help the studies in which enzymes are chemically and/or enzymatically manipulated *in vitro* before delivered to cells for functional test.

An interesting and novel application for protein delivery with cationic liposomes was introduced by Gao and Huang with gene therapy in mind (48). A well known barrier to DNA delivery is the nuclear uptake of DNA after the DNA reaches the cytosol (49). This problem was tackeled by a cytoplasmic expression system borrowed from bacteriophage T7 which encode for an enzyme (T7 RNA polymerase) that has been shown to transcribe DNA into RNA in the cytosol of mammalian cells (48). Gao and Huang mixed a purified recombinant T7 RNA polymerase with DNA coding for a reporter gene under the control of T7-specific promoter and complexed the protein/DNA mix to cationic liposomes. The ternary complex was incubated with mammalian cells for transfection. Cationic liposomes containing 3β [N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and DOPE as well as Lipofectin™ were used

successfully in this study. Reporter gene expression was shown to occur with faster kinetics than the expression of the reporter gene under the control of a nuclear promoter which is nonresponsive to T7 polymerase. This was indicative of rapid cytoplasmic gene expression independent of nuclear transcription factors. The short half-life of this gene expression under the control of the T7 system was also consistant with the notion of cytoplasmic expression since nuclear gene expression can last at least twice as long as the T7 system did. The T7 system described can be a useful new tool for gene therapy applications where a fast, transient, but robust level of transgene expression is prefered.

4. Gene Therapy Using Cationic Liposomes

One of the most important applications of gene transfer studies is gene therapy of human diseases (1). In spite of the fact that gene therapy was born in an effort to cure inherited diseases, gene therapy is now targeted toward many diseases existing with or without available therapy. This wide range of diseases spans genetic diseases such as cystic fibrosis (CF), cancer diseases including benign and malignant tumors, and infectious diseases such as AIDS, among others (1,7-9).

The idea behind exploiting cationic liposomes for gene therapy was born as an alternative to other gene therapy protocols that had potential toxicity, biohazard, and immunogenicity (7-9). Cationic liposomes showed

minimal toxicity effects in cell culture systems and were not expected to be immunogenic since they are composed of nonimmunogenic lipids.

Organ distribution, gene expression, and toxicity studies using cationic liposomes:

The first report on the use of cationic liposomes for gene delivery in animals came from Brigham *et al* in 1989 (50). Mice were used for testing the delivery of a reporter gene DNA complexed to Lipofectin™ to lung tissue. Delivery of complexed DNA intraveneously or intratracheally, but not intraperitoneally, resulted in gene expression in the lung with no detectable activity in the liver or spleen. Gene expression was persistant for at least one week from the time of injection. The data presented an opportunity to use cationic liposomes as a DNA carrier for gene therapy since all previous work was performed on cultured cells.

Another *in vivo* study took advantage of Lipofectin™ to deliver a reporter gene to lungs of mice (51). Aerosolization of complexed DNA was the route of delivery employed in this study which allowed lung-specific targeting of DNA. Gene expression persisted for at least 21 days after administration of reporter gene. *in situ* immunostaining also detected the reporter gene expression in the majority of epithelial and alveolar lining cells in the lung. No injury was detectable with histological screening in spite of the extensive gene expression in the lung sections.

These results lend more support to gene therapy with cationic lipid vehicles.

In addition to the previous two studies (50, 51) and to set the stage for the first human cancer gene therapy clinical trial using cationic liposomes, five years after the first introduction of cationic liposomes as a mediator of gene delivery (23), animal testing studies were initiated in mice by Nabel *et al* using DC-Chol cationic liposome (52,53). The first objective of these studies was the evaluation of the toxicity and organ distribution of DNA/DC-Chol cationic liposome complex using different assays (52). The route of complex delivery was by intravenous or direct intratumor injection. The majority of DNA was localized in the heart and lung tissues, analyzed by quantitative polymerase chain reaction (PCR) assays, 9-11 days after i.v. injection of complex. Intratumor injection resulted in detection of DNA in the tumor injected. To assess the toxicity of the complex, histological examinations were performed in addition to assays examining tissue-specific serum enzymes, biochemical analysis, and electrocardiographic monitoring of treated mice. None of the toxicity tests performed showed any abnormalities caused by the inclusion of liposomes in the treatment.

The initial studies showing lack of toxicity with liposomes mandated further tests with other animals such as pigs and rabbits (53). No detectable histological toxicity or serum biochemical abnormalities were

seen. Pertinent to the long-term expression of the transgenes delivered were tests aimed at immunologic responses potentially causing autoimmune damage, potential gonadal localization of injected complex, and possible toxic effects if animals other than mice were used (53). Histological testing included all major organs of the mouse revealing no detectable immunopathological damage to the organs. PCR analysis also could not detect any gonadal localization of transfected genes including testes and ovaries but could easily detect the genes in lung, kidney, spleen, and liver. The data from all the biodistribution and toxicity studies support the use of DC-Chol cationic liposomes for DNA delivery in a human cancer gene therapy protocol (18).

A surprisingly efficient DNA delivery by cationic liposomes was reported by Zhu *et al in vivo* in 1993 (54). A single direct injection of Lipofectin™-complexed DNA coding for a reporter gene into mice, via the tail vain, led to transfection of almost all mouse tissue including vascular endothelium, lung, spleen, heart, liver, kidney, bone marrow, and lymph nodes detectable by immunohistoanalysis.The treatment protocol showed no toxicity and gene expression was efficiently extended to at least 9 weeks after the injection. These unexpected results delivered a new hope for gene therapy aimed toward targeting expression to all the major organs in the body. However, more reports in the future are required to confirm the study.

Another *in vivo* study described the transfection of splenic T lymphocytes by intraperitoneal injection of a reporter gene DNA complexed to three different types of cationic liposomes in mice (55). In addition to lymphocytes, bone marrow-derived hematopoietic cells were also transfected. Gene expression lasted for at least 2 weeks with no detectable toxicity. This study introduces the opportunity to use gene therapy to combat diseases affecting the immune system.

Local transfection of tissues in an organ was shown recently by Roessler *et al* in a study describing the injection of DNA, coding for a reporter gene, complexed to Lipofectin™ into the mouse brain (55). Plasmid DNA expression lasted at least 3 weeks after transfection. The results were accumulated through histochemical analysis of tissue sections of the treated brains. This report was subsequent to the preliminary and similar report by Ono *et al.* who also described the same protocol resulting in up to 9 days of gene expression post-injection of the complex into a neonatal mouse brain (56).

One of the most recent reports described an *in vivo* transfection with cationic liposomes by demonstrating gene transfer into the lungs of rabbits (57). The animals were injected with DNA, coding for a recombinant human α1-antitrypsin gene, complexed to Lipofectin™ intravenously or by aerosol. The human gene was expressed efficiently regardless of the injection route for at least 7 days post-injection. The

intravenous injection route led to gene expression in the pulmonary endothelium while the aerosol route led to alveolar epithelium expression. Expression in the airway epithelium was a result of both routes of injection. Radiolabeling of DNA injected allowed its detection in endothelial cells in the lung. These data set a precedent for targeting DNA delivery to specific subsets of cells in the lung by a selective route of delivery.

Cationic liposome-mediated gene delivery was compared favorably to the biolistic particle delivery system in a report by Hui *et al* (58). DC-Chol:DOPE liposomes were used to deliver either a reporter gene or an allogeneic class I MHC gene to tumor cells *in vivo*. A strong CTL response was generated against tumor cells expressing the MHC gene after the injection of DNA/liposome complex directly into mouse spleen. This strategy should lend support to a therapy protocol which employs a direct injection of the spleen of cancer victims in order to activate an immune response against tumor cells.

Gene therapy in mice by gene transfer with cationic liposomes:

Cancer immunotherapy in an animal model (mouse) using cationic liposome mediated gene transfer was the final step before the actual human gene therapy trial. Nabel *et al* introduced DNA complexed to DC-Chol cationic liposomes into mice bearing malignant tumors (melanoma) by

direct injection of the complex into the tumor lesions (59). The delivered DNA codes for a foreign MHC class I antigen gene which is expected to stimulate tumor rejection by allospecific CTL if the gene is expressed. Expression of the gene induced a specific CTL response to the cells expressing the foreign gene in addition to a general immune response to other tumor cells not expressing the gene. As a result of the specific and general immune response, tumor growth was hindered and in some cases a complete tumor regression was observed.

Two other animal studies reported recently were aimed toward gene therapy of CF in a mouse model of the disease (60,61). In the first report, a transgenic mouse with a disrupted (mutated) cystic fibrosis transmembrane conductance regulator (CFTR) gene were used to test the delivery of wild type CFTR gene via Lipofectin™ (60). The DNA/Lipofectin™ complex was delivered intratracheally which allowed transfection of the epithelia of the airway and alveoli deep in the lungs. CFTR gene expression and correction of the ion conductance defects in the trachea were demonstrated by *in situ* hybridization and voltage clamping techniques.

The second report used CFTR mutant mice and human CFTR gene complexed to either Lipofectin™ or DOTAP cationic liposomes (61). The complexes were delivered into the airways by nebulization, a treatment that resulted in 50 % correction of the deficient ion transport involved in CF in

some of the mutant mice. The human CFTR gene expression was detected by reverse transcriptase PCR. The success of these two reports paved the road for human CF gene therapy clinical trials using cationic liposomes (20).

Human gene therapy trials using therapeutic DNA complexed to cationic liposomes:

The significant results with animal studies prompted Nabel *et al* to pursue a human cancer gene therapy clinical trial using DC-Chol cationic liposomes as a carrier for therapeutic genes (19). Five patients with melanoma refractory to all available therapy participated in this study which aimed at demonstrating gene transfer, gene expression and safety (19). All patients received injections of DNA/DC-Chol liposome complexes intratumorly. The DNA injected codes for a foreign MHC protein, HLA-B7. All patients were HLA-B7-negative. Three to seven days after injection tumor biopsies were tested with PCR for the presence of injected DNA which was detected in the tumor nodules but not in the serum. Gene expression was also detected using immunostaining for HLA-B7 protein. In addition, immune responses to HLA-B7 protein were detected in injected patients. No toxicity was detected in any of the five patients. A significant finding was the regression of two injected tumor nodules at different sites in one of the treated patients. Moreover,

regression of uninjected tumor nodules at a distant site were also observed in the same patient after treatment.

CF was the second target disease for human gene therapy using cationic liposomes as a carrier for therapeutic DNA. This human clinical trial also used DC-Chol cationic liposomes (20). The DNA/liposome complex was delivered to the nasal epithelia of CF patients as part of the phase I clinical trial (20). The trial is still ungoing and the results will be reported in the near future. As more of these clinical trials are approved, the efficacy and long term safety of the treatment should become more established.

5. Conclusions

It is evident from this introduction that the field of cationic liposomes mediated delivery has progressed tremendously since the first report of a cationic lipid formulation for gene delivery (23). It is also evident that the progress was much more rapid in the applications of cationic liposomes than basic research and formulation development. This is mainly due to the demanding field of gene delivery and therapy which is in favor of novel delivery systems devoid of undesirable side effects such as toxicity, biohazard, and immunogenecity. Preliminary studies with DC-Chol liposomes to address the issues of toxicity and immunogenecity (19,50,51) clearly established the safety of cationic lipid carriers for gene

therapy. Yet to be demonstrated is the efficacy of these carriers in different disease models and in repeated tests in animals and humans. These tests should be imminent since the preliminary success with cationic liposomes have attracted the attention of many clinicians interested in using the liposomes in human clinical trials around the world.

Another important field is yet to benefit from cationic liposome mediated gene delivery (i. e. biotechnology). This field has produced a large number of transgenic animals for a variety of applications which have proven to be extremely useful in basic research as well as in biomedical research (62,63). Delivery of engineered genes into embryonic cells of animals in order to produce a transgenic animal is challenging and is certainly in need of improved DNA delivery vehicles. Cationic liposomes may have future application in this area provided that delivered DNA is stably expressed in the transgenic tissue. Stable expression of a transgene delivered by cationic liposomes is a nascent area of study and is quickly developing. One can envision cationic liposomes mediated delivery of protein co-factors which may control the integration of a co-delivered transgene, which codes for a stable expression DNA element. This type of stable delivery system is being explored in our laboratory as part of our effort to control gene expression by co-delivery of protein factors with DNA complexed to cationic liposomes.

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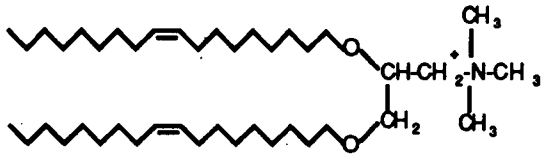

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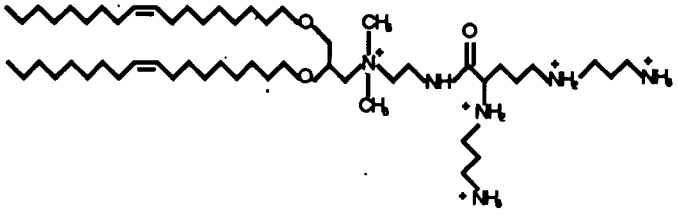

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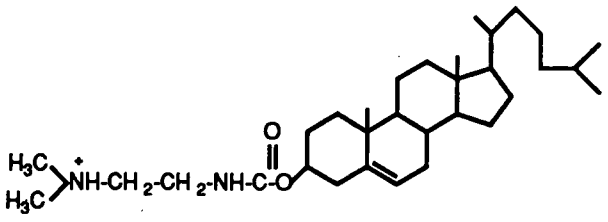

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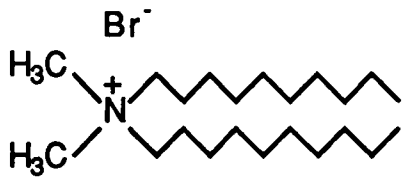
Figure 1. Cationic lipids (1-7) and phospholipids (DOPC and DOPE) structures. 1, DOTMA; 2, DOSPA; 3, DC-Chol; 4, DDAB; 5, DOGS; 6,DOTAP; 7, DMRIE (borrowed from Frank Sorgi).



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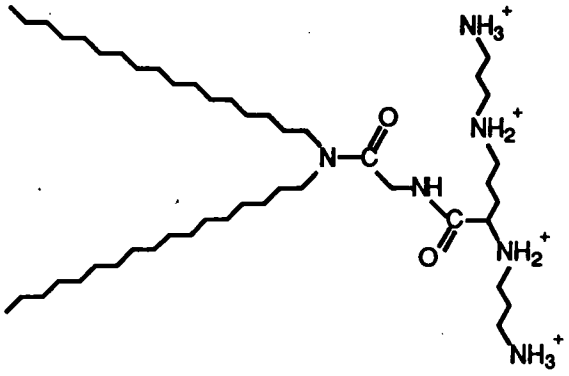

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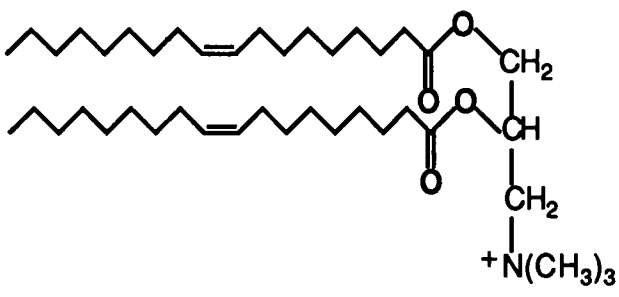

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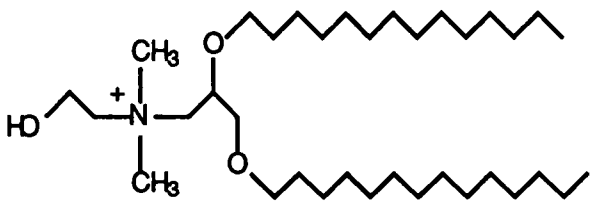

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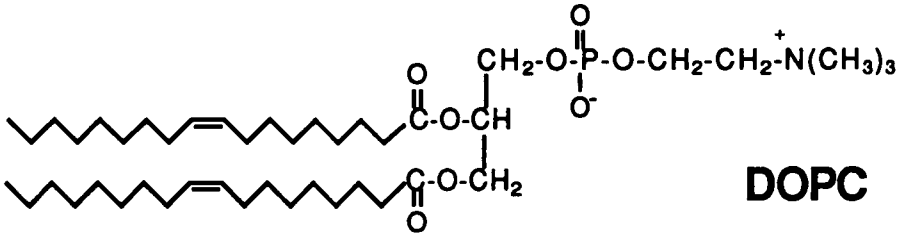
Figure 1 (continued)



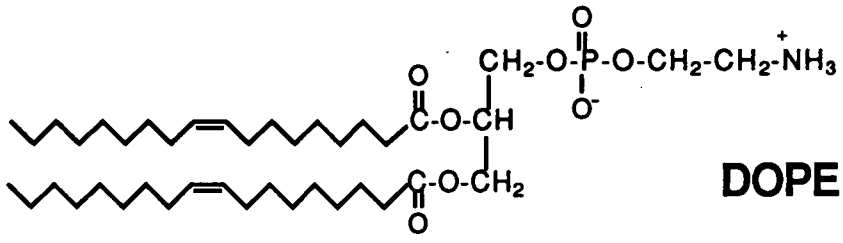


Figure 1 (continued)

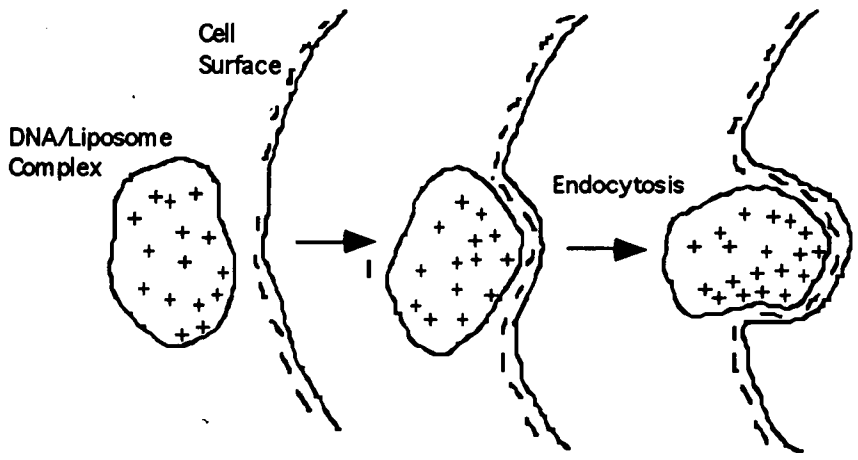


Figure 2. A model for DNA delivery by cationic liposomes (Modified from reference 33, Behr *et al*).

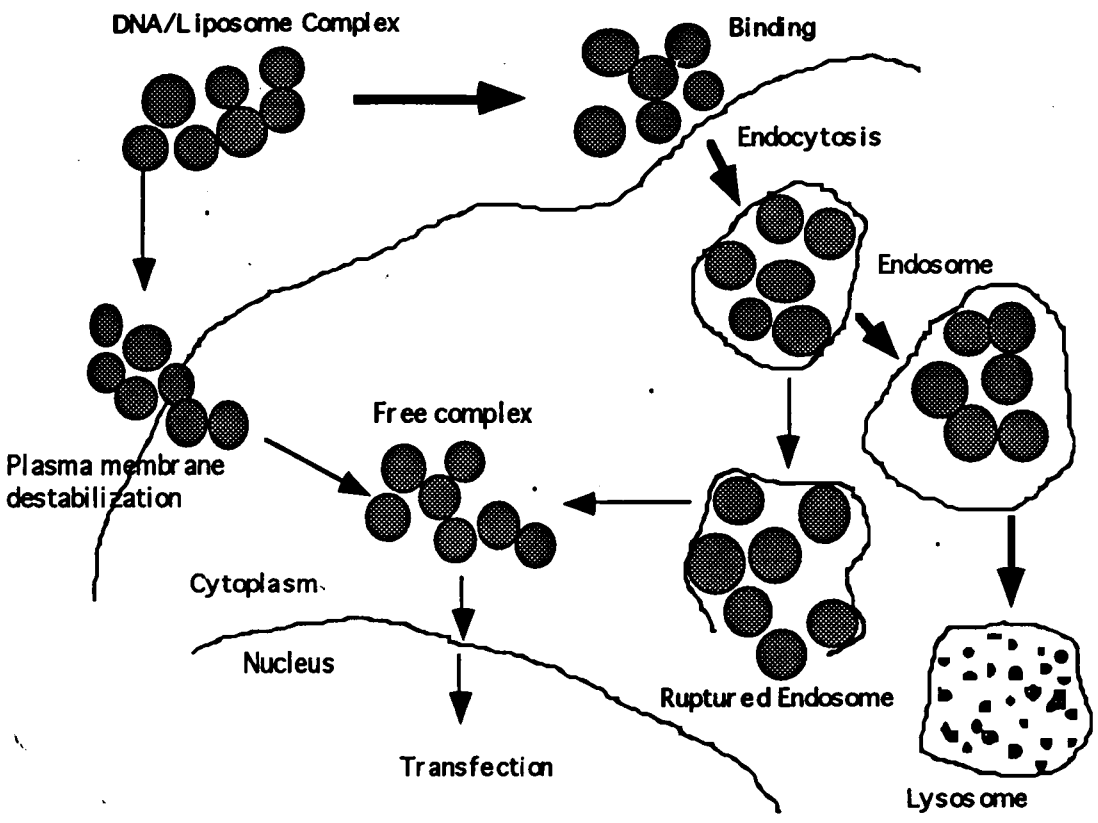


Figure 3. A Model for delivery of DNA by cationic liposomes (Modified from reference 35, Zhou *et al*). Thick arrows indicate the major pathway.

