To the Graduate Council:

I am submitting herewith a dissertation written by Chen-Yen Wang entitled "Cytoplasmic Delivery of Macromolecules via pH-Sensitive Liposomes." I have examined the final 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 Biochemistry.

Leaf Huang

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We have read this dissertation
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CYTOPLASMIC DELIVERY OF MACROMOLECULES VIA pH-SENSITIVE LIPOSOMES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Chen-Yen Wang

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ABSTRACT

In this dissertation, it has been demonstrated that liposomes composed of dioleoylphosphatidylethanolamine, cholesterol and oleic acid (4:4:2) can effectively deliver the entrapped calcein to the cytoplasm of carrot protoplasts. These experiments were performed by using a fluorescent dye, calcein, as a marker of cytoplasmic delivery. The optimal ratio for uptake of pH-sensitive liposomes by protoplasts was 0.45μmole phospholipid per 6 X 10^5 protoplasts. Addition of 11% PEG 6000 was essential for the optimal uptake of liposomes by plant protoplasts. The superiority of the new liposome composition over the conventional compositions was correlated with its high sensitivity to acidic environment.

These pH-sensitive liposomes were also used to deliver plasmid DNA to target mammalian cells in tissue culture or in a mouse model. In these studies, a cAMP regulatory sequence on the upstream of the rat PEPCK gene was used to control the expression of an exogenous gene in the plasmid. For tissue culture cells, HSV- thymidine kinase gene was delivered to mouse Ltk^- cells so that growth of the cells in the HAT medium was completely cAMP dependent. Maximal short-term expression of the viral thymidine kinase gene was obtained from the liposomes prepared with a pH-sensitive lipid composition, i.e. DOPE:chol:OA (4:4:2), and coated with monoclonal antibody specific to the target cells. The transformation efficiency mediated by the pH-sensitive immunoliposomes was significantly higher than that of the conventional

calcium phosphate precipitation method. The long-term transformation efficiency via these liposomes was about 9%.

RDM-4 lymphoma cells grown in the peritoneal cavity of the nude mouse were used as a target for the immunoliposomes to deliver the bacterial chloramphenicol acetyltransferase (CAT) gene. Intraperitoneally injected liposomes, although not exclusively accumulated in the target cells, mediated a significant expression of the CAT activity in the target cells. Organs enriched with reticuloendothelial cells such as spleen did not show gene expression, despite significant uptake of the liposomes. Again, the pH-sensitive immunoliposomes mediated transformation gave higher CAT activity than the pH-insensitive ones did. The presence of the targeting antibody was also necessary for maximal gene delivery. The implications of this result in DNA mediated cancer chemotherapy and genetic therapy are discussed.

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ABBREVIATIONS

8BrC:	8-bromoadenosine 3':5'-cyclic monophosphate
BT:	N^6-2'-O-dibutyryladenosine 3'-5'-cyclic monophosphate plus theophylline
CAT:	chloramphenicol acetyltransferase
Chol:	cholesterol
CT:	cholera toxin
D:	dicetylphosphate
DOPE:	dioleoylphosphatidylethanolamine
DOPC:	dioleoylphosphatidylcholine
HAT:	Hypoxanthine + Aminopterin + Thymidine
HSV:	herpes simplex virus
LUV:	large unilamellar vesicles
OA:	oleic acid
PEG:	polyethylene glycol
PEPCK:	phosphoenolpyruvate carboxykinase
PGE:	prostaglandin E1
PS:	phosphatidylserine
SA:	stearylamine
SUV:	small unilamellar vesicles
TK:	thymidine kinase
Xan:	3-isobutyl-1-methylxanthine
PSL:	pH-sensitive liposomes
PSIL:	pH-sensitive immunoliposomes
PIL:	pH-insensitive liposomes
PIIL:	pH-insensitive immunoliposomes

CHAPTER I

INTRODUCTION

Liposomes have become an important carrier of biological molecules such as enzymes (1) and nucleic acids (2,3). Liposomes consist of one or more concentric lipid bilayers surrounding aqueous compartments making them effective tools for delivery of biological molecules (4). It has been demonstrated that liposomes are nontoxic and biodegradable and do not cause an immune response.