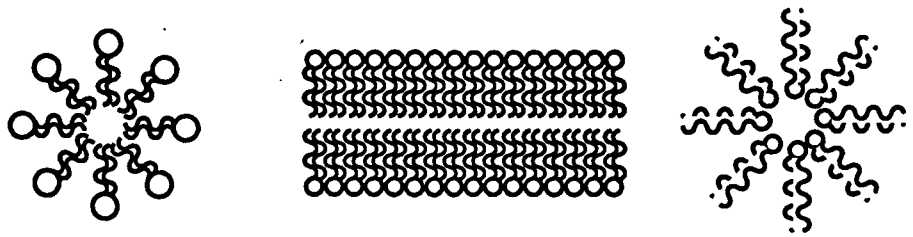


Figure 4. Different lipid phases structures (borrowed from Frank Sorgi).

PART II

EFFECT OF CATIONIC CHOLESTEROL DERIVATIVES ON GENE TRANSFER AND PROTEIN KINASE C ACTIVITY

1. Abstract

Four different cationic derivatives of cholesterol were synthesized which contain either a tertiary or a quaternary amino head group, with and without a succinyl spacer arm. Their ability to inhibit protein kinase C (PKC) activity was measured in a detergent mixed micellar solution. Derivatives containing a quaternary amino head group were effective inhibitors (K_i 12 and 59 μM) of PKC and derivatives containing a tertiary amino head group were approximately 4 to 20-fold less inhibitory. Liposomes containing an equimolar mixture of dioleoyl phosphatidylethanolamine (DOPE) and a cationic cholesterol derivative were tested for the DNA mediated transfection activity in mouse L929 cells. Highest activity was found with the derivative with low PKC inhibitory activity and with a succinyl spacer arm. The transfection activity of this tertiary amine derivative, N,N-dimethylethylenediaminyl succinyl cholesterol was dependent on DOPE as a helper lipid; liposomes containing dioleoyl phosphatidylcholine and this derivative had little activity. The transfection protocol of this new cationic liposome reagent was optimized with respect to the ratio of liposome/DNA, dose of the complex and time of incubation with cells. Several adherent cell lines could be efficiently transfected with this liposome reagent without any apparent cytotoxicity. However, the transfection activity was strongly inhibited by the presence of serum components.

2. Introduction

DNA mediated transfection has become an important tool in modern biology. Among the conventional reagents such as calcium phosphate, DEAE-dextran and other particulate reagents, liposomes have become increasingly acceptable as a convenient and reproducible reagent for DNA mediated transfection. There are generally two classes of liposomal transfection reagents: those which are cationic (1-9) and those which are anionic (10-16). Transfection with anionic liposomes generally requires that the DNA be entrapped in the internal aqueous space of the liposomes although one exception has been reported (17). Because of the relatively time-consuming protocol for DNA entrapment, anionic liposomes are not widely used for transfection. Transfection with cationic liposomes, on the other hand, does not require DNA entrapment in the liposomes. Instead, complexes of DNA and liposomes can be easily prepared by simple mixing and reasonable transfection efficiency is reproducibly obtained for many different cell types. The most widely used cationic liposome reagent is lipofectin which is composed of an equal molar mixture of DOPE and DOTMA (1). DOTMA is a double-chain amphiphile which contains a positively charged, quaternary amino head group (18). Although lipofectin is effective in delivering DNA, RNA, (1,19-21), and negatively charged proteins (22) into cells, its use is often limited by the toxicity to the treated cells and the relatively high cost of the reagent.

Cationic amphiphiles are often good stabilizer molecules for the L_α phase of unsaturated PE (3). Amphiphiles containing a quaternary amino group show higher stabilization activities than those containing a tertiary amino group (23). This is related to the difference in the charge content of the amphiphile at neutral pH. Higher level of positive charge content of the quaternary amino group brings more interfacial hydration to stabilize the L_α phase of unsaturated PE (23). On the other hand, amphiphiles containing quaternary amino groups are effective inhibitors of protein kinase C in addition to being better L_α phase stabilizers (23).

We describe here the protein kinase C inhibition activities of several cationic amphiphiles which contain a common hydrophobic moiety, i.e. cholesterol, and compare their activities to promote the DNA mediated transfection in mammalian cells. Furthermore, we have studied in detail the transfection activity of one of the cationic cholesterol derivatives containing a tertiary amino head group. The amphiphile, N,N-dimethyl ethylene diaminyl succinyl cholesterol, when mixed with DOPE to form liposomes, is highly effective in promoting the DNA mediated transfection. Furthermore, the new transfection reagent is not toxic to the treated cells. The preparation and characterization of the new liposome reagent are described in this report.

3. Materials and Methods

Materials:

DOPE and DOPC were purchased from Avanti Polar Lipids (Alabaster, AL). DNAse I was purchased from Sigma. Lipofectin was purchased from Bethesda Research Laboratory.

Synthesis of cationic cholesterol derivatives:

The synthesis of cholesteryl-3β-carboxyamidoethylenedimethylamine (IV), cholesteryl-3β-carboxyamidoethylene-trimethylammonium iodide (III), cholesteryl-3β-oxysuccinamidoethylenedimethylamine (II) and cholesteryl-3β-oxysuccinamido-ethylenetrimethylammonium iodide (I) is described in reference 23. In that paper compounds IX, VIII, IV and III refer to compounds I, II, III and IV in the present work.

Isolation of protein kinase C:

Protein kinase C was purified from rat brain to near homogeneity by a modification of a published method (24). 25 brains from Sprague-Dawley rats (150-200g) were removed, washed, homogenized and applied to a DEAE Sepharose, Phenyl Sepharose and polylysine agarose columns. The uncontaminated fractions containing PKC were pooled and salts were

removed using Amicon YM-10 ultrafiltration. The enzyme gave a specific activity of 200 nmoles phosphate incorporated per min per mg of protein in a histone phosphorylation assay using the triton mixed micelle assay with 6.5 mole % phosphatidylserine, 2.5 mole % DAG, and 100 μM calcium present. Specific activities ranging from 30 nmoles/min/mg (25) to 600 nmoles/min/mg (26) have been observed for PKC using the Triton mixed micelle assay under the same conditions.

Mixed micelle assay for protein kinase C:

The Triton X-100 assay previously described by Bell and coworkers was used to measure enzyme activity (27). Phosphatidylserine and 1,2-diolein with and without additive were dissolved in a solution of chloroform/methanol (2:1, v/v). Solvent was evaporated with a stream of nitrogen and last traces removed using a vacuum desiccator at 40°C. The lipid films were then solubilized by the addition of 3% Triton X-100, vortexed vigorously for 30 sec and then incubated at 30°C for 10 min to allow for equilibration. A 25 μl aliquot of this solution was used in a final assay volume of 250 μl, containing 20 mM TRIS-HCl, pH 7.5, 10 mM $MgCl_2$, 200 μg/ml histone III-S, 100 μM $CaCl_2$, 10 μM [γ-^{32}P] adenosine 5' triphosphate, 2.75 mM Triton X-100, with 300 μM (6.5 mol %) phosphatidylserine and 107 μM (2.5 mol %) 1,2-diolein. For controls, 25 μl of 20 mM EGTA replaced the $CaCl_2$. To initiate the reaction, 150 ng of protein was added. After brief mixing, the tubes were incubated for 10

min at 30°C. The reaction was terminated by adding 1 ml of cold 0.5 mg/ml BSA and 1 ml of cold 25% trichloroacetic acid. This mixture was passed through a GF/C Whatman filter and washed five times with 2 mL of 25% trichloroacetic acid. After drying, the filters were counted with 6 ml ACS scintillation fluid.

Liposome preparation:

DOPC or DOPE were combined with cationic cholesterol derivatives in chloroform and dried with N_2 gas to remove the chloroform solvent. The dried lipid film was vacuum desiccated for at least half an hour and suspended by vortexing in dH_2O. After this hydration step, the samples were vortexed briefly and sonicated in a bath type sonicator (Laboratory Supplies, Hicksville, NY) to generate small unilamellar vesicles. Vesicles were stored at 4°C at a total lipid concentration of 3.5 mM.

Cell culture:

L929 mouse fibroblasts and Vero monkey kidney cells were routinely cultured in McCoy's 5A modified media (Gibco) supplemented with 10% bovine calf serum. HeLa cells were cultured in Eagles' MEM media with Earle's salts supplemented with 10% fetal calf serum (Hyclone). A431 human epidermoid carcinoma (from G. Carpenter, Vanderbilt University), FAO human liver cell line (a gift from Dr. John Koontz at

University of Tennessee at Knoxville), and A375 human malignant melanoma were routinely cultured in DMEM with L-glutamine, 4.5g/L glucose and 10% fetal calf serum. CCRF-CEM human T lymphoblastoid cells (obtained from ATCC) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. OKT4 hybridoma (obtained from American Type Culture Collection) was cultured in Iscove's modified Dulbecco medium with 20% fetal calf serum. All media contained antibiotics:100 units/ml, penicillin, 100 µg/ml streptomycin and 10 µg/ml Gentamycin. Cells were cultured in 5% CO_2 and 95% humidified air.

Transfection and CAT assay:

Plasmid pUCSV2CAT (a gift from T. Hazinski) containing the E. coli CAT gene driven by the SV40 virus early promoter was used in this study. Plasmid DNA was purified by standard method (28). Five µg plasmid DNA (1.75 mg/ml) in 1X TE buffer was mixed gently with liposomes at room temperature in 1 ml serum-free McCoy's medium and incubated for 10 min before adding to cells. Cells at 50-80% confluency in 100 mm plastic plates were washed once with serum-free McCoy's medium after which 2 ml of the same medium was added per plate. DNA-liposome complex was added to the cells in a total incubation volume of 3 ml and was incubated at 37°C with 5% CO_2 for 5 h. Transfection media was removed and the cells were incubated in the growth medium containing serum for 48 h before the CAT assay which was performed as described

(28). Basically, 60 min reaction time, 0.1 μCi [^{14}C]chloramphenicol (53 mCi/mmole), and 4 mM acetylCoA were used per sample. Equal amounts of protein per sample were used for the assay as quantitated by the Bradford microprotein assay (BioRad). Percent conversion of chloramphenicol to acetylated forms was normalized to mg proteins used per sample. The data in the figures represent an average of two experiments with duplicate samples per data point. The variability in CAT expression was reproducible under identical conditions and was eliminated when liposomes were used immediately after sonication. Variability is further decreased when the cells are transfected at the same degree of confluency.

4. Results

Cationic derivatives of cholesterol:

We have synthesized four derivatives of cholesterol, all containing an amino head group (Fig. 1). Compounds I and II contain a succinyl spacer arm between the substituted ethylene diamine and cholesterol; whereas compounds III and IV do not. Compounds I and III are quaternary amines and compounds II and IV are tertiary amines.

Inhibition of PKC:

PKC activity was assayed in Triton mixed micelles containing various amounts of the cationic cholesterol derivatives. As can be seen in Table 1, derivatives I and III, both containing a quaternary amino group, were effective inhibitors. Ki values of these compounds were in the low μM range. On the other hand, derivatives II and IV containing a tertiary amino group were weaker inhibitors. Their Ki values were about 4-20 fold higher than those of derivatives I and III.

Liposome preparations:

Cationic cholesterol derivatives did not form a stable dispersion by themselves at physiological pH. It was necessary to add at least 10% phospholipid, either DOPE or DOPC, to obtain a stable liposome dispersion by sonication. We routinely prepare liposomes with an equimolar mixture of a cationic cholesterol derivative and DOPE. Furthermore, the liposomes were prepared in deionized water or TE buffer to avoid aggregation due to the high ionic strength of the medium. The average size of the liposomes was 147 ± 5 nm as measured by dynamic light scattering.

Transfection activity and cellular toxicity of cationic cholesterol derivatives:

Liposomes containing an equimolar mixture of DOPE and a cationic cholesterol derivative were tested for the transfection activity in mouse L929 cells. Plasmid DNA was mixed with liposomes and the CAT activity of the cell extracts were determined by a standard protocol. As can be seen in Table 1, derivatives II and IV showed positive transfection activity with the activity of II much higher than that of IV. Derivatives I and III, both quaternary amines, did not show any appreciable transfection activity. Furthermore, the toxicity of the liposome/DNA complex to L929 cells was also measured by trypan blue exclusion assay. It is clear from the data in Table 1 that complexes containing quaternary amine derivatives (I and III) were more toxic to the treated cells, whereas those containing the tertiary amine derivatives (II and IV) were less toxic. These results indicate that liposomes containing the tertiary amine derivatives warrant further studies. We have decided to concentrate on the derivative II and to characterize in detail the transfection activity of the liposomes containing this derivative.

Transfection activity as a function of phospholipid composition:

Liposomes containing mixtures of II and DOPE, or II and DOPC were tested for transfection activity on the mouse L929 cells. The relative proportion of the phospholipid, either DOPE or DOPC in the liposomes

was varied from 10 to 90%. Fig. 2 shows the result of this experiment. Obviously, DOPE liposomes containing 20-50% II induced very high levels of CAT activity in the treated cells, whereas DOPE liposomes containing either too little (10%) or too much (>90%) II did not. Also shown in Fig. 2 is the observation that liposomes containing II in DOPC were not active in transfection, regardless of the concentration of the cationic amphiphile in liposomes. Thus, the transfection activity of II depended on the nature of the helper phospholipid in the liposomes. Furthermore, optimal activity of the DOPE liposomes was in those containing 20-50% II. The transfection activity of liposomes containing equimolar mixture of DOPE and II were used for further studies, because these liposomes appeared homogenous in size. However, the transfection activity of these liposomes decayed with storage (4°C) with an estimated half life of only 2 days.

Effect of lipid concentration on the transfection activity:

Liposomes composed of an equimolar mixture of II and DOPE were used to study the lipid concentration dependence of the transfection activity. In the experiment described in Fig. 3, 5 μg DNA was mixed with various amounts of liposomes and added to the L929 cells. The transfection activity showed a sudden increase at about 60 nmol total lipid above which maximal levels of CAT activity were found in the treated cells. Also shown in Fig. 3 is the comparison of transfection activity of a well-established liposome reagent, lipofectin, also using 5 μg DNA for the

assay. It is clear that lipofectin had a higher activity than the cationic liposomes described here when a sub-optimal lipid concentration was used. However, the activity of II/DOPE liposomes was much higher than that of lipofectin if higher lipid concentrations were used. Lipofectin-mediated transfection at concentrations greater than 40 nmol is not shown due to observed cytotoxicity of the treated cells.

Effect of DNA concentration on transfection activity:

In an experiment in which the total lipid concentration was kept at 70 nmol and the amount of DNA varied from 0 to 20 μg, maximal transfection activity of the treated L929 cells was found to be at 5 μg DNA (Fig. 4). Higher or lower DNA concentrations resulted in lower activities, producing a bell-shaped curve shown in the figure. From the results of this experiment and those of Fig. 3, it was decided that a ratio of 70 nmol total lipids to 5 μg DNA gave optimal transfection activity for liposomes containing an equimolar mixture of II and DOPE. The ratio of positive to negative charges of the complex at this ratio can be calculated to be approximately 2.2.

Electrophoretic characterization of liposome/DNA complexes:

We have characterized the liposome/DNA complexes by agarose gel electrophoresis (data not shown). Incubation mixtures containing

increasing amounts of liposomes showed decreasing intensities of free DNA bands and a concomitant increase in the amount of DNA on the top of the gel which did not enter the gel during electrophoresis. Furthermore, all of the uncomplexed, free DNA could be digested by DNAse, but only a small portion of the complexed DNA was digested. At the optimal liposome/DNA ratio for transfection, all DNA was complexed with liposomes.

Effect of the complex dose on transfection activity:

Complex of liposomes and DNA was prepared at the optimal ratio of 70 nmol lipid and 5 μg DNA. Various amounts of the complex were added to L929 cells to study the dose effect on transfection activity. The result shown in Fig. 5 clearly indicates that the transfection activity increased with the dose until 280 nmol total lipid and 20 μg DNA was added. Higher doses did not produce any significant increase in transfection activity (data not shown). Minimal cytotoxicity of the treated cells was observed even at the highest dose used. CAT activities shown in the figure were lower than normal because the liposomes used were not prepared on the day of the experiment.

Effect of serum in transfection activity of the complex:

The transfection activity of the liposome/DNA complex was sensitive to the presence of serum (Fig. 6). As little as 5% of calf bovine serum inhibited transfection activity by approximately 85%. No CAT activity could be detected in the cell extract if 20% serum was included in the incubation medium. Thus, the optimal condition for transfection with this novel liposome reagent should not include serum in the incubation medium.

Effect of incubation time course of liposome/DNA complex on transfection activity:

Using optimal complex (70 nmol lipid and 5 μg DNA), we have incubated the L929 cells with complex in the absence of serum for different periods of time. Data in Fig. 7 show that the degree of transfection increased with time for up to 9 h. Longer incubation times resulted in even higher levels of CAT activity in the treated cells (data not shown). However, since the incubation was carried out in the absence of serum, we have chosen 5 h as the standard condition to assure good cell viability during the incubation. The cells were approximately 95% viable by the trypan blue exclusion test under these conditions (Table 1).

Test of the transfection activity of the complex on other cell lines:

Several cell lines, both adherent and in suspension, were tested for transfection with the complex using the conditions optimized for the L929 cells (Table 2). It is clear that three other cell lines, i.e. A431 epidermal cells, Vero Kidney cells and HeLa, could be transfected at efficiencies more than 2-fold higher than that of L929 cells. However, not all adherent cells showed high transfection activity because FAO hepatoma cells could only be moderately transfected. Two suspension cell lines, CEM and OKT4, were hardly transfected at all. These results indicate that there is a large difference between the transfectability among different cell types and cell lines using the liposomes composed of II and DOPE. We have found a difference in the transfection response of different cells under different transfection conditions such as the ratio of DNA to cationic lipid. However, it may be possible to obtain better transfection results if the conditions were optimized for cells that did not transfect well such as FAO cells.

5. Discussion

It is clear from the data shown in Table 1 that cationic cholesterol derivatives are inhibitors of PKC, with quaternary amino derivatives showing stronger inhibitory activities than the tertiary amino derivatives. This observation is consistent with the previous conclusions that cationic

amphiphiles are generally inhibitors of PKC (23), probably as analogs of sphingosine, an endogenous negative effector of the enzyme (29). Also shown in Table 1 is the gene transfer activity of these derivatives when mixed with DOPE to form cationic liposomes. Tertiary amino derivatives (II and IV) showed much stronger activities than the corresponding quaternary amino derivatives (I and III) on a per mg protein basis. This is perhaps related to the cellular PKC activity which may be crucial for the mechanism with which the plasmid DNA is delivered to cells via the cationic liposomes. Furthermore, the expression of the reporter gene as controlled by the SV40 early promoter is stimulated by an activation of the PKC activity (30). When PKC is inhibited by the quaternary amino derivatives, some yet unidentified step(s) in the delivery and expression of the reporter gene may be inhibited. This is supported by the preliminary results which showed that the transfection activity of liposomes containing DOPE and derivative II can be enhanced by including phorbol myristate acetate in the liposomes (data not shown). Recently, it has been shown that transfection by the calcium phosphate method can also be significantly enhanced by PKC activators (31).

Liposome/DNA complexes containing derivatives I and III were more toxic to the treated cells than those containing II and IV (Table 1). This is may be also related to the fact that I and III are stronger inhibitors of PKC than II and IV. The amounts of I and III used in the transfection experiments were close to their Ki values, thus bringing the strong

possibility that the cellular PKC was inhibited throughout the period of incubation. Of the two tertiary amino derivatives, II was more effective than IV in the transfection activity (Table 1). This is probably due to the fact that II contains a longer spacer arm between the amino group and the hydrophobic anchor (Fig. 1) which would provide a better binding to the positively charged amino group with the negatively charged DNA liposomes containing ligands in the longer spacer arm bind to receptors more readily than those with a shorter arm. Since liposomes containing derivative II showed little or no toxicity to the cells and facilitated a strong transfection activity, detailed studies were done with this derivative.

Essentially, the transfection activity of II was observed only when it is mixed with DOPE but not DOPC (Fig. 2). Other cationic lipids also show a higher transfection activity when they are used to prepare liposomes with DOPE than with DOPC (1). Unsaturated PE has a high propensity to form the inverted hexagonal (H_{II}) phase at the physiological conditions. Although stable bilayer liposomes can be obtained when DOPE was mixed with an appropriate amount of II, aggregates of liposomes with DNA could contain some non-bilayer structures due to phase separation. The presence of these non-bilayer structures, which would be absent in liposomes containing DOPC, could be important for the entry of the complex into cells. Although fusion of the liposomes with cell membrane has been proposed as the mechanism of DNA delivery by the cationic liposomes (1), it is more likely that the liposome/DNA complex enters the

cells by endocytosis (3). Formation of the H_{II} nonbilayer phase in the acidic endosomes/lysosomes would probably destabilize the membrane of these endocytic vesicles, resulting in the release of the DNA into the cytoplasm. This mechanism, has been demonstrated for the action of another cationic liposome formulation containing lipopolylysines (32). as well as some other PE-containing liposomes (33,34).

The transfection protocol was optimized with respect to both the liposome and DNA concentrations (Figs. 3 and 4). Optimal concentrations were found such that the positive-to-negative charge ratio of the complex was approximately 2.2, i.e. there was an apparent excess of liposomes. However, it is unlikely that all negative and positive charges in the complex are involved in the ionic interactions because both the supercoiled DNA and liposomes are bulky enough to provide steric hindrance preventing close contact of each other. The excess positive charge content of the complex will certainly allow an efficient binding, and perhaps internalization, of the complex by the cells. Preliminary results using cells incubated with a fluorescently labeled complex have confirmed this hypothesis.

When the incubation conditions were optimized with respect to the concentration of the liposome/DNA complex (Fig. 5) and the incubation time (Fig. 7), the transfection activity of the DOPE/II liposomes was about 3 fold greater than that of lipofectin using L929 cells and 23 μM total

lipids (Table 1). The principal factor which contributed to this difference is the fact that the DOPE/II liposomes are not toxic to cells when complexed with DNA, whereas the complexes with lipofectin at the similar concentrations showed a high level of cytotoxicity to the treated cells (Table 1). The PKC inhibitory activity of the cationic lipid (DOTMA) in lipofectin showed a relatively lower value of Ki in comparison to that of II probably due to the presence of a quaternary amino group in DOTMA (1). The lack of toxicity and the excellent transfection activity on many different cell lines (Table 2) of the cationic liposomes composed of DOPE and II have made this liposome composition highly desirable as a reagent for *in vitro* transfection of mammalian cells. In addition, this liposome composition may be useful for stable transfection efficiency of cultured cells. A stable transfection efficiency of 3×10^{-4} was observed in LMTK$^-$ cells using HSVTK plasmid DNA and following the transient transfection protocol. However, the *in vivo* use of the reagent will be limited due to the fact that the transfection activity is strongly inhibited by the presence of serum (Fig. 6). Large amounts of negatively charged serum proteins probably also complex with the liposomes and interfere with the binding with cells. In this regard, the behavior of the new transfection reagent is similar to what has been reported for lipofectin (1). Another drawback of the new liposome reagent is that it is relatively unstable. This is probably due to the fact that II contains a ester bond which can be readily hydrolysed. The instability of liposomes containing II is reflected in the variation in CAT expression in the figures presented. The older the

liposomes are, the less efficient they are in delivering DNA to the cells. These liposomes are most efficient if used within a few hours after sonication. We have recently described a cationic cholesterol derivative (DC-chol) of similar structure but containing a more stable carbamoyl bond. Liposomes containing DC-chol are indeed much more stable than those containing II (6).

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Table 1. Inhibition of PKC and promotion of DNA mediated transfection activities of cationic cholesterol derivatives

Derivative	PKC Inhibition Ki(μM)	CAT Activity[a] mU/mg protein	Cellular Toxicity[b] % viable cells	
			70 μM	93 μM
I	59	5	85.0 ± 3.6	74.3 ± 2.5
II	191	143	94.7 ± 3.1	90.0 ± 2.6
III	12	4	78.3 ± 5.0	69.0 ± 5.0
IV	258	56	92.0 ± 3.6	86.0 ± 3.0
DOTMA	95	40	63.7 ± 7.6	44.0 ± 6.6

[a] 23 μM liposomes (70 nmol lipid) containing an equimolar mixture of DOPE and cationic derivative were mixed with pUCSV2CAT DNA (5 μg) and added to L929 cells. After 5 h incubation at 37°C, cells were washed and incubated in fresh medium containing 10% serum for 2 days before assayed for CAT activity which is shown as the average of three different experiments.

[b]Liposome/DNA complexes were prepared as described in footnote a. After 5 h incubation of L929 cells with the complex, cell viability was determined by trypan blue assay. Percent viable cells is shown as mean ± s.d. of three determinations. Control cells which received no complex showed 98% viability.

Table 2. Transfection efficiency of the DNA/liposomes complex on different cell lines.[a]

Cell line	CAT activity (mU/mgprotein)
HeLa	61.2
Vero	43.5
A431	30.9
L929	14.8
FAO	3.3
CEM	0.2
OKT4	0.1

[a] Five μg DNA and 70 nmol total lipids (liposomes composed of equimolar mixture of II and DOPE) were used. Transfection incubation time was 5h. CEM and OKT4 cells are suspension cells and the rest are adherent cells.

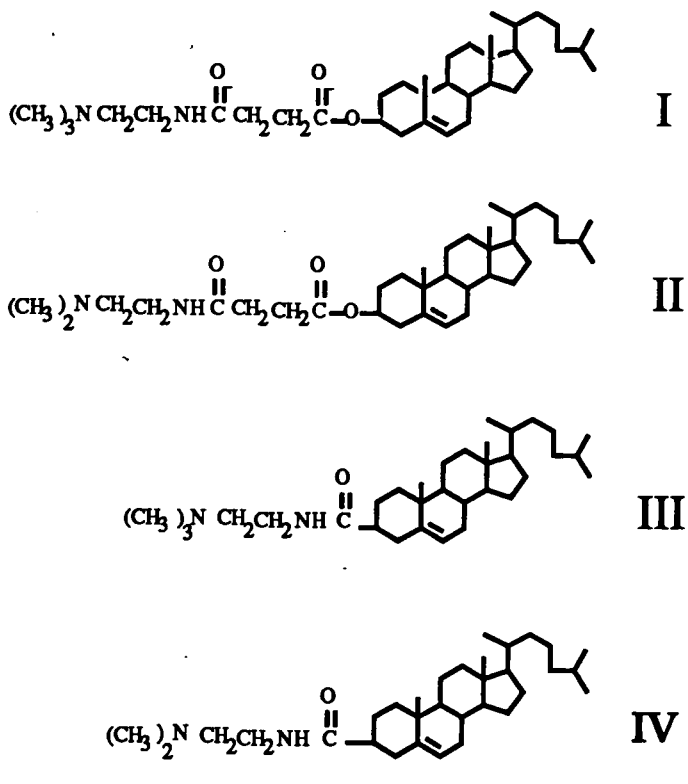

Figure 1. The structure of the four cationic cholesterol derivatives.

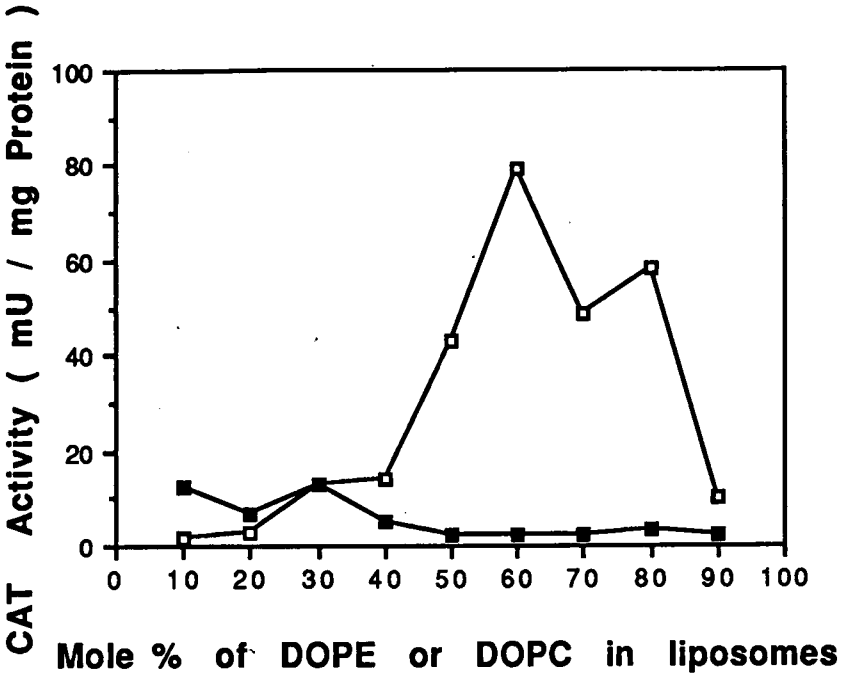

Figure 2. The effect of phospholipid composition of cationic liposomes on the transfection activity. Liposomes were prepared with derivative II and various % mole amounts of DOPE (❑) or DOPC (■). Liposomes (23 μM total lipids) were mixed with 5 μg DNA and added to L929 cells in serum-free McCoy's media. Transfection was allowed for 5 h after which the cells were washed and incubated at 37°C in serum-containing media for 2 days before CAT activity was measured.

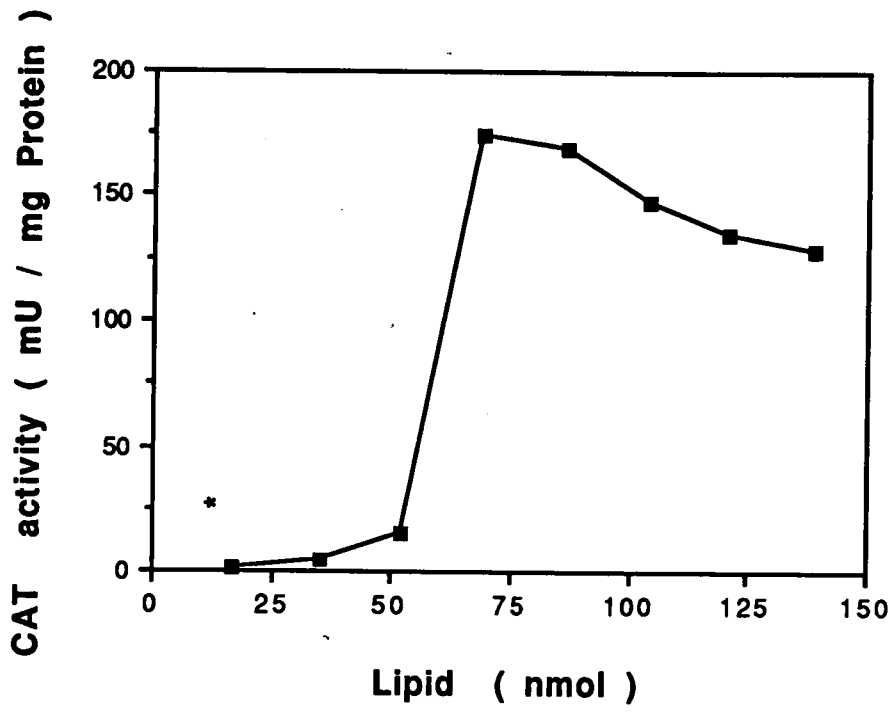

Figure 3. The effect of total lipid concentration on transfection activity. Different amounts of liposome (composed of an equimolar mixture of derivative II and DOPE) (■) were mixed with 5 μg DNA and used for transfection of L929 cells as described in figure 2 legend. Lipofectin (* data point) was also used for comparison of transfection activity under conditions identical to those used for the cationic liposomes.

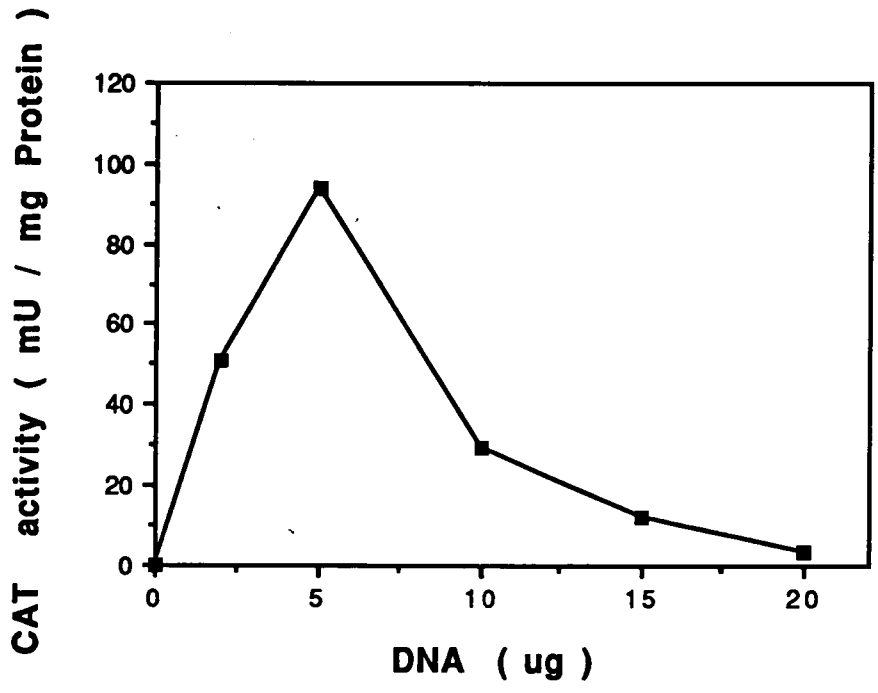

Figure 4. The effect of DNA concentration on transfection activity. Zero to 20 μg DNA was mixed with liposomes composed of equimolar mixture of II and DOPE (70 nmol) and transfection of L929 cells was carried out as described in figure 2 legend.

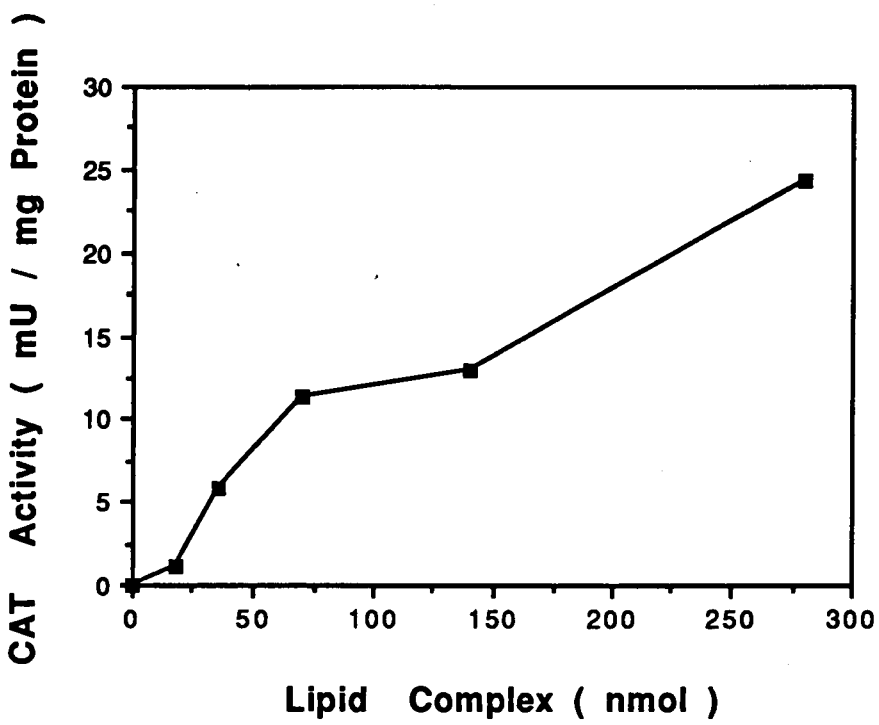

Figure 5. The effect of dose of the DNA/liposome complex on transfection activity. A ratio of 70 nmol lipid (an equimolar mixture of II and DOPE) and 5 μg DNA was maintained for transfection at different doses of complex. 0, 1.25, 2.5, 5, 10 and 20 μg DNA was used with 0, 17.5, 35, 70, 140 and 280 nmol lipids, respectively. Transfection conditions are the same as described in figure 2 legend, with the exception that the initial time of incubation with the DNA/liposome complex was varied as indicated.

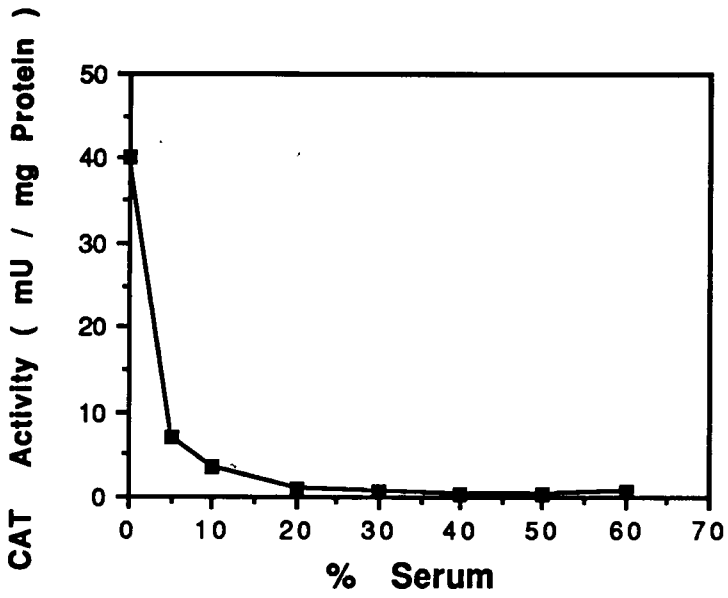

Figure 6. The effect of serum on transfection activity. Zero to 60% calf serum was added to the transfection media. Five μg DNA and 70 nmol lipid were used. Other conditions were the same as described in figure 2 legend.

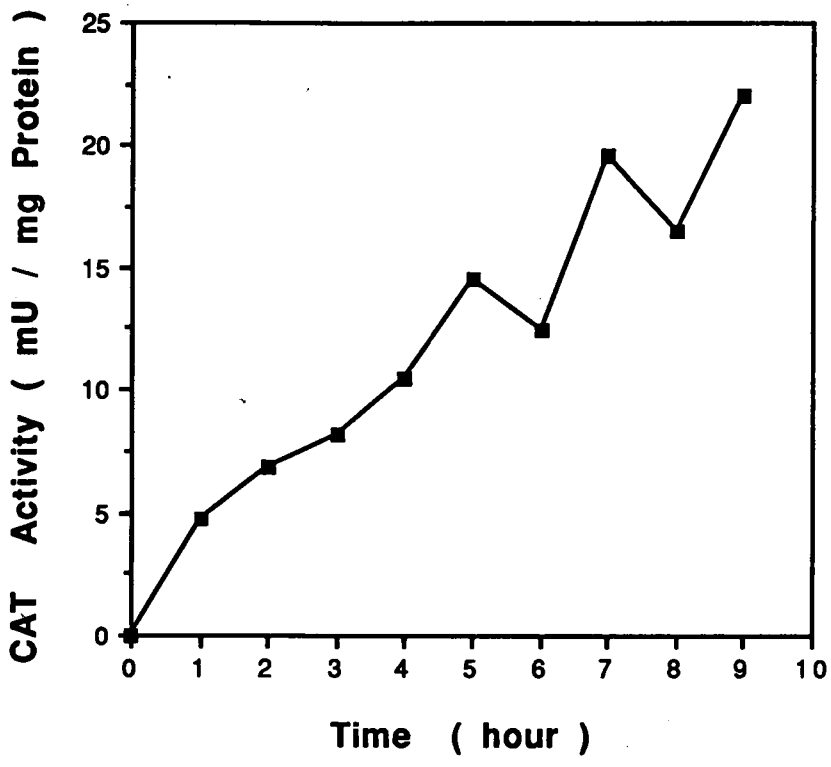

Figure 7. The effect of transfection incubation time on transfection activity. Five μg DNA was mixed with 70 nmol total lipid composed of equimolar mix of derivative II and DOPE and added to cells as described in figure 2 legend.

PART III

THE ROLE OF DIOLEOYL PHOSPHATIDYLETHANOLAMINE IN CATIONIC LIPOSOME MEDIATED GENE TRANSFER

1. Abstract

In a reporter gene assay, cationic liposomes containing the cationic lipid 3β [N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and a neutral phospholipid dioleoyl phosphatidylethanolamine (DOPE) showed high transfection activity. DNA/liposome complex which contained low amount of liposomes could bind to the cell surface but failed to transfect the cells. We have designed a two-step protocol to examine this phenomenon in more detail. A431 human cells were incubated on ice (pulse) with DNA complexed to a low level of cationic liposomes. The cells were washed and incubated at 37°C (chase) with or without free cationic liposomes of various composition (helper liposomes). Only liposomes enriched with DOPE showed helper activity; liposomes containing dioleoyl phosphatidylcholine (DOPC), a structural analog of DOPE, had no helper activity. The delivery was inhibited by the lysosomotropic agent chloroquine and was optimal if the helper liposome chase was initiated immediately after the pulse. An endocytosis model of DNA delivery by cationic liposomes is proposed in which the principal function of the chase liposomes is to destabilize the endosome membrane and allow the release of DNA into the cytosol. This model is consistant with the known activity of DOPE to assume non-bilayer structures, hence destabilizing the endosome membrane.

2. Introduction

Cationic liposomes are commonly used for mediating gene transfer, i.e. transfection, to mammalian cells (for recent reviews, see 1-4). Their popularity is justified by their high transfection efficiency, low toxicity and commercial availability among others. In practice, they represent the simplest methodology known for DNA delivery. Simply, pure DNA of any size or shape is mixed with the cationic liposomes and incubated with cells for a few hours followed by a gene expression assay in one or two days.

One of the critical elements for efficient DNA delivery is the lipid composition of cationic liposomes (1,3). The cationic lipid component is amphipathic and can vary in its chemical structure. Each cationic lipid may contain single or multiple cationic charges and the overall positive charge must be preserved. However, not all cationic lipids are capable of DNA delivery. Some cationic lipids with protein kinase C inhibition activity have no transfection activity (5).

Most cationic liposomes, with some exceptions (6,7), have a common neutral phospholipid component in addition to the cationic lipid component (1). The phospholipid is needed for stabilizing most types of cationic lipids in a lipid bilayer and may provide the cell penetration function of cationic liposomes. A neutral phospholipid, dioleoyl phosphatidylethanolamine

(DOPE), serves as the second lipid component of cationic liposomes (1). DOPE is critical for transfection because replacement of DOPE with another neutral phospholipid of the same acyl chain composition, dioleoyl phosphatidylcholine (DOPC), abolish most of the transfection activity of cationic liposomes (5,8-10). DOPC contains a choline head group instead of the ethanolamine head group on DOPE. This substitution changes many properties of the phospholipid (11). One of the major differences between DOPE and DOPC is the high non-bilayer forming activity of DOPE and the absence of this activity with DOPC (11). DOPE is a strong destabilizer of lipid bilayers (11). Recent work in our group have shown the effect of DOPE vs DOPC in delivering DNA to the cytosol of cells transfected with cationic liposome complex (9). Electron microscopy observations clearly showed the endosome destabilizing effect of DOPE-containing cationic liposomes. In comparison, DOPC-containing cationic liposomes showed no effect on endosomes (9).

This work is aimed at the elucidation of the role of DOPE in the cationic liposome mediated gene delivery. A new pulse-chase protocol has been designed to dissect the process of transfection and to allow more detailed examination of each step in the transfection. The studies presented here support a major role for endocytosis in the uptake of DNA/cationic liposome complex.