In the last decade, liposome-mediated DNA transfer has been achieved in plant protoplasts (5), E. coli (6), mammalian and avian cells in culture (7,8,9). Previous liposomes were composed of phosphatidylcholine, phosphatidylglycerol (10,11), phophatidylserine (12), stearylamine (13,14), or their combinations (15,16). All of these liposomes are able to deliver encapsulated DNA by fusion or by endocytosis. However, the transfection efficiencies of the exogenous DNA were in the range of 10^{-5}, indicating that one cell receives exogenous gene in 10^{5} treated cells (17).

Recently, pH-sensitive liposomes which become unstable and release their contents at low pH have been developed by Connor in our laboratory and in Papahadjopoulos' laboratory (18,19). We demonstrated that pH-sensitive liposomes composed of phosphatidylethanolamine (PE) and oleic acid (OA) or palmitoylhomocysteine (PHC) can deliver entrapped contents into the cytoplasm of plant protoplasts (20), mouse L-929 cells (21) and thymidine kinase defective mouse (Ltk^{-}) cells

(unpublished result). The thymidine kinase gene which was delivered into the Ltk^- cells expressed thymidine kinase with the transformation efficiency in the range of 10^{-2} to 10^{-1} transformant/treated cell.

The ability of phosphatidylethanolamine (PE) to form non-bilayer structure under physiological conditions (22,23) is essential for the design of pH-sensitive liposomes. PE molecules prefer the hexagonal phase because the size of their lipid head group is small compared to size of the acyl chains. Thus, increased interaction between adjacent lipid head groups (24) with intermolecular hydrogen bonding (25,26) is allowed. It is possible to form a stable PE-containing liposome by the addition of a second amphipathic component. Palmitoylhomocysteine and oleic acid meet the requirements as the second component. They contain a negative charge on the head group at neutral pH which reduces the intermolecular interactions between PE by providing an electrostatic repulsion. When the pH is lowered, the negative charge is neutralized and the PE reverts to the hexagonal phase. Thus, the bilayer vesicle releases its contents. In addition, liposomes enriched with PE tend to fuse with other membranes (27).

The PE-containing liposomes deliver biologically functional molecules by endocytosis which is presumably followed by an acid-induced fusion between endosomal membrane and liposome membrane (87). This mechanism provides a novel pathway for the cytoplasmic delivery of the entrapped contents other than the lysosomal pathway. To enhance the efficiency of delivery, monoclonal antibodies were used to increase specific binding of liposomes to target cells (28). In the experiments

described in this dissertation, i.e., transformation of mouse L cells and RDM4 cells, the anti H-2k^k antibodies specific for H-2K^k antigen expressed on the surface of L and RDM-4 cells were derivatized with palmitic acid by reacting with the N-hydroxysuccinimide ester of palmitic acid (29). The hydrophobic anchor allowed the insertion of antibodies into the lipid bilayer without compromising their ability to interact with antigen. In these experiments, plasmid DNA was successfully delivered into mouse L cells in vitro or RDM4 cells in vivo. These results provide new evidence for the potential use of pH-sensitive immunoliposomes as a vector for gene therapy.

Gene Therapy

The central theme of gene therapy is to insert a copy of a normal gene into a gene-defective cell. Hopefully, this inserted gene can express in the host at the level in need. Based on these requirements of gene transfer therapy, genetic engineers have been looking for a vector to carry a human gene into a human chromosome. Gene therapy has been applied in fruit flies (30) and in mice (31). The first clinical application of gene therapy was performed, although without success, in a child with severe combined immune deficiency (SCID) in 1968 (32). Some diseases are caused by monogenic defect such as diseases shown in Table 1. For those diseases, gene therapy can be carried out by either repairing the defective gene in situ, or inserting a functional gene elsewhere in the genome.