3. Materials and Methods

Materials:

The lipids 1, 2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) and 1, 2-dioleoyl-sn-glycero-sn-3-phosphatidylcholine (DOPC), were purchased from Avanti Polar Lipids. 3β [N-(N', N'-dimethylamino-ethane)-carbamoyl] cholesterol (DC-Chol) was synthesized as described by Gao and Huang (12). Dulbecco's Modified Eagle Medium (DMEM) was from Life Technologies, Inc. Fetal bovine serum was from Hyclone Laboratories, Inc. Acetyl-coA and chloramphenicol were from Sigma. [^{3}H] acetyl-coA (3-6 Ci/mmol) was from Amersham. Betamax was from ICN Biomedical, Inc.

Plasmid DNA:

Plasmid pUCCMVCAT contained the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the human cytomegalovirus immediate early promoter. The plasmid was constructed using standard molecular cloning techniques (13). Briefly, the supercoiled plasmid vector pUCSV2CAT (a gift from Dr. T. Hazinski) was linearized with HindIII restriction enzyme to release the simian virus 40 promoter. A HindIII DNA fragment coding for the CMV promoter was ligated to the linearized vector and the correct orientation for gene expression was

selected from transformed bacterial colonies. Supercoiled plasmid DNA was cloned in E. coli and purified by alkaline lysis and cesium chloride gradient centrifugation as described (13). Plasmid DNA was radioactively labeled with ^{32}P using a nick translation kit (Promega) and [^{32}P] dCTP.

Liposomes:

Cationic liposomes containing DC-Chol and DOPE or DOPC were prepared by 5 min sonication, in a bath sonicator, of mixed and dried lipids in 20 mM HEPES buffer as described (12). The liposomes, having an average diameter of 150 nm, were stored at 4°C. The lipid composition of liposomes is indicated by molar ratio or mole %.

Cell culture and transfection:

Human epidermoid carcinoma cell line A431 (a gift from Dr. G. Carpenter, Vanderbilt University) were cultured in growth medium (DMEM with 10% fetal bovine serum, L-glutmine, high glucose, penicillin and streptomycin). Twenty four-well plates were used for all the experiments. Cells were plated two days before transfection which was perfomed at 70-80% confluency. Two methods were used to transfect the cells with DNA. In one method plasmid DNA was complexed to cationic liposomes in DMEM at room temperature for 10-15 min and then the complex was incubated with cells for 4 h at 37°C. Cells were washed and

cultured for another 20 h in growth medium at 37°C before the CAT assay. The second transfection method involved a two step procedure where the cells are initialy incubated with DNA/liposome complex on ice (pulse) followed by an incubation with free liposomes at 37°C (chase). In the first step, DNA was mixed with cationic liposomes in ice-cold DMEM without serum and incubated on ice for 30 minute to form a complex. Chilled complex was added to the washed cells and incubated on ice for 30 min (pulse). The cells were then washed three times with chilled DMEM followed by one wash with warm DMEM and a chase with free cationic liposomes in warm DMEM. The chasing liposomes added had different compositions and they were added at different time points after the pulse with the complex. The liposome chase was maintained for 4 h (unless indicated otherwise) at 37°C followed by washing with warm DMEM and incubation of cells with growth medium for another 20 h before lysis and CAT assay. To assay for DNA binding to cells, ^{32}P-labeled DNA was complexed to cationic liposomes and incubated with cells at 37°C for 4 h (Fig. 1) or at 4°C for 30 min followed by washing with cold DMEM 3 times, scraping of cells, and counting the scraped cells in a liquid scintillation counter.

CAT assay:

The CAT assay (12) was done with modifications. The cells were washed once with PBS and lysed with 0.1% Triton X-100 in 0.25 M Tris-

HCl buffer (pH 7.8) at room temperature for 10-15 min. The samples were heat-inactivated at 65°C for 10 min and centrifuged in a microfuge at high speed at 4°C for 5 min. The CAT reaction was performed at 37°C for 1 h using 60 μg protein of cellular lysate (Table 1 and 2 and Fig. 2-4) or for 15 min using 15 μg protein (Fig. 1 and 5) unless otherwise noted. The reaction conditions were as follows: 100 μl total volume containing 1 mM chloramphenicol, 0.1 mM acetyl-CoA, 0.1 μCi [^{3}H] acetyl-coA and cell lysate in 0.25 M Tris-HCl, pH 7.8. The reaction was stopped by rapid freezing and products of CAT reaction were extracted with 600 μl toluene. Three ml of organic liquid scintillation cocktail BetaMax was added to the organic phase of the extract and counted in a Beckman liquid scintillation counter. CAT activity is expressed as % acetylation of chloramphenicol (% conversion per 60 or 15 μg protein as described in the CAT reaction conditions above). Proteins were quantitated using a BIO-RAD microprotein assay (Bio-Rad Laboratories) and bovine serum albumin was used as a standard.

4. Results

In order to understand the mechanism of DNA delivery by cationic liposomes we have studied the effects of liposome concentration, lipid composition, and other parameters on the transfection activity and cellular uptake of DNA.

Effect of cationic liposome concentration on transfection activity and DNA uptake by cells:

To verify the effect of different concentrations of cationic liposomes (DC-Chol:DOPE, 1:4 mole ratio) on the cellular uptake of DNA, a range of concentrations of the liposomes were used for delivering a constant amount of ^{32}P-labeled plasmid DNA (pUCCMVCAT). The complexes were incubated with cells for 4 h at 37°C to allow continuous cellular uptake of DNA. The results shown in Fig. 1 indicate that cell associated DNA increased with increasing amount of liposomes, saturating at 10 nmole total lipids (40 μM) per 1 μg DNA. Any increase in liposome concentration beyond the optimal concentration did not result in further increase in DNA uptake by the cells. To verify the effect of liposome concentration on functional DNA delivery (transfection activity) we repeated the same experiment shown in Fig. 1 using unlabeled DNA for delivery. In addition, the cells were incubated for an additional 20 h at 37°C in growth medium to allow for expression of the reporter gene (quantitated by a CAT assay) delivered by the liposomes. The results of this experiment are also shown in Fig. 1. There was a close correlation between the transfection activity and total cellular uptake of DNA. The ratio of 10 nmole lipids (40 μM) per 1 μg DNA allowed both maximum transfection and maximum cellular uptake of DNA. This result suggests that the efficiency of transfection is determined by the total DNA uptake.

Effect of cationic liposome concentration on DNA binding to the cell surface:

There are many steps involved in the process of DNA uptake. To examine the initial step of uptake, i.e. the binding of DNA to the cell surface, we have incubated cells with DNA/liposome complex at 0°C to avoid the subsequent step of internalization. DC-Chol:DOPE (3:2) liposomes of various concentrations were mixed with a fixed amount of ^{32}P-labeled DNA and the complexes were incubated with cells on ice for 30 min followed by washing. The short incubation time was necessary to insure high viability of cells which do not tolerate cold temperature for an extended period of time. Preliminary experiments showed that 30 min was sufficient to allow for maximal binding of DNA/liposome complex to cells (data not shown). To compare the DNA binding with the transfection activity after the low temperature incubation we have also used unlabeled DNA in a separate experiment for the initial period of 0°C incubation for 30 min. After washing, the cells were incubated with growth medium at 37°C for an additional 20 h before the CAT assay was done. As can be seen in Fig. 2, DNA binding to the cell surface was not well correlated with the transfection activity of the bound DNA. Maximal DNA binding was observed at liposome concentration of 10 μM or higher; whereas the maximal transfection activity required at least 4-fold more liposomes, i.e. 40 μM total lipid. Apparently, the DNA bound to the cell surface via low amounts of liposomes (<40 μM lipid) was not available for gene

expression. Only the DNA/liposome complexes containing more liposomes were active in both cell binding and transfection. Therefore, the data indicate that at low liposome concentration maximal DNA binding to cells is allowed but one or more of the subsequent events, such as internalization or release of DNA into the cytosolic compartment, might be inhibited.

Effect of helper liposome on DNA delivery:

The results from Fig. 2 clearly established the requirement for a minimal dose of cationic liposomes for optimal DNA delivery. We raised the question whether the addition of an extra dose of free cationic liposomes would help DNA delivery after DNA was already bound to cell surface using a low concentration of complexing cationic liposomes at low temperature. Adding free liposomes to deliver DNA may allow us to study the effect of phospholipid composition and mole ratio on the delivery of the DNA which is already bound to cell surface. Thus, DNA/liposome complex containing a relatively low amount (20 μM) of liposomes composed of DC-Chol:DOPE (3:2) or DC-Chol:DOPC (3:2) was incubated with cells on ice for 30 min (pulse) followed by washing and warming to 37°C with or without the addition of free liposomes (chase). Free cationic liposomes containing different ratios of DC-Chol to DOPE or DOPC were used in the chase to study the helper (free) liposome activity. It is clear from Table 1 that only DOPE-containing liposomes were capable of helping DNA delivery when DNA was complexed to either DOPE or

DOPC complexing liposomes. DOPC-containing free liposomes had essentially no detectable activity in this assay system. Helper activity of DOPE liposomes increased with increasing DOPE content in the liposomes with 80% DOPE content being optimal (8.4 fold enhancement in activity). Interestingly, liposome containing excess DOPE (i.e. 90% DOPE) showed no helper function at all (Table 1). This is probably related to the instability of these liposomes due to their high content of DOPE, a phospholipid with high tendency to form non-bilayer structures (11). If 10% of DOPE was replaced with DOPC to improve the liposome stability, the helper function of the liposomes was again maximal. Further degree of DOPE substitution with DOPC (20-30% DOPC) resulted in decreased helper activity, indicating that the helper function is closely associated with the DOPE content. Other liposome compositions were also tested and the results (not shown) indicate that as little as 5% DC-Chol in the liposomes enriched with DOPE was sufficient for a significant helper activity. These data, taken together, indicate that the most important lipid ingredient in the helper liposomes is DOPE; only minimal amount of cationic lipid is needed probably to provide a means for the binding of liposomes to the negatively charged cell surface.

The effect of helper liposome dose on DNA delivery:

The effective dose of helper liposomes needed for DNA delivery was determined (Fig. 3). Cells were pulsed with DNA/DC-Chol:DOPE (3:2)

liposome complex and chased with increasing concentrations of free helper liposomes containing 20% DC-Chol and 80% DOPE or DOPC. DOPE liposomes were found to perform a detectable helper function at concentrations as low as 1 μM and optimal delivery at 20-40 μM. Free DOPC-containing cationic liposomes showed no detectable helper activity at all concentrations used.

Time course of DNA delivery by helper liposomes:

Chasing the bound DNA with helper liposomes is critically dependent on the initiation time of the chase after the pulse with DNA/liposome complex. Helper liposomes were added at different time points after the initiation of the chase (Fig.4). Complexing liposomes were DC-Chol:DOPE (3:2), and helper liposomes were either DC-Chol:DOPE (3:2) or DC-Chol:DOPE (1:4). Data in Fig. 4 showed that the DNA delivery activity decreased gradually as the addition of helper liposomes was delayed, with half of the original activity found when the helper liposomes were added 70-80 min after the initiation of the chase. Similar to the results in Table 1, Fig. 4 shows that free cationic liposomes with higher DOPE content (80%) performed better DNA delivery function than free cationic liposomes with lower DOPE content (40%). In this experiment, the chase incubation period lasted for 4 h for every time point listed. The next experiment was to investigate the length of chase period required for optimal DNA delivery when all chases were initiated

immediatly after the pulse. The results showed that even 15 min of total chase time (followed by washing off the helper liposomes) was sufficient to achieve a substantial level of helper activity (Table 2).

Effect of chloroquine on DNA delivery:

Chloroquine is a lysosomotropic agent known to interfere with endocytosis by raising (neutralizing) endosomal and lysosomal pH and by inhibiting maturation of endosomes (14). Therefore, we decided to test the effects of chloroquine on transfection and on helper liposome activity in the pulse/chase experiments shown above. Cells were transfected with DNA/DC-Chol:DOPE (3:2) liposomes or DNA/DC-Chol:DOPC (3:2) liposomes at 37°C for 4 h with or without 200 μM chloroquine (Table 3). Alternatively, cells were pulsed with DNA/DC-Chol:DOPE (3:2) liposomes on ice for 30 min, washed and chased with free DC-Chol:DOPE (1:4) liposomes with or without 200 μM chloroquine at 37°C (Table 3). Chloroquine addition inhibited over 95 % of the transfection activity associated with both transfection and helper liposome activity. Regardless of the type of phospholipid used in the liposome formulation (DOPE or DOPC), DNA delivery was abolished by the chloroquine treatment (Table 3).

Ratio of cationic lipid to phospholipid in cationic liposomes for transfection:

DNA delivery by cationic liposomes is largely affected by the critical ratio of cationic lipid to phospholipid present in the liposome formulation (5,9,12). The ability to complex DNA and to bind with the cell surface, which is negatively charged, is dependent on the liposome content of cationic charges. To establish the optimal ratio of phospholipid to cationic lipid (DC-Chol) for optimal transfection, cells were transfected with a complex of cationic liposomes and DNA for 4 h at 37°C. The liposomes contained different ratios of DC-Chol to DOPE or DOPC. The results of this experiment are shown in Fig. 5. At all lipid ratios used, DOPE cationic liposomes were superior in DNA delivery when compared to DOPC cationic liposomes. DOPC liposomes had detectable transfection activity at 30-50% DOPC content but had none above 50%. This is in contrast to the easily detectable DNA delivery at 30-80% DOPE. The optimal DC-Chol content in the DOPE liposomes was about 50-60%.

5. Discussion

It has been shown by us (9) and others (8,10) that endocytosis of DNA/liposome complex is the major route of DNA uptake by cells during transfection. The complex first adsorbs to cell surface by charge interaction. The surface bound complex is then internalized by endocytosis

into endosomes and lysosomes. A small portion of the endocytosed DNA is released into the cytosol from which the DNA must enter into the nucleus for transcription. The majority of the internalized DNA stays in the endocytic compartments and is eventually degraded. This is a complicated mechanism which involves multiple steps. The fact that the transfection activity of DC-Chol/DOPE liposomes closely correlates with the total cellular uptake of DNA (Fig. 1) strongly suggests that one or more of the above mentioned steps may control the amount of DNA arriving at the final transcription compartment which in turn determines the transfection activity of the liposomes. Ideally, it is important to dissect the overall uptake process into individual steps and analyze the contribution and control of each step. As the first step of this approach, we have used a pulse/chase protocol and examined the relationship between the amount of DNA bound to the cell surface and the final activity of transfection. Data presented in Fig. 2 indicate that only the complexes containing sufficient amount of liposomes could eventually arrive at the final destination for transfection. Those DNA/liposome complexes with suboptimal amount of liposomes, although bound to cells efficiently, were not active in transfection. This observation gave us an opportunity to examine if additional liposomes added at the onset of chase incubation could improve the transfection activity of the DNA/liposome complex already bound to the cell surface. Our hypothesis was that these complexes are internalized into the endosomes but not released into the cytosol. Co-internalization of

helper liposomes of proper composition may enhance the release of DNA into the cytosol and increase the transfection activity.

This hypothesis is supported by several lines of observation. First, only liposomes enriched with DOPE showed strong helper activity (Table 1). DOPE is a phospholipid which exhibits a high tendency to form inverted hexagonal (H_{II}) phase particularly at acidic pH (11). DOPC, a structural analog of DOPE, has no activity to form H_{II} phase under physiological conditions (11). The inverted hexagonal phase may destabilize acidic endosomes by fusion of the hexagonal phase lipids with the endsomal membrane creating leaky holes in the process. Cationic liposomes containing DOPC showed no helper activity (Table 1). Zhou and Huang (9) have previously shown by transmission electron microscopy that only DNA/liposome complex containing DOPE can destabilize the endosome membrane and escape into the cytosol; complex containing DOPC has no such activity. Apparently, in our present study complexes containing a low level of DOPE may have been incapable of destabilizing the endosome membrane. Only when additional helper liposomes enriched with DOPE are co-localized into the same endosome, the complex could then be released into the cytosol after the endosome membrane is destabilized. That the action of the helper liposomes is in an intracellular compartment is also supported by the data in Fig. 4. Delayed addition of helper liposomes would not result in the co-localization with DNA/liposome complex in the same endosome, thus showing reduced or

diminished helper activity. Furthermore, the lysosomotropic agent, chloroquine, completely inhibited the activity of helper liposomes (Table 3), indicating that endocytosis is a necessary element for the helper liposome activity. The proposed mechanism of action is also consistant with the data in Table 2 which showed that cells only needed to be exposed to the helper liposomes for a short period of time to exhibit an enhanced transfection activity. Only the helper liposomes initially bound to the cell surface would have a chance to be co-internalized together with the DNA/liposome complex. Prolonged incubation with the helper liposomes would not significantly enhance the transfection because the helper liposomes bound to the cell surface at the later time could not be co-localized with the complex in the same endosome. Taken together, the data presented here and published by others (8-10) strongly support the role of DOPE in the cationic liposome mediated gene transfer as that of an endosomolytic agent, similar to that of the adenovirus (15-18) and fusion peptides (20) used to enhance the transfection activity of molecular conjugates.

If the optimal helper liposomes are those enriched with DOPE, then why were the optimal transfection liposomes those relatively enriched with DC-Chol as shown in Fig. 5 ? Obviously, there are steps in the transfection process other than the release of DNA from endosome in which DC-Chol plays an important role. For example, liposomes with sufficient amount of DC-Chol may be required for the formation of proper complex with DNA.

It is likely that the negatively charged DNA is coated with a layer or more of positively charged lipids such that the net charge of the complex is neutral or positive. Preliminary results from this lab (Sorgi et al, unpublished data) indicate that the optimal transfection complex contains a collection of heterogeneous structure including tubes and fused liposomes. The role of DC-Chol, or other cationic lipids, in the formation of transfection complex is presently unknown, but must be important in the determination of the final transfection activity. Obviously, more work is needed to answer this question.

Finally, liposomes containing DC-Chol and DOPC, although weak in activity, were active in transfection (Fig. 5). Since the activity was inhibitable by chloroquine, endocytosis may also be involved in the mechanism of action of these liposomes (Table 3). This and other cationic lipid formulations (6,7), which are completely devoid of DOPE, obviously rely on other unknown mechanism(s) to escape the endosome or lysosome. Destabilization of plasma membrane is another possible mechanism for DNA entry into cells. However, we have not performed any experiments to support or rule out this possibility. This interesting aspect of liposome mediated gene transfer will be an important subject for future studies.

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Table 1

Effect of lipid composition of helper liposome on DNA delivery [a]

Helper liposome (mole ratio) DC-Chol : DOPE : DOPC			CAT activity (% conversion) Complexing liposome DC-Chol : DOPE (3:2 mole ratio)	CAT activity (% conversion) Complexing liposome DC-Chol : DOPC (3:2 mole ratio)
-	-	-	2.5 ± 0.6	0.2 ± 0.1
6	-	4	4.2 ± 0.3	0.2 ± 0.1
2	-	8	2.4 ± 1.4	0.3 ± 0.2
6	4	-	8.2 ± 0.6	1.8 ± 0.2
4	6	-	12.8 ± 0.4	2.8 ± 0.4
2	8	-	21.0 ± 0.5	6.0 ± 0.5
1	9	-	2.8 ± 0.5	0.3 ± 0.1
1	8	1	24.7 ± 2.0	-
1	7	2	14.5 ± 2.5	-
1	6	3	10.6 ± 2.3	-

[a] A431 cells were incubated (pulse) for 30 min on ice with pUCCMVCAT DNA (1 μg) and DC-Chol:DOPE (3:2) or DC-Chol:DOPC (3:2) complexing liposomes (20 μM), washed, warmed, and incubated (chase) for 4 h at 37°C with free helper liposomes (40 μM) containing different lipid composition as shown. After the chase, cells were washed and cultured in growth medium for 20 h at 37°C before the CAT assay.

Table 2

Effect of chase incubation time on DNA delivery by helper liposomes [a]

Helper liposome (μM)	Chase incubation time (h)	CAT activity (% conversion)
0	4	2.9 ± 0.17
40	0.25	17.7 ± 1.2
40	0.5	16.0 ± 0.6
40	1	18.9 ± 3.4
40	2	19.4 ± 0.9
40	3	20.3 ± 1.2
40	4	16.2 ± 1.6
40	5	14.9 ± 0.7

[a] A431 cells were incubated (pulse) for 30 min on ice with pUCCMVCAT DNA complexed to DC-Chol:DOPE (3:2) complexing liposomes, washed, warmed, and incubated (chase) for different length of time at 37°C with DC-Chol:DOPE (1:4) free helper liposomes. After the chase, cells were washed and cultured in growth medium at for 20 h at 37°C before the CAT assay.

Table 3

Effect of chloroquine on DNA delivery

Chloroquine treatment[a] (μM)	CAT activity (% conversion)		
	Pulse/chase[b]	Transfection[c]	
		DOPE liposomes	DOPC liposomes
0	21.9 ± 2.0	17.0 ± 1.1	4.4 ± 0.2
200	0.8 ± 0.1	0.3 ± 0.0	0.2 ± 0.0

[a] Chloroquine was added during incubation at 37°C.

[b] A431 cells were incubated (pulse) with pUCCMVCAT (1 μg) DNA/DC-Chol:DOPE (3:2) liposome (20 μM) complex on ice for 30 min, washed, and incubated (chase) with 40 μM of free helper liposomes (DC-Chol:DOPE (1:4) at 37°C for 4 h. Cells were then washed and incubated at 37°C in growth medium for 20 h before the CAT assay.

[c] A431 cells were transfected with pUCCMVCAT (1μg) DNA/DC-Chol:DOPE (3:2) or DNA/DC-Chol:DOPC (3:2) liposome (40 μM) complexes at 37°C for 4 h. Cells were then washed and incubated at 37°C in growth medium for 20 h before the CAT assay.

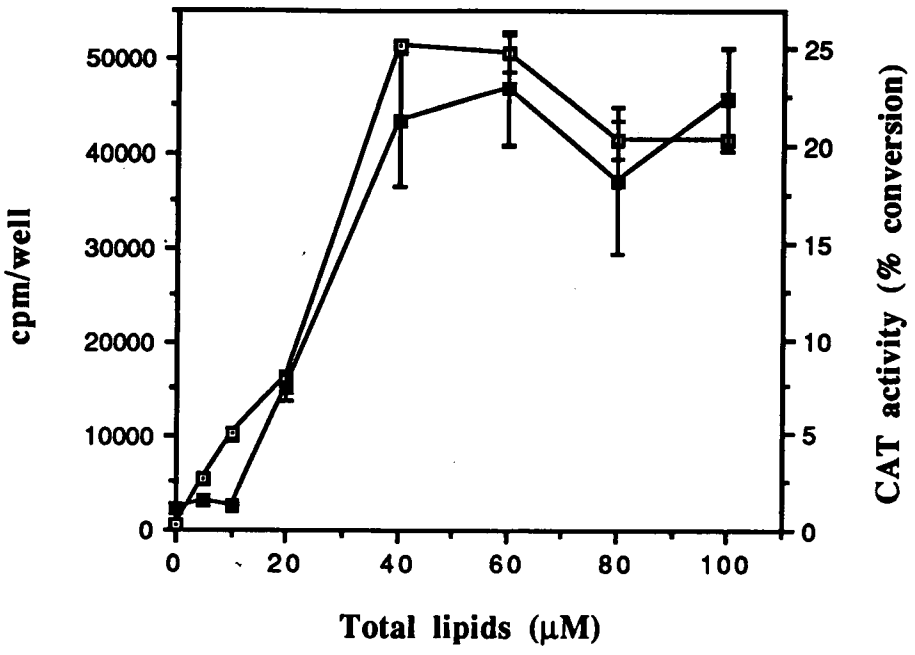

Fig. 1. Effect of cationic liposome concentration on transfection and DNA uptake by cells. One μg ^{32}P-labeled (❑) or unlabeled (■) pUCCMVCAT plasmid DNA was complexed to different amounts of cationic liposomes (DC-Chol:DOPE, 3:2 mole ratio) and incubated with A431 cells for 4 h at 37°C. Thereafter, cells were harvested for counting (❑) or incubated for 20 h before the CAT activity (■) was assayed. Total volume of incubation was 250 μl/well.

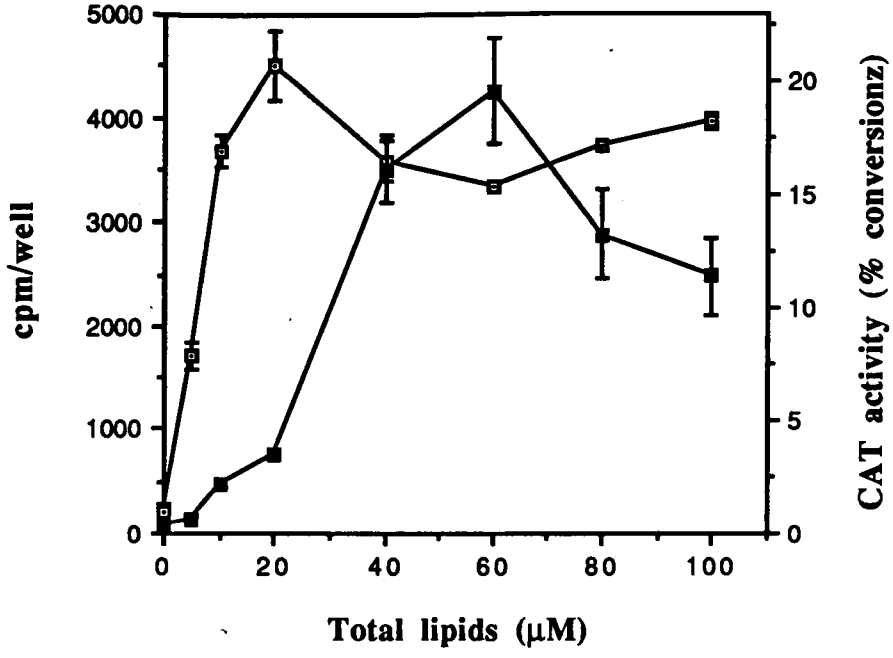

Fig. 2. Effect of cationic liposome concentration on transfection and cell binding. One μg ^{32}P-labeled (❑) or unlabeled (■) pUCCMVCAT plasmid DNA was complexed to different concentrations of cationic liposomes (DC-Chol:DOPE, 3:2 mole ratio) and incubated with A431 cells for 0.5 h on ice. Thereafter, cells were washed and harvested for radioactiviy counting (❑) or incubated in growth medium at 37°C for 20 h before the CAT activity (■) was assayed. Total volume of incubation was 250 μl/well.

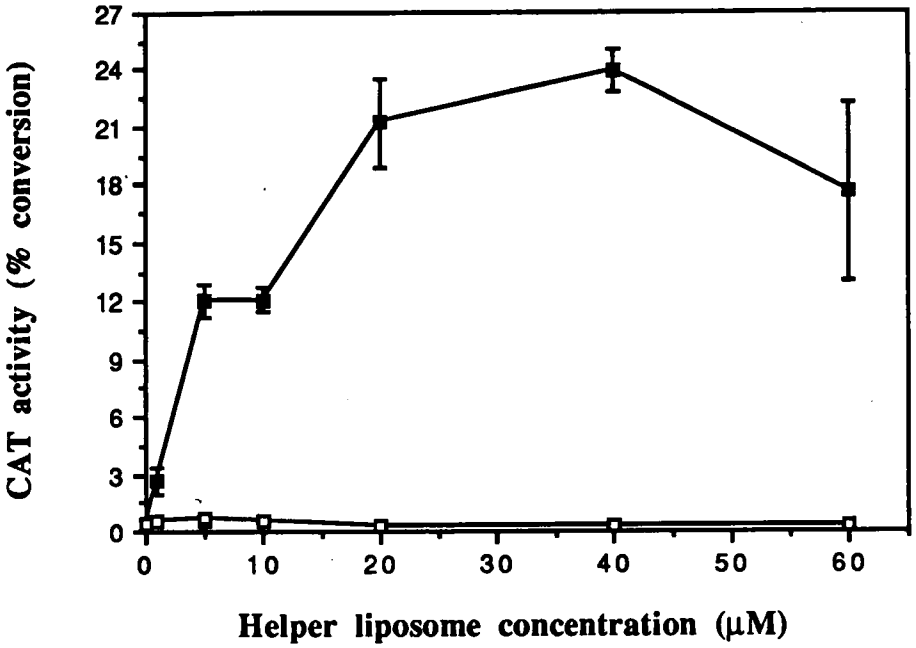

Fig. 3. Effect of helper liposome concentration on DNA delivery. A431 cells were pulsed with pUCCMVCAT DNA complexed to DC-Chol:DOPE (3:2) liposomes and chased with increasing concentrations of DC-Chol:DOPE (1:4) (■) or DC-Chol:DOPC (1:4) (❑) liposomes. The cells were then washed and incubated at 37°C in growth medium for 20 h before harvested for CAT assay.

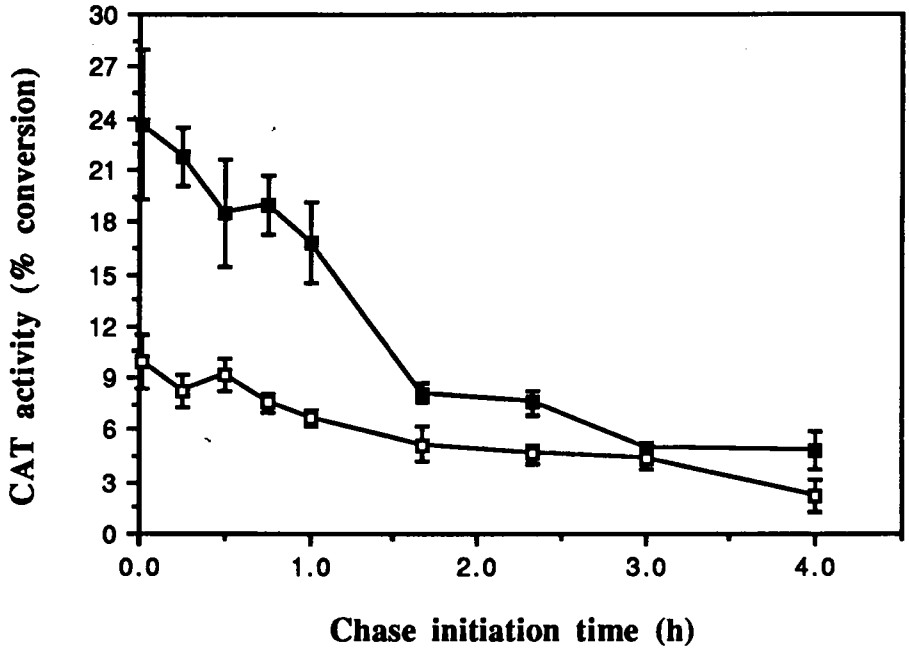

Fig. 4. Time course of helper liposome chase. A431 cells were pulsed with pUCCMVCAT DNA complexed to 20 μM of DC-Chol:DOPE (3:2) liposomes and chased with 40 μM of DC-Chol:DOPE (1:4) (■) or DC-Chol:DOPE (3:2) (❑) liposomes at the indicated time points after the completion of the pulse. The cells were then washed and incubated at 37°C in growth medium for 20 h before harvested for CAT assay.

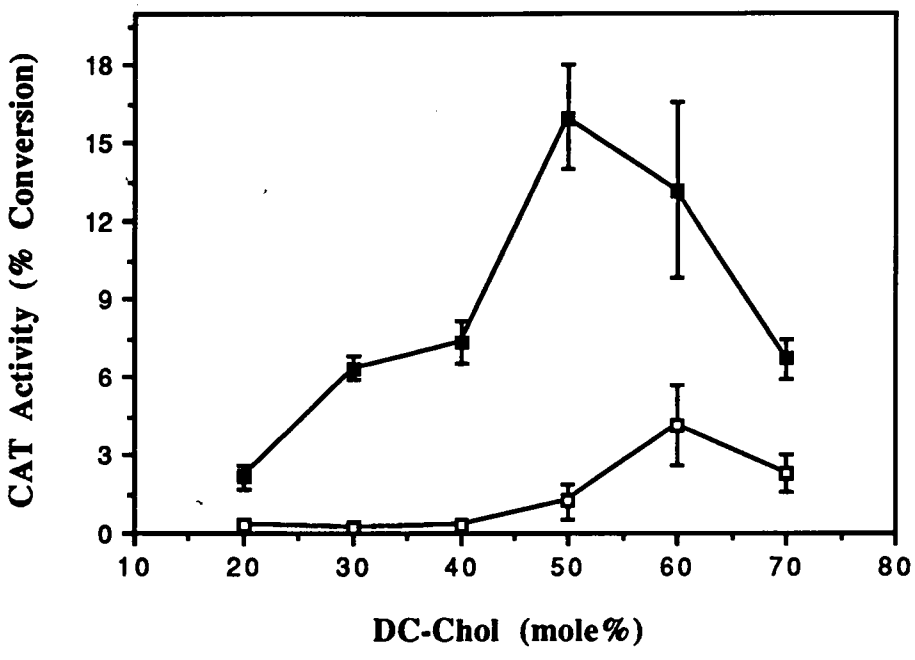

Fig. 5. Effect of lipid composition of liposomes on transfection. A431 cells were transfected for 4 h at 37°C with 1 μg pUCCMVCAT plasmid DNA complexed to 40 μM total lipids of liposomes containing different mole % of DC-Chol in DOPE (■) or DOPC (❑) liposomes. Cells were lysed and assayed for CAT activity 20 h after transfection.

PART IV

DELIVERY OF HUMAN IMMUNODEFICIENCY VIRUS-1 TRANS-ACTIVATOR PROTEIN (TAT) TO MAMMALIAN CELLS BY CATIONIC LIPOSOMES

1. Abstract

Cellular uptake of the human immunodeficiency virus-1 trans-activator protein tat is known to be mediated through its binding to a putative cell surface receptor followed by endocytosis and lysosomal degradation. However, tat entry into cells is inefficient and often requires a toxic treatment such as chloroquine to enhance its delivery. We have explored the use of cationic liposomes to deliver tat using a reporter gene expression assay. Phosphatidylcholine containing cationic liposomes had no detectable tat delivery activity. In contrast, delivery of tat was enhanced by up to 150-fold using cationic liposomes enriched with dioleoyl phosphatidylethanolamine (DOPE). This phospholipid-rich formulation showed no toxicity at concentrations sufficient for maximal delivery of tat. The results of mechanistic studies indicated that tat and liposomes simultaneously bound to the cell surface and were co-internalized. Due to the non-bilayer forming activity of DOPE, liposomes may allow the release of tat into the cytosol by destabalizing the endosomal membrane.

2. Introduction

Human immunodeficiency virus type-1 (HIV-1) transcriptional activator (tat) protein is a well known potent inducer of HIV-1 gene expression. Tat activates HIV by promoting transcription of HIV genes from the HIV-1 long terminal repeat (LTR) promoter. The transcriptional

activity of tat was thoroughly reviewed recently (1-4). Tat is unusual as a transcription factor because it behaves as a cell surface ligand that may bind to a putative cell surface receptor recently found on various cell types (5-8). Surface bound tat undergoes adsorptive, or receptor-mediated, endocytosis and some of the internalized tat molecules manage to evade lysosomal degradation and reach the cytosol (6,9). Once in the cytosol, tat can translocate to the nucleus and transactivate the HIV promoter (9). Moreover, tat is suspected to commute between cells (10) and induce the growth of Kaposi cells (derived from Kaposi's tumors in AIDS patients) in culture (11,12). Finally, tat has been shown to activate or suppress the expression of a variety of cellular genes in various cell types (13-20).

Tat protein may provide a useful system for intracellular protein delivery. The undesirable activities of tat, such as HIV activation, can be completely eliminated by producing mutations or deletions in the recombinant tat protein (21-23) or through chemical synthesis of the relevant tat peptides (24,25). Several of these genetic and chemical modifications preserve tat's cell binding, internalization and nucleus translocation functions. This strategy was successfully used to carry proteins, conjugated to tat polypeptides lacking HIV transcriptional activation functions, efficiently into mammalian cellular compartments such as the cytoplasm, nucleus and nucleolus (26,27). However, since the level of spontaneous penetration of tat into the cytosol is often too low, assistance is usually needed to ensure that tat escapes intracellular lysosomal

degradation (9). Different reagents and methods have been used to deliver tat protein intracellularly. Chloroquine and other lysosomotropic reagents (9), the scrape-loading technique (9) and electroporation (28) have been successfully used to deliver tat polypeptide to different cell types. Albeit efficient, these methods can be toxic and injurious to the treated cells. Another delivery method is a simple incubation of large doses of tat protein with cells (9,24). Although not toxic, this method consumes large quantities of tat protein.

Delivery of tat protein has been of interest to us because of tat's ability to pass through the cellular compartments which represent the major barrier for delivering macromolecules into the cells. Recently, tat was shown to deliver several different functional proteins which were chemically conjugated to tat by simple incubation of tat-conjugates with the cells (27). These results prompted our attempts to improve the efficiency of tat delivery by using liposomes, which are well established vehicles for drug delivery. In this work, we describe a non-toxic and highly efficient method using cationic liposomes to deliver tat protein to a mammalian cell line responsive to tat-induced gene expression.

3. Materials and Methods

Materials:

The lipids 1, 2-dioleoyl-sn-glycero-3-phosphatidylethanol-amine (DOPE), 1, 2-dioleoyl-sn-glycero-sn-3-phosphatidylcholine (DOPC), and 1, 2-dioleoyl-sn-3-succinyl glycerol (DOSG) were purchased from Avanti Polar Lipids. Dulbecco's Modified Eagle Media (DMEM), Genitecin, Lipofectin™ and Lipofectamine™ liposomes were from Life Technologies, Inc. Fetal bovine serum was purchased from HyClone Laboratories, Inc. Acetyl-coenzyme A, chloramphenicol, poly-L-lysine, lysozyme, chloroquine and other chemicals were purchased from Sigma. [^{3}H]acetyl-CoA (3-6 Ci/mmol) was purchased from Amersham and Iodine-125 was from NEN DUPONT. IODOGEN was from Pierce. BetaMax was from ICN Biomedical, Inc. pZ523 columns were from 5 Prime to 3 Prime, Inc. 3β [N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) was synthesized as described by Gao and Huang (29).

Liposome preparation:

Cationic liposomes containing DC-Chol and DOPE, DOPC, or both were prepared by 1 min vortexing followed by 5 min sonication of mixed and dried lipids in 20 mM HEPES buffer. The liposomes, with mean

diameter of 150 nm, were stored at 4°C and were stable for over 6 months.

Recombinant tat protein expression and purification:

The work described in this paragraph was completely performed by our collaborator Dr. James Barsoum in Biogen Inc. Plasmid pTAT72, obtained from Dr Alan Frankel (9), codes for the first exon of HIV-1-tat gene spanning amino acids 1 through 72. The sequence encoding amino acids 23-37 was deleted from tat (1-72) by PCR deletion mutagenesis of pTAT72. One primer (AGCCCAGCGCGTCGGCCGCC-ATG) covered the Eag1 site upstream of the tat gene while the other primer (TTTTTACGGCCGTAAGAGATACCTAGGGCTTTGGTGATGAAGCA CGCGGTTTT) covered the Eag1 site within tat and deleted tat residues 23-37. The PCR product was digested with Eag1 and the 765 bp fragment was cloned into pTAT72 which had been digested with Eag1 (the 4057 bp large fragment). The tat coding region is controlled by the bacteriophage T7 promoter inducible with isopropyl β-D-thiogalactopyranoside. The recombinant tat protein was purified using a modified procedure (30). Briefly, after induction E. coli was suspended in 10 volumes of 25 mM Tris-HCl (pH 7.5), 1 mM EDTA and lysed in a French press followed by centrifugation at 10,000 x g for 1 h. The supernatant was loaded on to a Q Sepharose Fast Flow (Pharmacia LKB, Piscataway, NJ) ion exchange column (20 ml resin / 60 ml lysate). 0.5 M NaCl was added to the flow-

through fraction to precipitate tat protein followed by centrifugation at 35,000 rpm, in a 50.2 rotor, for 1 h. The pellet was dissolved in 6 M guanidine-HCl and recentrifuged. The supernatant was loaded on to a Biogel A 0.5 agarose gel filtration column equilibrated with 6 M guanidine-HCl, 50 mM sodium phosphate (pH 5.4), 10 mM dithiothreitol, and then eluted with the same buffer. The eluted fractions were loaded onto a C4 reverse phase HPLC column and eluted with a gradient of 0-75% acetonitrile in 0.1% trifluoroacetic acid. Twenty mg of tat protein was produced per liter of cultured E. coli (6 g of cells per liter). Tat protein constituted 5% of total E. coli protein. Tat protein ran as a single 10 kd band on SDS-PAGE. Tat protein was iodinated using ^{125}I and Iodogen reagents as described (9).

Plasmid DNA:

Plasmid pUC-BENN-CAT which codes for the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of HIV-1 LTR promoter was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (deposited by Dr. Malcolm Martin). Plasmid pSV2-neo which codes for the bacterial neomycin phosphotransferase II (neo) gene, and gives resistance to G418 (Genitecin), under simian virus 40 promoter control was from American Type Culture Collection. Both plasmids were amplified in E. coli and purified by pZ523 columns following the manufacturer's instruction.

Cell culture and transfection:

A431 human epidermoid carcinoma cells (from Dr. G. Carpenter, Vanderbilt University) were maintained in DMEM supplemented with 10% FBS, L-glutamine, glucose, penicillin and streptomycin. Cells stably transfected with CAT gene controlled by HIV-1 LTR promoter were obtained by co-transfection of A431 cells with pSV2-neo and pUCBENNCAT plasmids at a ratio of 1:10, respectively, using DC-Chol cationic liposomes. Two days after transfection, the cells were incubated with 750 μg/ml of G418 and two weeks later surviving clones were separated by limiting dilution. One clone (termed A431LTRCAT) of cells, which expressed CAT activity only after trans-activation with a delivered tat wild type sequence (or a tat expression vector, data not shown) was used for all the tat protein delivery experiments. Twenty four-well plates were used for all experiments. Cells were plated in the wells two days before protein delivery and were always treated with tat and liposomes at 70-80% confluency. Protein delivery protocol was as follows: tat protein was added to 250 μl of warm serum-free DMEM in microfuge tubes and liposomes were added immediately afterwards. The mixture was added to each well containing the cells after removal of growth medium from the cells. Cells were incubated for 5 h, or as indicated, at 37°C in 5% CO_2 and 95% air after which the tat-containing medium was removed and fresh growth medium was added. Cells were further incubated for 20 h at 37°C

before the CAT assay, described below. All samples were performed in triplicate and experiments were repeated at least twice.

CAT assay:

The assay was performed as described (29) with modifications as follows: twenty four h after tat addition, cells were washed once with PBS and lysed with 0.1% Triton X-100 in 0.25 M Tris-HCl buffer (pH 7.8) at room temperature for 10-15 min. This was followed by heat inactivation at 65°C for 10 min and centrifugation for 5 min at the highest speed of a microcentrifuge. About 1/5 of the lysate supernatant (equivalent to about $1x10^5$ cells or 30 μg protein) was used for each sample in a 15 min CAT reaction at 37°C. All reaction conditions were adjusted such that the activity was linear with time. The reaction volume was 100 μl containing lysate and CAT reaction mix (1 mM chloramphenicol, 0.1 mM acetyl-CoA and 0.1 μCi [^{3}H]acetyl-coA in 0.25 M Tris-HCl, pH 7.8). The CAT reaction was stopped by rapid freezing and products were extracted with 1 ml toluene. One half the organic extract was counted for radioactivity in 3 ml BetaMax liquid scintillation cocktail. CAT activity is expressed as percent acetylation (% conversion to products). The BIO-RAD microprotein assay (Bio-Rad Laboratories) was used to quantitate proteins and bovine serum albumin was used as a standard.