Table 1. Genetic diseases as potential targets of gene therapy.*

Abnormal gene	Disease	Primary target organ
β-globin	sickle cell disease	blood
β-globin	β-thalassemia	blood
α-globin	α-thalassemia	blood
Pyruvate kinase	PK deficiency	blood
Glucocerebrosidase	Gaucher's disease	blood, bone
Adenosine deaminase	Immunodeficiency	liver, spleen, immune system
Hypoxanthine-guanine phosphoribosyl transferase	Lesch Nyhan Syndrome	nervous system
Factor VIII	Hemophilia	blood
Factor IX	Christmas Disease	blood
Phenylalanine hydroxylase	Phenylketonuria	nervous system

*Reference: Martin, J. Chine, M.D. (1984) Molecular and Cellular Biochemistry 59, 3-10.

Since functions of most enzymes in vivo are not limited at maximum concentration, replacement of enzymes by gene therapy does not have to achieve normal enzyme concentration. However, it is important to produce these compounds in the necessary amounts in response to the right physiological stimuli. In human gene therapy, there are five preconditions such as a high-titer vector; highly efficient transfection of target cells; stable integration into the genome; adequate expression; and safety for the patient and others. From the clinical point of view, the first logical application of gene therapy in humans will be in the genetic diseases of the blood. For the moment, gene therapy of genetic disorders has been carried out only in the bone marrow (Figure 1) (34).

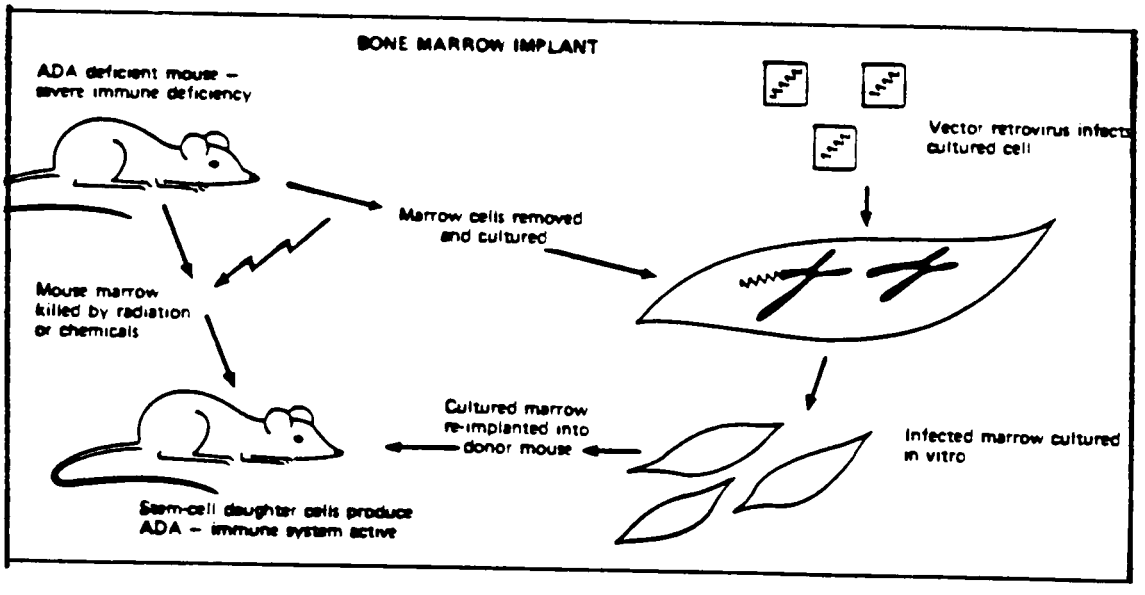


Figure 1. Bone Marrow Implant.

Ref. McCormick D. (1985) Biotech. 3, 689-693.

Since erythrocytes and leukocytes are derived from marrow stem cells, the genetic defects in the blood, bone or immune system are possibly cured by gene therapy of stem cells.

Plant Cell Transformation

Advances in recombinant DNA technology have allowed the transfer, integration, and expression of foreign genes into either single cells (protoplasts) or intact tissues. For a successful transformation of protoplasts by foreign DNA, it is necessary to reach a balance between maximizing the transformation frequency and maintaining an acceptable level of protoplast viability (105). To date, at least 7 different techniques have been described for introducing genes into plant cells (Table 2) (34-47). Although each method has certain advantages, their practical application is unlikely because of low efficiency (liposomes), or introduction of undesirable genetic information (agrobacterium). Therefore, we developed an improved system by which exogenous DNA can be delivered to the cytoplasm of plant protoplasts with higher transformation efficiency.