4. Results

Cationic liposomes vs. chloroquine for tat delivery:

Recombinant HIV-1 tat protein (exon 1 encoding amino acids 1-72) was used for all the experiments described. It is known that tat exon 1 codes for a functional tat protein which can trans-activate gene expression from the HIV-1 LTR promoter (9). Cationic liposomes composed of DC-Chol and DOPE in buffer, were used at 40 μM concentration for delivery of tat protein to A431LTRCAT cells in culture. These are A431 cells stabely expressing a CAT gene under the control of the HIV-1 LTR promoter element. Fig. 1 shows the results of tat protein delivery to A431LTRCAT cells in culture in a reporter gene (CAT) activation assay. Various concentrations of tat protein alone or mixed with DC-Chol:DOPE liposomes (1:4 mole ratio) or chloroquine (250 μM) were incubated with A431LTRCAT cells for 5 h at 37°C. Twenty h later the cells were lysed and assayed for CAT. The delivery was tat dose dependent and transactivation was specific to tat wild type sequence because a deletion mutant (Δ23-37 amino acids) of tat showed no transactivation function. The domain deleted in the mutant tat corresponds to the cysteine-rich domain which is important for the trans-activation function of tat. This deletion doesn't interfere with tat's cellular uptake function known to be present on the remainder of the undeleted portion of tat polypeptide (27). Using this formulation of cationic liposomes, 150-fold enhancement in tat

delivery was observed as compared to using tat alone. The most effective chloroquine dose (250 μM) was used to achieve the best tat delivery in this cell line which was 10-fold less than that achieved with the cationic liposomes. In addition, chloroquine killed more than two-thirds of the cells (in the presence or absence of wild type tat protein) while the liposomes had no detectable toxic effects as revealed by the change in cell morphology and protein of the treated culture.

An interesting result in Fig. 1 was the decrease in CAT activity seen with high concentrations (>5 μg/ml) of wild type tat observed only with cationic liposome delivery. This was the result of toxicity (detected visually and with protein quantitation) due to the high level of wild type tat delivered into the cells. Appropriate controls such as liposomes alone or with the tat (Δ23-37) deletion mutant at different doses had no detectable toxic effects on the cells (data not shown). Tat wild type sequence is toxic when present intracellularly at high doses and this toxicity is related to its wild type functional sequence (unpublished data). The results indicate that cationic liposomes were a potent tat-delivery vehicle and efficient enough to allow complete cell death at tat doses higher than 10 μg/ml. Free tat showed no detectable toxicity at this concentration when the liposomes were absent and the delivery was hardly detectable.

Delivery of tat protein by different formulations of liposomes:

Fig. 2 shows a CAT assay summarizing the results of tat protein delivery to A431LTRCAT cells by increasing concentrations of different cationic liposomes and one anionic liposome formulation (0-120 μM). All liposome formulations contained DOPE. One μg/ml of tat protein was used for all samples. The data show that anionic liposomes (DOSG 20%:DOPE 80%) were not active in delivering tat, but all cationic liposome formulations used were capable of delivering tat. Notable is the decrease in delivery activity of DC-Chol cationic liposomes upon decreasing the mole ratio of DOPE to DC-Chol from 4:1 to 2:3. There is an increase in delivery activity upon treatment with cationic liposomes in a liposome dose-dependent manner (Fig. 2). Tat protein alone at a concentration of 1 μg/ml could not be delivered in a detectable manner without cationic liposomes in this cell line. An excessive dose of cationic liposomes can be toxic to the cells and may be responsible for the lack of increase in delivery activity at the higher liposome doses used. This was clear when proteins in the cellular lysates were quantitated. The toxicity of liposomes correlated well with the ratio of cationic lipid to DOPE and increased with an increase in liposome concentration and increased cationic lipid content in the liposomes (data not shown). DC-Chol:DOPE (1:4) liposomes showed the least toxicity (not shown) and most efficient tat delivery (Fig. 2). Two commercially available liposome formulations (both containing DOPE), lipofectin and lipofectamine, were also tested.

Lipofectamine, which is highly efficient in delivering DNA, was not very efficient in delivering tat as compared with other formulations (Fig. 2).

Effect of DOPE vs DOPC on tat protein delivery:

The results from Fig. 2 prompted a study of the optimal ratio of cationic lipid (DC-Chol) to DOPE phospholipid. Additionally, the activity of DOPE was investigated by replacing it with another neutral phospholipid, i.e. DOPC. DOPC lacks fusogenic activity (31) and is inefficient in DNA delivery in contrast to DOPE (32). DOPE is believed to help DNA delivery through its fusogenic activity. Cationic liposomes made with DC-Chol cationic lipid and either DOPE or DOPC, at different molar ratios of DC-Chol to the phospholipid were used to deliver tat protein (1 μg/ml) to A431LTRCAT cells. Cationic liposome concentration was kept constant at 40 μM and the results of the experiment are shown in Fig. 3. Delivery activities of DC-Chol liposomes containing DOPE were readily detectable; whereas liposomes containing various amounts of DOPC essentially had no detectable activity. Liposomes containing higher DOPE (60-80%) and lower DC-Chol content exhibited higher delivery activities. However, cationic liposomes with excess DOPE (90%) showed a lower level of activity probably as a result of the instability of these liposomes (data not shown). If a small portion (10%) of DOPE was replaced with DOPC to increase the liposome stability, the stabilized liposomes again showed high delivery activity (Fig. 3).

Effect of cationic liposomes on the cellular uptake of tat:

It is possible that the enhanced delivery of tat by cationic liposomes is due to enhanced cellular association of tat. To test this hypothesis, tat protein was radiolabeled with ^{125}I and incubated at increasing concentrations with cells at 37°C for 5 h with or without DOPE/DC-Chol (1:4) cationic liposomes (40 μM). There was a concentration dependent increase in tat association with the cells (Fig. 4). Cationic liposomes brought about only a modest (< 20%) increase in the tat association with cells. This level of enhancement is obviously not enough to account for the 150-fold increase in the tat delivery activity at the same liposome concentration (see Fig. 2). However, it is possible that less tat is degraded in the lysosomes and more tat is delivered, when tat is is delivered with liposomes. This possibility can not be destinguished using the binding assay shown in Fig. 4.

Co-delivery of tat with cationic liposomes is critical for efficient tat delivery:

Since cationic liposomes did not significantly improve the cellular association of tat, we speculated that liposomes may enhance intracellular delivery by allowing escape from endosomal compartments. This speculation is supported by the known endocytic route of tat internalization by cells (6,9) and can be further supported by co-delivering tat with

liposomes or separating the delivery of tat and liposomes temporally. It is expected that intracellular delivery through an endosomal compartment mandates the co-localization of tat and liposomes in the same compartment. This means that co-delivery of tat and liposomes should give the optimal delivery results. We performed an experiment in which tat (1 μg/ml) was added to the cells first and the addition of liposomes (DC-Chol 20%:DOPE 80%) was delayed for various period of time. The total length of incubation time for both tat and liposomes was kept at one hour. The data in Fig. 5 indicate that when liposomes were added together with tat, i.e. they are co-delivered to the cells, the delivery efficiency was the highest. The delayed addition of liposomes caused a progressively decreased delivery efficiency with 50% efficiency occurring when liposomes were added between 45-60 min after the initiation of tat incubation. If liposomes were added after one hour, at which time any free tat was washed away, the delivery efficiency was further decreased. It is clear that optimal tat delivery is associated with a longer co-incubation time of tat and liposomes. Another experiment was done in which liposomes were added first and incubated with the cells for one hour. Cells were washed and tat was added at different times afterward. Again, the results showed greatly reduced tat delivery efficiency (data not shown). We conclude that tat may enter the cells together with liposomes for maximal delivery efficiency.

Competition of polylysine with liposome-mediated tat delivery:

Since cationic liposomes are multivalent in their cationic charge we tested other multivalent cationic macromolecules in the tat delivery assay. Increasing concentrations of the cationic polypeptide poly-L-lysine (MW 3,500) and the cationic protein lysozyme were used for tat delivery with or without cationic liposomes (DC-Chol 20%:DOPE 80%). The results shown in Fig. 6 indicate that polylysine inhibited tat delivery by liposomes at increasing polylysine concentrations from 20 to 80 μM. However, at lower polylysine concentrations tat delivery was enhanced up to 50%. In comparison, polylysine had no tat delivery activity by itself at any concentration. On the other hand, lysozyme had no inhibition or enhancement effect on tat delivery at any concentration tested with or without liposomes.

Presumably, polylysine inhibits the binding of tat or cationic liposomes or both to the cell surface. To determine which cationic entity polylysine was competing with, we performed the following experiment. Cells were incubated with tat for 1 hour, washed, and then treated with liposomes for an additional hour before washing and returning to the growth medium. The advantage of this experimental design is that it temporally separates tat delivery from liposome delivery so they can be competed out by polylysine (80 μg/ml) separately. The results in Table 1 clearly show that tat delivery was only inhibited if polylysine was added

together with tat, but not with liposomes. This suggest that polylysine inhibits tat binding but not the binding of cationic liposome with cells.

5. Discussion

In our continuous effort to study and improve the intracellular delivery of proteins we chose HIV-1 tat protein delivery as a useful model for several reasons: First, tat can be easily produced as a recombinant protein and can be readily purified in the large quantities needed for physical and delivery studies (9). Second, mutant tat can be readily prepared which allow testing of functional domains. We have produced a deletion in tat which eliminates the cysteine domain critical for tat trans-activation function (Fig. 1). Third, tat biological function, i.e. trans-activation of HIV-1 promoter, can be easily assayed with a reporter gene assay (29). Fourth, tat is known to enter cells through an endosomal pathway (6,9). Since endocytosis is the major route of delivery of macromolecules, such as proteins, nucleic acids, viruses, liposomes, and other targeted carriers (33), tat delivery represents a general model of macromolecular delivery by endocytosis.

To enhance the delivery of tat through the endocytosis route we used different formulations of cationic liposomes containing DOPE (Fig. 2). Liposomes containing DOPE have been shown by our group to deliver macromolecules such as DNA (32) and protein (34), by destabilizing

endosomal compartments after endocytosis. Electron microscopic observations have shown a clear distinction between the endosome destabilizing effect of DOPE liposomes and the lack of destabilization effect with DOPC liposomes (32). This is related to the unique property of DOPE to assume nonbilayer structures at physiological condition (31), particularly in an acidic pH environment such as that of an endosome (35). Data presented here are consistent with the notion that cationic liposomes and tat bind to the cell surface independently and are co-internalized into the same endosomal compartment. The membrane destabilizing activity of DOPE may cause the rupture or lysis of the endosome membrane and allows the release of tat into the cytosol. Tat could then enter the nucleus via the action of its nuclear localization signal to activate gene expression. This model is supported by a number of observations. First, data in Fig. 2 and 3 indicate that only liposomes enriched with DOPE show enhanced activity of tat delivery. Liposomes containing DOPC are not active because DOPC does not form a non-bilayer structure under physiological conditions and thus does not destabilize membranes (32). Second, anionic liposomes, although enriched with DOPE are not capable of binding efficiently to the negatively charged surface and were not active in delivering tat (Fig. 2). Third, maximum delivery efficiency was observed when tat and liposomes were co-incubated with cells (Fig. 5) to facilitate co-internalization and co-localization in the endosomes. Fourth, the overall liposome-assisted tat uptake by cells was only 20% greater than the unassisted uptake (Fig. 4), indicating that the action of liposomes was in an

intracellular compartment, presumably endosomes. Finally, the model is consistent with the established data of others (6) which show that tat is rapidly endocytosed at 37°C. Lysosomotropic agents such as chloroquine, although toxic to the treated cells, can enhance the delivery of the internalized tat as shown by others (9) and us (Fig. 1). Whatever the mechanism of delivery of tat by cationic liposomes is, it is possible that free tat delivery into cells may be through a different route than from the one of tat with liposomes. The experiments provided can not distinguish this possibility whether it is a subtle or major difference between the two mechanisms of tat delivery. To better understand the route of delivery of tat or other proteins, other experimental approaches should also be tested. One common and useful approach to dissect the endocytic pathway is the use of mutant cell lines known to exhibit a deficiency in one of the steps involved in endocytosis. Another approach is the use of drugs that specifically inhibit membranes fusion or hexagonal$_{II}$ transformation. It is imortant to use such drugs with minimal toxicity to cells to pinpoint the route of delivery without toxic inhibition of vital cellular functions of cells, as may be the case for chloroquine.

The activity of cationic liposomes in enhancing tat delivery is similar, although many folds higher in magnitude, to that of chloroquine (Fig. 1). Chloroquine and other weak bases work by accumulating in the acidic endosome/lysosome compartment due to acid-base equilibrium, raising the endosome pH, and blocking the degradation of tat in the

lysosomes (36). This action often leads to swelling of endosomes/lysosomes and toxicity to the treated cells (37). There is no evidence that cationic liposomes can change the endosomal/lysosomal pH. They certainly do not cause swelling of the organelle (32), and are thus much less toxic than chloroquine. The action of liposomes is to promote the destabilization of the endosomal membrane. Thus, its action is similar to that of adenovirus which exhibits a well defined endosomolytic activity as part of the viral uncoating process (38). Adenovirus, native or inactivated, has been used to enhance the delivery of DNA (39), and other macromolecules with high efficiency. Unlike adenovirus, cationic liposomes are not immunogenic and are thus more suitable for repeated administration *in vivo* (40).

The absence of tat delivery activity shown when polylysine, lysozyme, or DOPC liposomes are co-delivered with tat (Fig. 3 and 6) can be understood by the common absence of endosomolytic activity of the three reagents. DOPE-containing liposomes were the only reagent, in addition to chloroquine, capable of tat delivery (Fig. 2). However, low concentrations of polylysine enhanced tat delivery in the presence of DOPE liposomes (Fig. 6). This improved tat delivery might be the result of enhanced endocytosis triggered by polylysine. Protamine was described as a tat delivery enhancer molecule (6). Both protamine and polylysine are cationic polypeptides and may act similarly. High doses of polylysine were shown to inhibit tat delivery through a possible inhibition of tat binding

without affecting the binding of cationic liposomes to the cells (Fig. 6 and Table 1). It is conceivable that a relatively small molecule such as polylysine can compete with the binding of tat with the cell surface receptor, whereas the much larger liposomes would not be capable of doing so due to steric hindrance.

A potentially important application of the work described here is the delivery of therapeutic or diagnostic macromolecules such as oligopeptides or proteins conjugated chemically or genetically to tat polypeptide. Recently, Fawell *et al.* have described the delivery of four different proteins by conjugation to tat polypeptide devoid of a trans-activation function (27). Two of these proteins were naturally toxic to the cells and the others were reporter proteins. The toxic proteins killed the cells and the reporter proteins stained the cell interior only when chemically conjugated to tat. Co-delivery of tat with unconjugated proteins showed no delivery activity of these proteins. The high efficiency of tat delivery by cationic liposomes may further enhance the delivery of the tat-conjugated protein or peptide. In addition, cationic liposomes may facilitate the delivery of cationic proteins other than tat. Presumably, any macromolecules internalized via the endocytic pathway, either mediated by specific receptor or otherwise, could be co-internalized with cationic liposomes. Their penetration into the cytosol may then be facilitated by the membrane destabilization activity of liposomes in a manner similar to that of adenovirus. These hypotheses will be tested in the future.

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Table 1. Competition of poly-L-lysine with tat delivery[1].

Pulse	Chase	CAT activity (% conversion)
tat [a]	liposome [b]	4.24 ± 0.43
tat + PLL [c]	liposome	0.22 ± 0.01
tat	liposome + PLL	4.37 ± 0.49

1. A431LTRCAT cells were incubated (pulsed) in DMEM for 1 h at 37°C with the indicated reagent(s). After the pulse, cells were washed and incubated (chased) in DMEM with the reagent(s) indicated for 1 h at 37°C followed by washing, 20 h incubation in growth medium and a CAT assay as in fig. 1 legend.

[a] 1 μg/ml tat protein was used.

[b] DC-Chol:DOPE (1:4 mole ratio) cationic liposomes were used.

[c] 80 μg/ml poly-L-lysine was used.

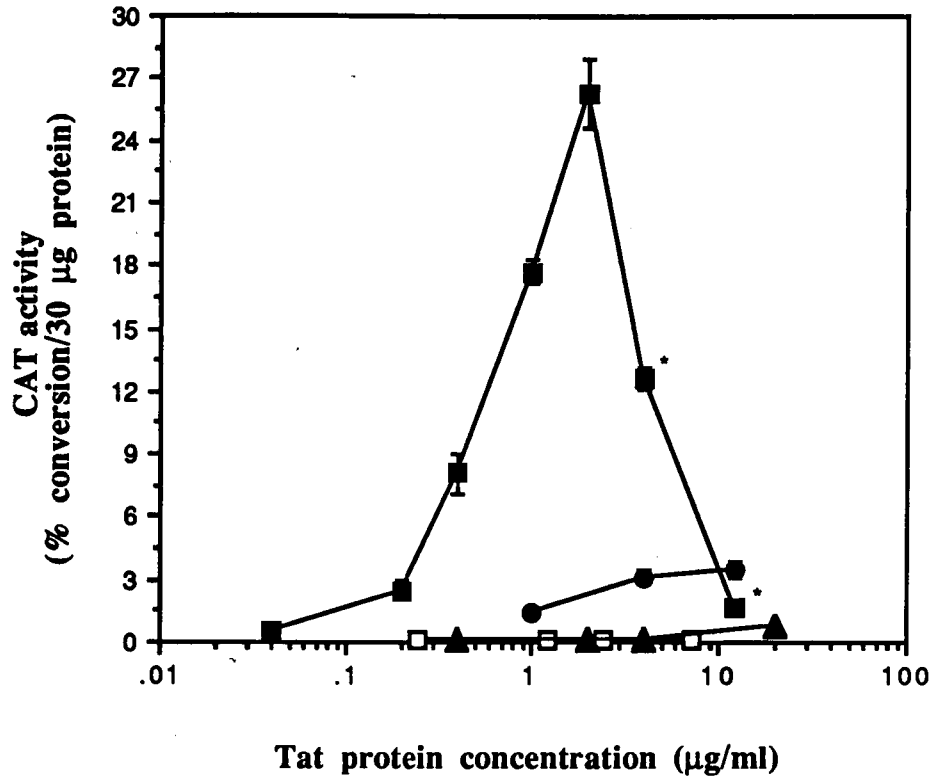

Fig. 1. Effect of tat protein dose and delivery method on tat delivery. Increasing concentrations of tat recombinant protein containing amino acids 1-72 (closed symbols) or tat Δ23-37 deletion mutant (open symbol) were mixed with 40 µM of DC-Chol:DOPE (1:4 mole ratio) cationic liposomes (■), 250 µM chloroquine (●) or none (▲) in DMEM media and incubated with A431LTRCAT cells. The cells were washed and incubated in growth media at 37°C for 20 h before assayed for CAT activity. Samples marked with an asterisk showed significant cytotoxicity.

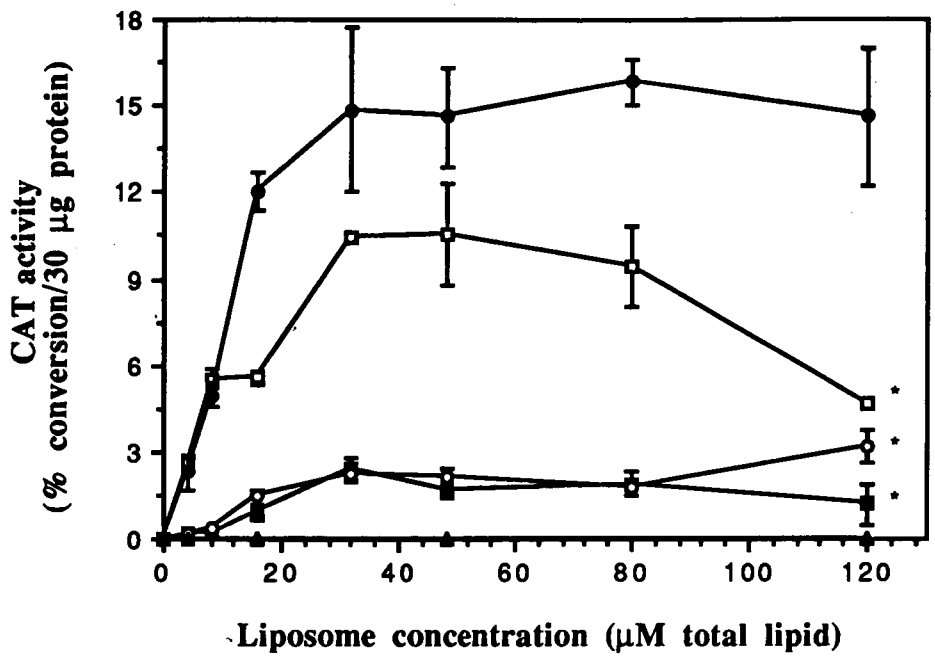

Fig. 2. Delivery of tat protein with different liposome formulations. Tat protein (1 µg/ml final concentration) was mixed with increasing concentrations of different liposome formulations including DC-Chol 20%:DOPE 80% (●), DC-Chol 60%:DOPE 40% (○), Lipofectin (❑), Lipofectamine (■), or DOSG 20%:DOPE 80% (▲) and incubated with A431LTRCAT cells. Cytotoxicity was evident in samples designated with an asterisk.

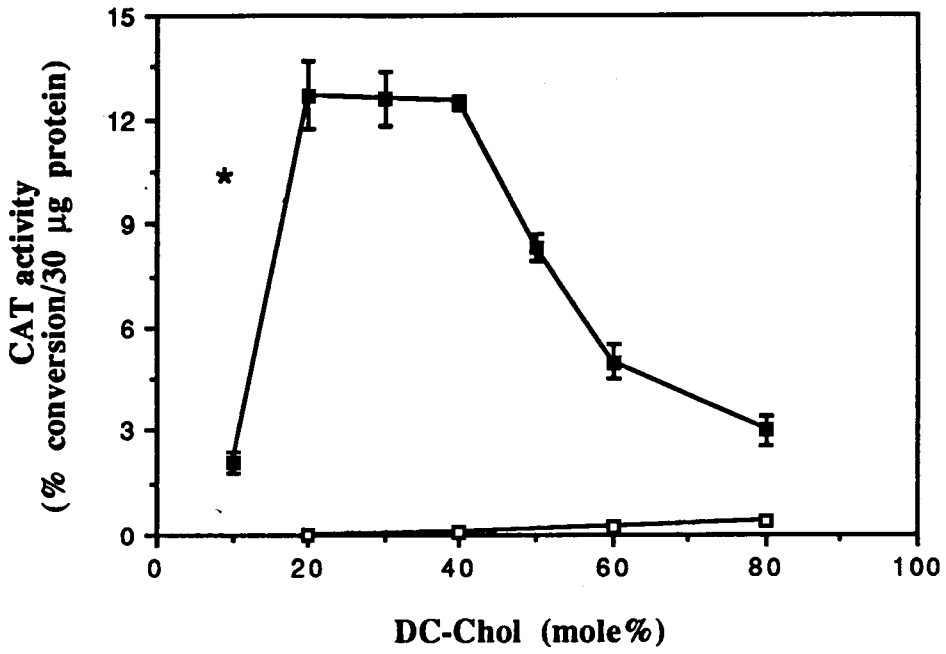

Fig. 3. Effect of lipid composition of cationic liposomes on tat protein delivery. Tat protein (1 µg/ml) was mixed with 40 µM of DC-Chol cationic liposomes containing different amounts of DOPE (■) or DOPC (❑). Tat protein was also mixed with cationic liposomes containing DC-Chol:DOPE:DOPC (1:8:1 marked as *). The tat/liposome mixtures were incubated with A431LTRCAT cells.

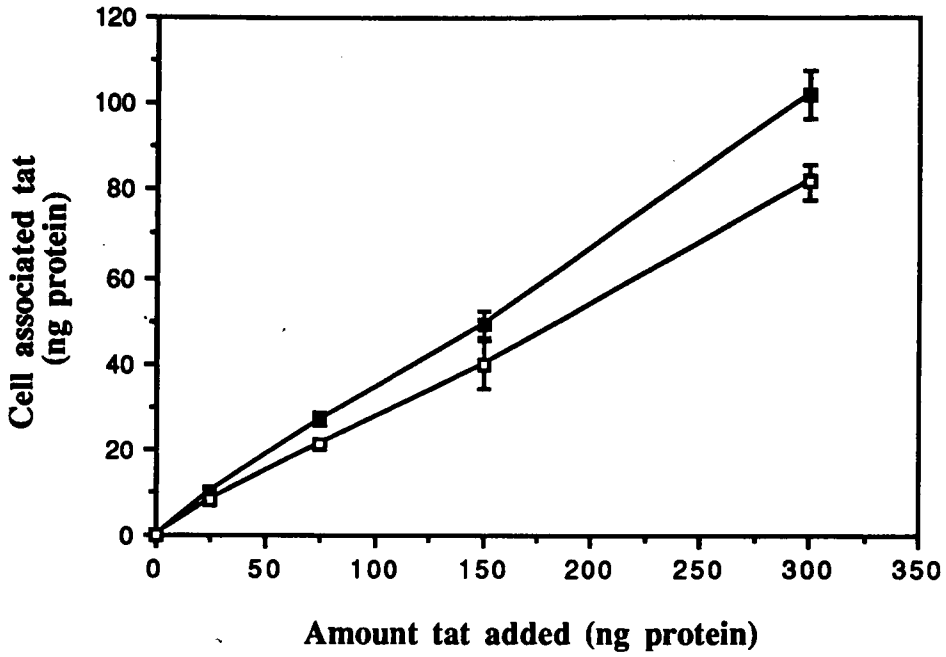

Fig. 4. Effect of cationic liposomes on tat protein uptake by cells. ^{125}I-labeled tat protein was incubated with A431LTRCAT cells with (■) or without (❑) DC-Chol:DOPE (1:4) cationic liposomes and incubated for 5 h at 37°C. Cells were washed and trypsinized and radioactivity of cell pellet was counted in a liquid scintillation counter.

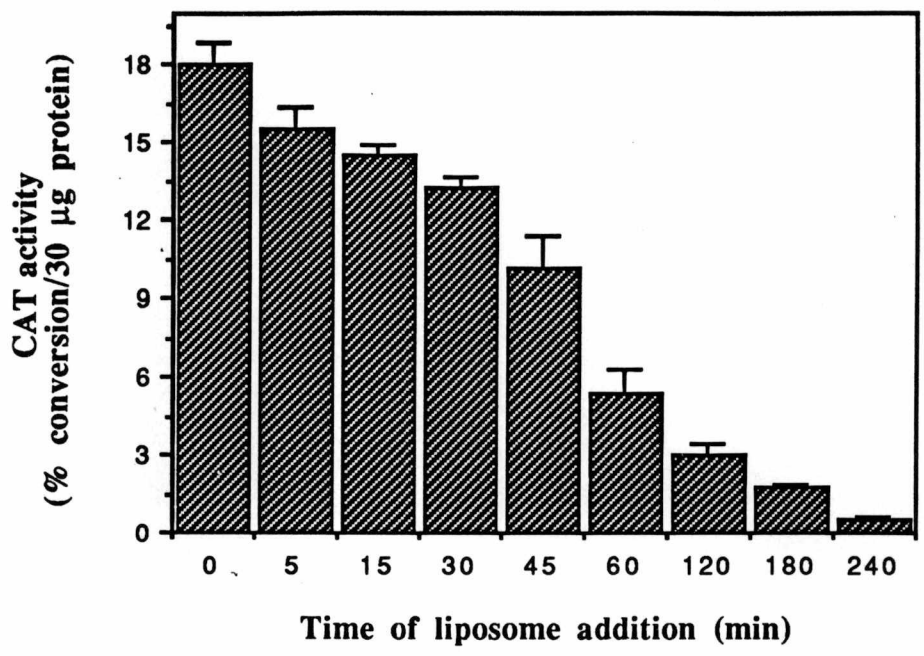

Fig. 5. Effect of co-delivery of tat protein with DC-Chol cationic liposomes on tat delivery. One µg/ml tat protein in DMEM was incubated with A431LTRCAT cells at 37°C for 1 h. DC-Chol:DOPE (1:4) cationic liposomes (40 µM) were added to the cells, for a total incubation time of 1 h at 37°C, at different time points after the initiation of the tat incubation. All cells were incubated at 37°C for 20 h in growth medium before the CAT assay.

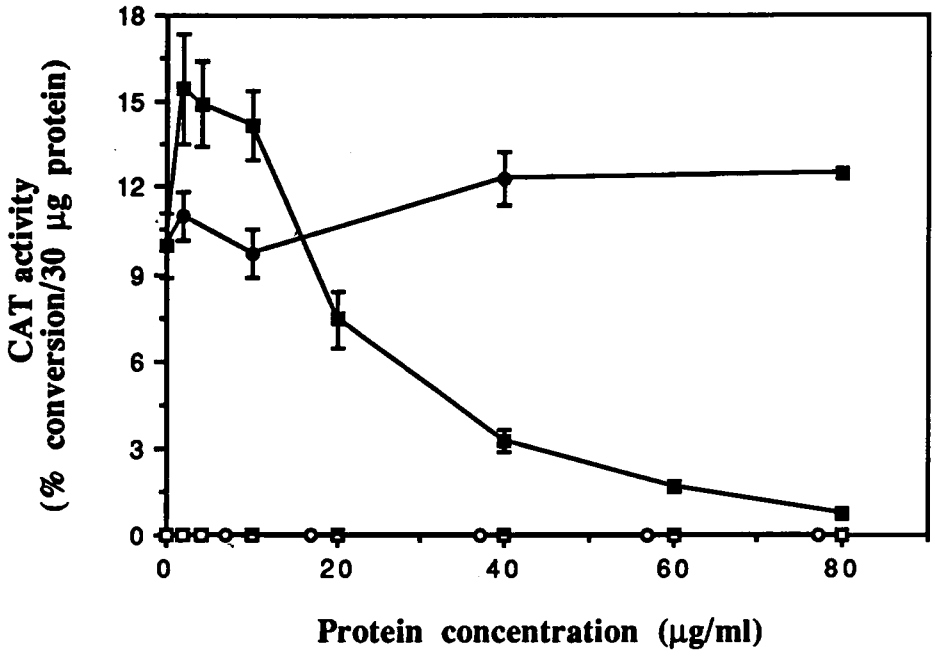

Fig. 6. Effect of cationic polypeptides on tat protein delivery. One µg/ml tat protein was mixed with increasing concentrations of poly-L-lysine (squares) or lysozyme (circles) with (closed symbols) or without (open symbols) DC-Chol:DOPE (1:4) cationic liposomes in DMEM and incubated with A431LTRCAT cells.

PART V

CO-DELIVERY TO MAMMALIAN CELLS OF A TRANSCRIPTIONAL FACTOR WITH CIS-ACTING ELEMENT USING CATIONIC LIPOSOMES

1. Abstract

The human immunodeficiency virus-1 transactivator protein (tat) was co-delivered efficiently with a reporter gene under the control of a tat-responsive DNA element using different formulations of cationic liposomes. Expression of a tat-responsive reporter gene was induced by incubating cells with a mixture of purified recombinant tat protein, reporter DNA and liposomes. Different cell lines were tested successfully as targets for the co-delivery. Tat was shown to trans-activate the co-delivered virus promoter specifically in the cells tested. Co-delivery of tat with DNA is a useful model for studying the function of *trans* -acting factors and their *cis* -acting DNA elements. The presently available methods such as foot-printing only reveal the binding, but not the functional consequence of the binding, of the factor with the element. In addition, this system may prove useful as a model for high level and regulated transgene expression in target cells.

2. Introduction

Regulation of transgene expression is a critical element in gene transfer studies. Additionally, the new field of gene therapy is highly dependent on the control of gene expression. *Cis* -acting elements composed of promoters and enhancers are commonly ligated to the transfected gene to control the induction of gene expression and/or to

enhance transcription and translation of the gene. However, this method of gene expression control does not allow the physical manipulation of the transcription or translation factors involved in gene expression. These factors may be modified intracellularly as a part of natural mechanism of controlling gene expression. Intracellular modifications include phosphorylation, dephosphorylation (1) and proteolytic cleavage (2) among others.

Extracellular manipulation of the gene expression machinery may allow unequivocal physical dissection and characterization of the post-translational modifications of the polypeptide of interest. Delivery of an *in vitro* -modified polypeptide to the cell should facilitate direct *in vivo* testing of the effect of the modification on the function of the protein (3). Furthermore, co-delivery of the modified factor with a *cis* -acting DNA element that can also be manipulated *in vitro* by a variety of molecular cloning techniques allows complete control of the modified factor and its target DNA element (3). Two methods are currently available, electroporation and microinjection, for co-delivery of a *trans* -acting factor with its cis-acting DNA element. Electroporation is an efficient delivery method for both DNA and proteins but is highly toxic, killing about 50% of the target cells in a successful delivery protocol (4). Microinjection on the other hand is a highly specialized technique and requires expensive specialized equipment and training which prohibit the use of the technique in most laboratories (5).

We have exploited cationic liposomes, commonly used for DNA delivery, to co-deliver a cationic polypeptide transcriptional factor, i.e. tat[1] of HIV-1, with a tat-responsive DNA element into mammalian cell lines. Tat was co-delivered with the LTR promoter/enhancer element of HIV-1 directing expression of a reporter gene using DC-Chol cationic liposomes.

3. Materials and Methods

Materials:

Dulbecco's Modified Eagle Media (DMEM), Lipofectin[2] and Lipofectamine[2] liposomes were from Life Technologies, Inc. Fetal bovine serum was from HyClone Laboratories, Inc. Acetyl-coenzyme A, chloramphenicol and other chemicals were from Sigma. [^{3}H]acetyl-CoA (3-6 Ci/mmol) was from Amersham. BetaMax liquid scintillation cocktail was from ICN Biomedical, Inc. pZ523 columns were from 5 Prime to 3 Prime, Inc.

Liposomes preparation:

Cationic liposomes containing 3β [N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), synthesized as described (6), and 1, 2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), from Avanti Polar Lipids, were prepared by a 5 min sonication of mixed and dried

lipids in 20 mM HEPES buffer as described (6). The liposomes, with mean diameter of 150 nm, were stored at 4°C.

Recombinant tat protein expression and purification:

Plasmid pTat72, from Dr. Alan Frankel (7), codes for the first exon of HIV-1-Tat gene spanning amino acids 1 through 72. The sequence encoding amino acids 23-37 was deleted from tat (1-72) by PCR deletion mutagenesis of pTAT72 using two primers. One primer (AGCCCAGCGCGTCGGCCGCCATG) spans an Eag1 site upstream of the tat gene. Another primer (TTTTTACGGCCGTAAGAGATA-CCTAGGGCTTTGGTGATGAAGCACGCGGTTTT) covers another Eag1 site within tat. The PCR product was digested with Eag1 and the 765 bp fragment was cloned into pTAT72 which had been digested with Eag1 (the 4057 bp large fragment). The tat coding region is controlled by the bacteriophage T7 promoter. Expression is induced with isopropyl ß-D-thiogalactopyranoside. The recombinant tat protein was purified using a modified procedure (8). Briefly, after induction E. coli was suspended in 10 volumes of 25 mM Tris-HCl (pH 7.5), 1 mM EDTA and lysed in a french press followed by centrifugation at 10,000 x g for 1 h. The supernatant was loaded onto a Q Sepharose Fast Flow (Pharmacia LKB, Piscataway, NJ) ion exchange column (20 ml resin / 60 ml lysate). 0.5 M NaCl was added to the flow-through fraction to precipitate tat protein followed by centrifugation at 35,000 rpm, in a 50.2 rotor, for 1 h. The

pellet was dissolved in 6 M guanidine-HCl and recentrifuged. The supernatant was loaded onto Biogel A 0.5 agarose gel filtration column equilibrated with 6 M guanidine-HCl, 50 mM sodium phosphate (pH 5.4), 10 mM dithiothreitol, and then eluted with the same buffer. The eluted fractions were loaded onto a C4 reverse phase HPLC column and eluted with a gradient of 0-75% acetonitrile, 0.1% trifluoroacetic acid. Twenty mg of tat protein was produced per liter of cultured E. coli (6 g of cells per liter). Tat protein constituted 5% of total E. coli protein. Tat protein was a single 10 kd band on SDS-PAGE.

Plasmid DNA:

pUC-BENN-CAT and pSVtat72 plasmid DNA was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (deposited by Dr. Malcolm Martin). pUCSV2CAT plasmid was from Dr. T. Hazinski. pCMVlacZ plasmid was a gift. All plasmids were cloned in E. coli and purified by pZ523 columns.

Cell culture and transfection:

A431 human epidermoid carcinoma (from Dr. G. Carpenter, Vanderbilt University), HeLa, 293, BHK, and CHO cells (all from American Type Culture Collection) were maintained in DMEM supplemented with 10% fetal bovine serum, L-glutamine, glucose,

penicillin and streptomycin. Cells were plated in multiwell (24 wells) plates one or two days before transfection and were always transfected at 70-80% confluency. DNA and tat protein or tat protein alone were added to 250 µl of warm serum-free DMEM in microfuge tubes and liposomes were added and mixed immediately afterwards. The mixture was incubated for 10-20 min at room temperature and then added to the cells after removal of growth media. Cells were incubated for 5 h at 37°C in 5% CO_2 after which the transfection medium was removed and fresh growth medium was added. Cells were further incubated for 20 h at 37°C before the chloramphenicol acetyltransferase (CAT) assay.

CAT assay:

Twenty five h after transfection, cells were washed once with PBS and lysed with 0.1% Triton X-100 in 0.25 M Tris-HCl buffer (pH 7.8) at room temperature for 10-20 min. This was followed by heat inactivation at 65°C for 10 min and high speed microcentrifugation for 5 min. About 1/10 lysate supernatant (equivalent to about $5x10^4$ cells or 15 µg protein) was used for each sample in a CAT reaction. The reaction volume was 100 µl containing 1 mM chloramphenicol, 0.1 mM acetyl-CoA, and 0.1 µCi [^{3}H]acetyl-CoA in 0.25 M Tris-HCl (pH 7.8) and cell lysate. CAT reaction was carried out at 37°C for 15 min and stopped by rapid freezing and products were extracted with 1 ml toluene. The organic extract was counted for radioactivity in BetaMax liquid scintillation cocktail. CAT

activity is expressed as % acetylation of chloramphenicol (% conversion/15 μg protein). The BIO-RAD microprotein assay (Bio-Rad Laboratories) was used to quantitate proteins and bovine serum albumin was used as a standard. All samples were performed in triplicates and all experiments were repeated at least twice.

4. Results

Tat specificity and dose-dependence effect on trans-activation:

Recombinant HIV-1 tat protein (exon 1 encoding amino acids 1-72) was used for all the experiments described. It is known that tat exon 1 codes for a fully functional tat protein which can trans-activate gene expression from the HIV-1 LTR promoter (7). Cationic liposomes, made by sonication of DC-Chol and DOPE in buffer, were used for delivery of DNA and tat protein to the cells in culture. Plasmid pUCBENNCAT, which contains a CAT gene under the control of HIV-1 LTR promoter, and various concentrations of tat protein were mixed with DC-Chol:DOPE (3:2 molar ratio) liposomes and incubated with A431 cells. Fig. 1 shows that CAT gene expression was stimulated up to about 50-fold when wild type tat protein was co-delivered with the plasmid DNA. The degree of activation was tat dose-dependent and was specific to tat because a non-functional deletion mutant (Δ23-37) of tat showed no trans-activation function. The

domain deleted in the mutant corresponds to the cysteine-rich domain which is important for the trans-activation function of tat (9).

Specificity of tat trans-activation to HIV-1 LTR promoter:

Plasmid DNA encoding a CAT reporter gene under the control of HIV-1 LTR promoter (pUCBENNCAT) or under the simian virus (SV40) promoter (pUCSV2CAT) were delivered, by DC-Chol liposomes, with or without tat protein, to A431 cells in culture. Tat protein and cationic liposome concentrations were kept constant in this experiment while DNA concentration was varied. The data in Fig. 2 show that tat protein did not activate the SV40 promoter but was a potent *trans* -activator of the HIV-1 LTR promoter. The activation was absent at higher DNA concentrations, because the delivery of high concentrations of DNA by cationic liposomes is usually inefficient (6). It can be seen that transfection of pUCSV2CAT at high concentrations was also inhibited (Fig. 2). It is also evident from the figure that the optimal ratio of DC-Chol liposomes to DNA was 40 μM to 2 μg/ml (which equals 10 nmoles total lipids to 0.5 μg DNA/well in a 24 well-plate), for A431 cells, regardless of the type of plasmid DNA delivered.

Co-delivery of tat protein by different formulations of cationic liposome:

Fig. 3 shows the co-delivery of tat protein and pUCBENNCAT plasmid DNA to A431 cells by different cationic liposomes. The data show that lipofectamine and lipofectin, both commercially available cationic liposome formulations, could also co-deliver DNA and tat protein in addition to DC-Chol liposomes (DC- Chol:DOPE, 3:2 mole ratio) in this cell type. Gene expression was induced in a cationic liposome dose-dependent manner. It is worth noting here that an excessive dose of cationic liposomes was toxic to the cells and may compete with DNA-complexed liposomes for cellular binding and uptake. The asterix symbols in Fig. 3. denote the toxic concentrations of liposomes which were manifested as death of some of the cells affected. The toxicity was particularly noticeable with lipofectamine liposomes at high concentrations. However it was possible to choose the optimal concentration of liposomes for all three formulations such that the trans-activation activity of tat was demonstrated without any significant cytotoxicity to the treated cells.

Co-delivery of tat and DNA to different cell lines:

Different cell lines can also be used as targets for co-delivery of tat protein with DNA by DC-Chol cationic liposomes, as shown in Table 1. Three human cell lines (A431, HeLa, and 293) and two hamster cell lines (BHK and CHO) were tested. The co-delivery of tat with HIV-1 LTR

promoter driving a CAT gene (pUCBENNCAT DNA) enhanced gene expression, manifested in the increased CAT activity, several fold in all cell lines tested (Table 1). In addition, the tat-induced HIV promoter showed 2-8 folds higher gene expression levels than the SV40 promoter in all cell types. It is worth mentioning that tat has been shown to be less active in rodent cells than in human cells (10), which explains the lower induction levels by tat in BHK and CHO cells.

Co-delivery of tat protein and DNA vs co-transfection of tat expression vector with DNA:

The delivery of tat protein with DNA was compared to the co-delivery (co-transfection) of a tat expression vector with DNA using the same DC-Chol liposomes reagent. An irreleavant plasmid DNA (pCMVlacZ) was included in the transfection mixture to make the total DNA concentration 2 μg/ml, because the DNA/liposome ratio is a crucial parameter determining the efficiency of transfection. Plasmid pSVtat72 contained a tat (1-72) cDNA driven by the SV40 promoter. Tat protein and tat DNA expression vector both induced reporter gene expression (Table 2). However, gene expression induced by tat protein was 2.6-fold higher than that induced by a tat expression vector. This result indicates that co-delivery of purified tat with a tat-responsive element can induce a high level of transgene expression which surpasses the conventional method of co-transfection with a tat expression vector.