Delivery Systems

One of the major problems in chemotherapy is the targeting of drugs to restricted sites and specific cells. It is therefore important to develop target-specific drug carriers. Numerous approaches to the problem of controlled drug delivery have been developed in the last decade. These approaches include polymeric

Table 2. Methods of insertion of new genetic information into plant cells.

Technique	Efficiency (transformant/treated cells)	Characteristics	Reference
1. Infection (Co-cultivation) of protoplast-derived cells with Agrobacterium.	$>10^{-1}$	less labor regeneration difficult	35,42
2. Chemically stimulated uptake of isolated DNA into protoplasts.	$\sim10^{-5}$	less contamination time-consuming	36,37
3. Fusion of bacterial spheroplasts with plant protoplasts.	$\sim10^{-5}$	overcome host range limitation	38,39
4. Liposome-encapsulated delivery of DNA	$\sim10^{-5}$	regeneration well time consuming for preparation of liposomes	40,41
5. Tumor-inducing (Ti)	$\sim10^{-5}$	broad host range time-consuming for isolation of Ti plasmid	43
6. *E. coli* plasmid	$\sim10^{-6}$	broad host range time-consuming for isolation of plasmid	44
7. Electroporation	$\sim3X10^{-3}$	high transformation efficiency high degree of protoplast damage	45,46,47

sustained release systems (48,49), liposomal drug carriers (50,51) and antibody drug carriers (52,53). Among these drug carriers, liposomes have received considerable attention as carriers of antitumor agents, antibiotics and hormones because they are non-toxic and biodegradable (54). In most cases the selectivity of these carriers can be enhanced by the coupling of specific ligands, such as haptens (55) or monoclonal antibodies (56).

Liposomes have been applied to enzyme delivery since Purdon in 1871 used certain proteolytic enzymes to inhibit bacterial infection during surgery (57,58). The use of L-asparaginase in the treatment of acute lymphocytic leukemia (ALL) was first conceived by Kidd in 1953 (59,60). There were many studies involving the delivery of enzymes or proteins. However, there are several important limitations against the use of enzymes as therapeutic agents in medicine (61). Three major ones are: 1) until recently many enzymes, especially in pure form, were not readily available. 2) enzymes as foreign proteins are highly immunogenic. 3) they are degraded quickly. The development of modern techniques in recombinant DNA for the production of animal or human gene products may alleviate the first limitation, but the immune response is difficult to avoid. Correction of protein defects by transferring a gene encoding normal intracellular proteins is therefore an attractive alternative since it can potentially avoid both limitations.

Recently, numerous studies have focused on the development of genetic transformation systems using plasmid vectors. Those techniques

have been developed to introduce biologically active DNA into eukaryotic cells, including (i) the chemically stimulated uptake of naked DNA or DNA precipitates with the aid of endocytosis/fusion enhancers such as PEG, dextran and Ca^{2+}-phosphate (62); (ii) viral vectors such as retroviruses (63) or adenovirus (64) mediated gene transfer; (iii) fusion/endocytosis of plasmid-containing bacterial spheroplasts or liposomes with animal cells or plant protoplasts (3,6,65); and (iv) the physical introduction of naked DNA by the techniques of microinjection (66) and electroporation (67,68). Martin (33) and Anderson (17) have reviewed the current methods of gene transfer and gave prospects for human gene therapy (Table 3).

Among these methods, the retrovirus system has received considerable attention for gene insertion into mammalian cells because of high transformation efficiency (79,80). A new recombinant technique is able to eliminate the critical encapsulation signal of viruses (83). Although the viral oncogene can be eliminated, the random insertion of retrovirus in the host genome is still an unresolved hazard. People also worry about the possibility of recombination between vectors and endogenous viral sequences in human cells. To improve the transfer efficiency and safety, pH-sensitive immunoliposomes have therefore been developed and examined for the delivery of biologically functional molecules.

Table 3. Methods of insertion of new genetic information into animal cells.