5. Discussion

Induction of trans-gene expression can be achieved by co-delivery of inducing protein factors with their cis-acting DNA element using cationic liposomes (3). Recently, our group extended this idea and used cationic liposomes to co-deliver a purified bacteriophage T7 RNA polymerase enzyme with its specific *cis* -acting DNA element, which is the bacteriophage T7 promoter, driving a reporter gene (11). The delivery system allowed cytoplasmic transcription of the trans-gene delivered which bypassed the need for nuclear uptake of DNA which is an inefficient process (12).

In the present work, we have further extended this line of work by co-delivering the recombinant HIV-1 trans-activator tat protein with a plasmid containing the tat responsive element from HIV-1 driving a reporter gene. The co-delivery is probably a result of the cationic nature of tat protein which allow it to bind to DNA. The binding presumably takes place by electrostatic interaction as indicated by an agarose gel DNA-retardation assay (data not shown). Tat was able to retard DNA migration in a dose-dependent manner. It is likely that tat enters into the cells by a piggy-back mechanism as a part of the DNA/liposome complex. This is supported by the fact that the tat/DNA co-delivery had a similar optimal condition as the delivery of DNA alone (Fig. 2). Although tat by itself can enter the cells by an adsorptive endocytosis pathway (7,9,13), this is not

likely the case because most, if not all, of the tat molecules are DNA bound under the condition used in this study.

The delivery conditions described here may be useful in the study of interaction of trans-acting factors with cis-acting elements in transcription. Current methods of investigation such as band-shift assay, and DNA footprinting only reveal the physical binding of the factor with the corresponding element, but not the functional consequence of the binding. It is conceivable that the tat-DNA complex could be delivered to intact mammalian cells using cationic liposomes and the formation of transcription product directly observed. If so, the role of post-translational modifications such as phosphorylation and dephosphorylation of the factor could also be studied, as could the role of any modification on the cis-acting element. Co-delivery of a transgene with transcriptional activator also has implications in the growing field of gene therapy. Direct gene transfer using DNA/liposomes complex has been used in a clinical trial of gene therapy for melanoma using the DC-Chol liposomes (14). Being able to co-deliver a strong transcriptional activator with the DNA has lead to an enhanced expression of the transgene (Table 2). The potential application in direct gene transfer is being studied in our laboratory.

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Table 1. Co-delivery of tat and DNA to different cell lines by DC-Chol cationic liposomes [a]

Cell line	CAT activity (% conversion)		
	LTRCAT[b]	LTRCAT + tat	SVCAT[c]
A431	3.0 ± 0.1	65.1 ± 12.8	9.4 ± 1.1
HeLa	4.7 ± 0.8	61.8 ± 11.9	7.4 ± 1.4
293	6.4 ± 0.7	41.3 ± 3.4	6.5 ± 0.7
BHK	3.1 ± 0.5	15.9 ± 0.3	6.1 ± 0.8
CHO	2.5 ± 0.5	19.7 ± 5.0	9.6 ± 1.9

[a] Different cell lines were transfected with plasmid DNA (2 μg/ml), with or without tat protein (1 μg/ml), complexed to DC-Chol:DOPE (3:2 molar ratio) cationic liposomes (48 μM total lipids) for 5 h at 37°C. Twenty four h after transfection, cells were lysed and assayed for CAT activity (± standard deviation).

[b] LTRCAT = pUCBENNCAT plasmid DNA

[c] SVCAT = pUCSV2CAT plasmid DNA

Table 2. Co-delivery of tat protein and DNA in comparison to co-transfection of tat expression vector and DNA[a]

Delivered molecules	CAT activity (% conversion)
LTRCAT[b]	4.5 ± 0.9
LTRCAT + tat protein	83.2 ± 11.5
LTRCAT + SVTAT[c]	32.3 ± 5.1

[a] A431 cells were transfected with plamid DNA (1 μg/ml pUCBENNCAT and 1 μg/ml pCMVlacZ), with or without tat protein (1 μg/ml) or tat expression vector (1 μg/ml pSVtat72), complexed to DC-Chol:DOPE (3:2 molar ratio) cationic liposomes (40 μM total lipid) as described in table 1 legend.

[b] LTRCAT = pUCBENNCAT plasmid DNA

[c] SVTAT = pSVtat72 plasmid DNA

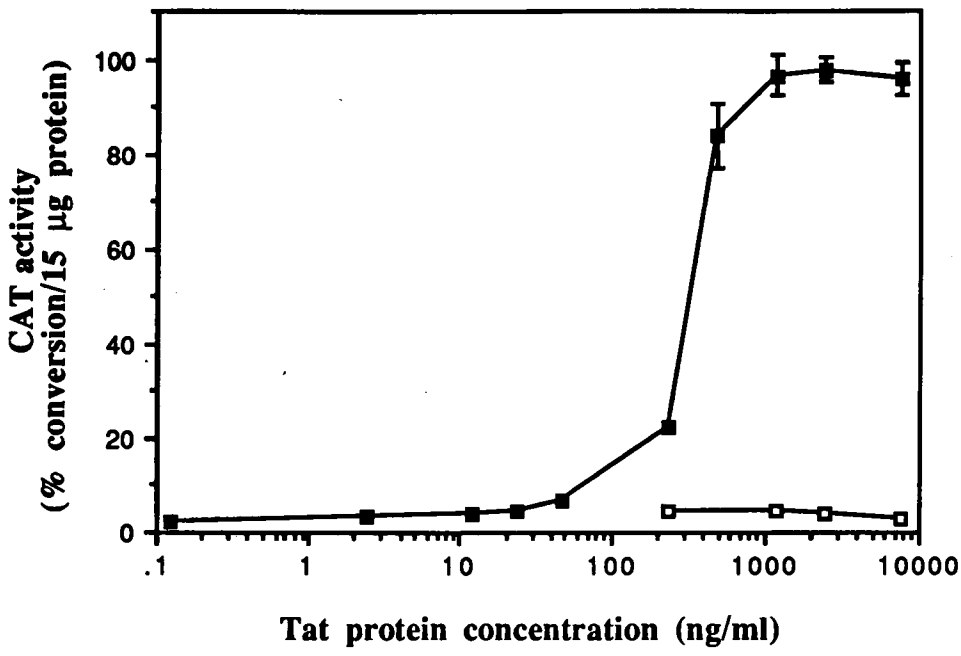

Fig. 1. Trans-activation activity at different concentrations of delivered tat protein. (A) 40 µM of DC-Chol:DOPE (3:2 molar ratio) liposomes were mixed with 2 µg/ml pUCBENNCAT plasmid DNA and increasing concentrations of tat protein in DMEM media. Recombinant tat protein encoding amino acids 1-72 (■) or the same tat protein with a deletion of amino acids 23-37 (❑) were delivered. The mixture was added to A431 cells for 5 h at 37°C and CAT activity was quantitated 20 h thereafter as detailed in the materials and methods.

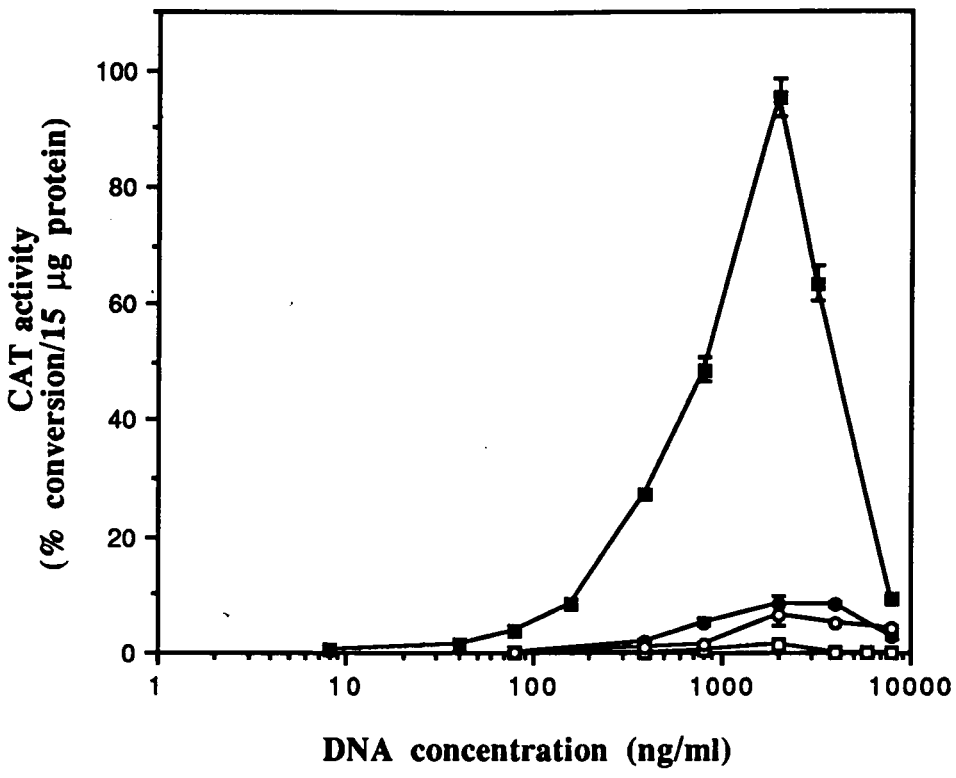

Fig. 2. Specificity of HIV-1 LTR promoter for tat trans-activation at different *DNA concentrations using DC-Chol liposomes and A431 cells.* DC-Chol:DOPE (3:2 molar ratio) liposomes at 40 µM concentration and tat protein (amino acids 1-72) at 1 µg/ml were mixed with increasing DNA concentration and added to A431 cells. pUCBENNCAT plasmid DNA with (■) or without (❑) tat protein or pUCSV2CAT plasmid DNA with (●) or without (○) tat protein were used.

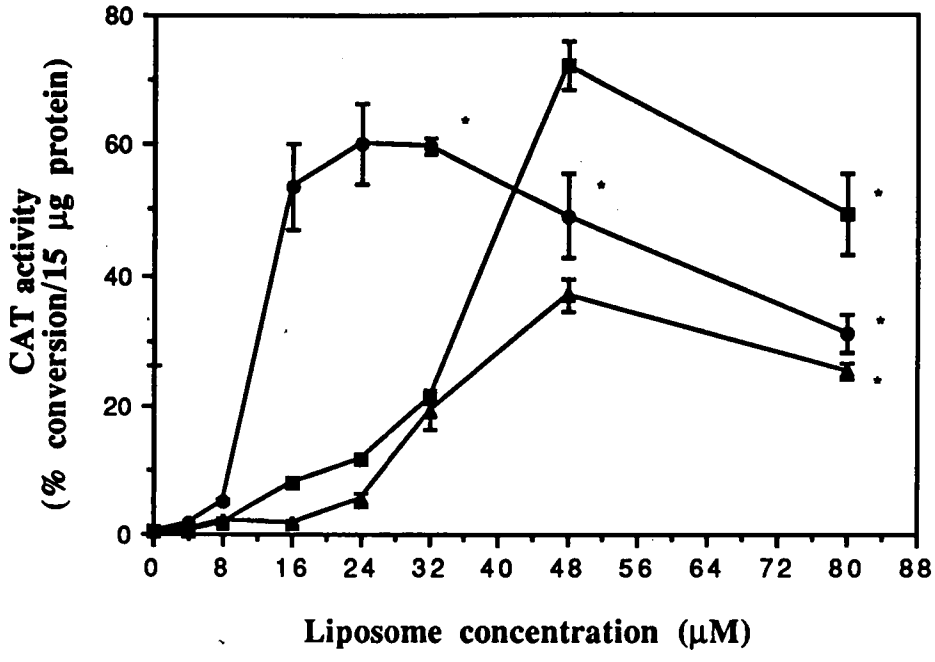

Fig. 3. Effect of different formulations and concentrations of cationic liposomes on tat protein co-delivery with DNA. A431 cells were transfected with different concentrations of DC-Chol:DOPE (3:2 mole ratio) cationic liposomes (■), Lipofectin cationic liposomes (▲), or Lipofectamine cationic liposomes (●) complexed with plasmid DNA (pUCBENNCAT, 2 µg/ml) and 1 µg/ml tat protein (amino acids 1-72). The results are shown in a CAT assay. The data points marked with * indicate observed toxicity.

PART VI

SUMMARY AND CONCLUSIONS

1. Summary

Prior to the work described in this dissertation, macromolecule delivery to mammlian cells in our laboratory was achieved by means of pH-sensitive anionic liposomes. Macromolecules such as DNA (1) or protein (2) were encapsulated in the liposomes at a relatively low efficiency. Recently, delivery of DNA was improved by complexing DNA to Lipofectin™: a cationic liposome made with a cationic lipid and a neutral phospholipid (3). This novel design of DNA carrier facilitated 100% DNA complexing with efficient DNA delivery activity to a variety of cell types. In addition, the cationic formulation was easy to prepare and highly reproducible. As a result of the difficulties associated with encapsulating macromolecules in liposomes in addition to their low encapsulation and transfection efficiency, a new cationic liposome formulation containing cationic detergent was tested by Pinnaduage *et al* in our laboratory (4). However, the results showed a high level of gene expression at the high cost of cellular toxicity. The toxicity was not surprising because of the inclusion of a cationic detergent in the liposome formulation. These results prompted us to formulate new cationic liposomes containing biodegradable and less toxic cationic component and which may improve the efficiency of transfection.

We used a reporter gene assay to test the DNA delivery activity of a series of cationic cholesterol derivatives, synthesized in Dr. R. M. Epand

laboratory (5), in a sonicated liposome formulation containing DOPE (6). The tested derivatives were composed of cholesterol linked with an amide or ester bond to a cationic head group containing a tertiary or quaternary amine (6). Data showed that derivatives with a tertiary amine head group were found to have efficient transfection activity, negligible cytotoxic activity, and a very weak PKC inhibition activity. In contrast, derivatives with a quaternary amine head group had no tranfection activity and showed higher toxicity in addition to strong PKC-inhibition activity. Therefore, a number of transfection optimization experiments were performed on the tertiary amine derivative containing a readily hydrolyzable ester bond, which is the derivative with no detectable toxicity. Data showed that the longer the incubation time of transfection complex with cells the higher the transfection activity. However, an extended period of incubation with the complex causes some toxicity to the cells. DNA delivery was accomplished only with attached cells. The inclusion of serum at 20% concentration or higher had detrimental effects on trasnfection possibly due to the presence of polyanions in the serum which can easily neutralize the net cationic charge of DNA/liposome complex. The ratio of DNA to liposomes was critical for transfection. An excessive DNA concentration abolished transfection activity mostly due to neutralization of liposome cationic charge needed for cell binding and due to an unneutralized net negative charge on DNA which also prohibit the complex from cell adsorption. Attempts to substitute DOPE with DOPC abolished the majority of transfection activity. DOPE exhibits membrane destabilization activity

because of its tendency to undergo a transition from a membrane stabilizing L_α phase to a membrane destabilizing hexagonal H_{II} phase under physiological conditions (7). The result of this structure/function/toxicity study has led to the synthesis of DC-Chol (8), which is now used routinely in this and many other labs.

The role of DOPE in enhancing DNA delivery was studied in more detail through binding and transfection assays using DC-Chol cationic liposomes. Complexed DNA was bound to cells at a low concentration of liposomes. However, DNA delivery did not take place at this low liposome dose. The finding that a minimal concentration of cationic liposomes was needed for efficient transfection led to the development of a new , 2 step (pulse/chase), DNA delivery assay. The assay confirmed a critical role for DOPE in the activity of cationic liposomes mediated gene delivery. The presence of DOPE *per se* and the concentration of DOPE were both essential to DNA delivery not through cell binding but through the uptake activity. DOPE was shown to be a major contributer to internalization of DNA into cells as the complete replacement of DOPE with DOPC could not eliminate DNA binding to cells but completely abolished DNA functional delivery. While complete replacement of DOPE with DOPC abolished DNA transfection, partial replacement had only partial elimination of transfection activity. Separating DNA binding from DNA uptake was possible only through the newly developed DNA delivery assay because a standard transfection could not distinguish the binding activity

from internalization since binding is continuously followed by uptake. The lysosomotropic agent chloroquine was used to test its effect on transfection. Chloroquine treatment resulted in complete inhibition of transfection suggesting that endocytosis must be the major route of DNA delivery by cationic liposomes. The essential role of DOPE in transfection also pointed toward the endocytosis mechanism since DOPE have membrane destabilization activity under low pH, a natural condition in endosomes (7).

The same cationic liposomes were also tested in the delivery of a model cationic protein to cells in culture. The model protein chosen for delivery was the human immunodeficiency virus-1 (HIV-1) trans-activator protein (tat), a transcription factor and a major inducer of the expression of genes under the control of HIV-1 promoter element. Tat was also known to undergo cell surface binding through specific cell surface receptors followed by adsorptive endocytosis and inefficient entry into the cytosolic compartment. The majority of tat protein, however, is naturally routed to the lysosomal compartment for degradation. To improve tat delivery through the endocytic route different cationic liposome formulations were tested in a reporter gene assay. A cell line that contained the HIV-1 promoter controlling a reporter gene was cloned. Tat delivery was successfully tested in this cell line with optimal delivery reaching 150-fold enhancement in tat delivery. Tat delivery was similar to DNA delivery in that the inclusion of DOPE, but not DOPC, in the liposome formulation was essential for the delivery process. A minimal

concentration of DC-Chol was also required for optimal tat delivery. Through a tat pulse and liposome chase assay, tat and cationic liposomes were shown to co-internalize into cells which implicates a common compartment for internalization. The endosome compartment is a possible candidate for the co-uptake of tat with liposomes, a conclusion supported by the known membrane disrupting function of DOPE (especially under low, endocytic pH) and the natural endocytic fate of tat.

Since both DNA and protein delivery were possible using available formulations of cationic liposome, the possibility of DNA and protein co-delivery was tested. Such a co-delivery strategy would be advantageous where transient and maximum gene expression, which can be dependent on the co-delivered protein factor, may be prefered in certain applications such as cancer gene therapy. Essentially, tat protein was complexed with DNA, coding for a reporter gene under the control of HIV-1 promoter, and cationic liposomes. Transfection of different cell lines with the ternary complex showed detectable reporter gene expression with tat protein concentrations as low as 10 ng/ml. High level of gene induction was demonstrated and was specific to the tat-responsive promoter. The co-delivery results reported (chapter 5) show much higher reporter gene activity than shown with tat protein delivery without a co-delivered DNA (chapter 4). This is most probably due to the use of A431LTRCAT cells, which may contain only a few integrated copies of tat-responsive sequence, for protein delivery without providing extra copies of DNA with the

delivered tat protein. On the other hand, the co-delivery system may deliver large quantities of DNA copies by transient transfection of cells with DNA complexed to tat protein and liposomes. It is expected to obtain higher transfection activity by delivering DNA with protein than delivery of protein alone.

2. Conclusions and future prospects

The studies presented in this dissertation demonstrate the flexibility of cationic liposome mediated delivery with respect to the type of macromolecule delivered. An additional level of flexibility was also demonstrated by co-delivery of two different types of macromolecules, i.e. DNA and protein. Presumably, RNA delivery by cationic liposomes, alone or in combinations with other macromolecules such as protein, can also be demonstrated, although not in this dissertation, and in fact has been in more than one report (8,9). This level of flexibility, combined with the simplified cationic liposome preparation procedure, the simplicity of the delivery protocol, the high reproducibility of delivery efficiency, and the possible application with most cell types and with all available cationic liposome formulations, place cationic liposomes as the carrier of choice for delivery of macromolecules in general. This conclusion, however, can not overlook the need for some improvements in the present cationic liposome formulations to allow further flexibility and higher efficiency in their design.

One area of improvment could be the targeting of cationic liposomes to specific organs and tissues. This targeting may be dependent on a targetable liposome carrier or on tissue-specific DNA control elements encoded in the delivered transgene sequence (10). The concept of targetable cationic liposomes have been recently addressed by Huang and colleagues using cell surface-specific antibodies chemically conjugated to a cationic polymer which allows DNA complexing (11,12). This DNA/antibody-polymer complex was additionally complexed to DC-Chol cationic liposomes to allow cellular uptake of the ternary complex. The complex was bound to cells specifically via the antibodies recognizing specific cell surface receptors. Gene expression was demonstrated in the target cells in a tissue culture system (12). Another targeting system could be antibodies directly conjugated to cationic liposomes such that DNA is complexed to a ternary conjugate without an additional subsequent complexing. This type of conjugate is yet to be developed.

Enhanced condensation of DNA by cationic liposomes is another area of improvement that cationic liposome design could benefit from. This may be possible through the inclusion of multiple cationic charges on the cationic lipid which are free to interact with DNA without the steric hinderance and rigidity that are probably imposed on cationic liposomes containing a cationic lipid with a single cationic charge per molecule (3,4,6). Lipofectamine™ is a commercial cationic liposome formulation with a cationic lipid carrying multiple cationic charges on each head group

which may be capable of efficiently condensing the complexed DNA. Our experience with the Lipofectamine™ in transfection experiments show that DNA/liposome complexes are very small and are barely visible by light microscopy. On the other hand, complexes containing cationic liposomes made with cationic cholesterol, which contain a single cationic charge per lipid molecule, can be seen as large aggregates of complexes observed with a light microscope. Lipofectamine™ is known to have superior transfection activity to all other cationic liposome formulations in most cultured cells. However, *in vivo* data have demonstrated the lack of transfection activity with Lipofectamine™ in contrast to efficient transfection activity with Lipofectin™ and DC-Chol liposome (J. Jessee, personal communications). Apparently, the condensed DNA/Lipofectamine complexes suffer from additional inhibitory factors when they are used *in vivo*. This and other problems of cationic liposome mediated delivery of macromolecules will be the subject of active investigations in the future.

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