Technique	Efficiency[+]	Reference
1. Cell fusion	$<10^{-6}$	69
2. Chromosome-mediated	$<10^{-6}$	70,71
3. Microcell-mediated gene transfer	$\leq 10^{-6}$	72,73
4. Liposome carrier of DNA	$\cong 2X10^{-4}$	9
5. Spheroplast fusion	0.1 to 0.3%	74,75
6. DNA-mediated gene transfer	10^{-3} to 10^{-7}	76,77
7. Direct injection of DNA	High: 1 to 10%	78
8. Infection with recombinant RNA viruses	High: 10 to 100%	64,79,80
9. Transfection with recombinant DNA viruses	Very high $\cong$ 100%	81,82,83

[*] Reconstructed from Martin (33) and Anderson (17).

[+] Units are transformants per treated cells.

Cytoplasm Delivery of pH-sensitive Liposomes

It has been demonstrated that PE liposomes can be stabilized with acylated amino acids such as palmitoylhomocysteine (PHC) (18) or fatty acids such as arachidonic acid (Kanda and Huang, unpublished data), oleic acid (19,84), palmitic acid (84), or a cholesterol derivative cholesteryl hemisuccinate (85) and become fusion active at a pH of below 6.5. Using this system, pH-sensitive liposomes deliver their contents into the cytoplasm instead of the lysosomes of the target cells (21). Calcein, Ara-C or FITC-dextran have been delivered to the cytoplasm of L-929 (86) and CV-1 cells (87). In addition, these liposomes were used to deliver diphtheria toxin A fragment to toxin-resistant murine cells (88).

This dissertation will document the application of the pH-sensitive liposomes for the delivery of intact DNA into animal cells *in vitro* and in an animal model system *in vivo*. To enhance the specific binding of pH-sensitive liposome to target cells, monoclonal antibodies were incorporated in liposomal bilayer.

Non-targeted Delivery by Liposomes to Plant Protoplasts

Since there is no available antibody for plant protoplasts, polyvinyl alcohol (PVA), polyethylene glycol (PEG), poly-L-ornithine (PLO) and high pH/Ca^{2+} solutions are usually used to enhance absorption of liposomes onto protoplast surface (12,40). Optimal conditions for the uptake of nucleic acids into plant protoplasts have been summarized by Freeman et al. (40). Generally 10-50% of supplied DNA becomes

protoplast-associated. Optimum delivery of plasmid DNA to protoplasts is achieved using high concentrations of PEG of high molecular weight (e.g. PEG- 6000). Liposome mediated delivery may be initiated by PEG enhanced adsorption of liposomes to the protoplast surface or, as reported by Fukunaga (12), endocytosis of liposomes by protoplast as induced by PEG.

CHAPTER II

GENERAL PROCEDURES, PREPARATION AND CHARACTERIZATION OF pH-SENSITIVE LIPOSOMES AND IMMUNOLIPOSOMES

Materials

Dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC) and brain phosphatidylserine (PS) were purchased from Avanti (Birmingham, AL). Oleic acid, cholesterol, dicetylphosphate, calcein, lysophosphatidylcholine, stearylamine and n-octylglucoside were obtained from Sigma. SM-2 beads were purchased from Bio-Rad Laboratories.

Liposome Preparation

Large unilamellar vesicles (LUV) were prepared according to Philippot, et al. (89) with modifications. Lipid films of various compositions were formed under a nitrogen stream and suspended in 10 mM Hepes 150 mM NaCl, 1mM EGTA and 183.3 mM D-glucose. Hexadecyl-^{3}H-cholestanyl ether was included in the lipid mixture to monitor the lipid (90). The lipid suspension was sonicated with a bath sonicator (Laboratory Supplies, Hicksville, New York) until the small unilamellar vesicles (SUV) were formed and the pH was adjusted to 8. After the small unilamellar vesicles (SUV) were formed by sonication, octylglucoside and calcein or DNA were added and the mixture was transferred to a dialysis bag. Octylglucoside was chosen because it has a high critical micellar concentration, which facilitates rapid removal from the mixed

micelle complex (91). The molar ratio of octylglucoside to lipid used in this study was 10 because optimal condition for the production of vesicles occurred when the detergent/lipid ratio was 10:1 (92). The concentration of calcein was 23.5mM in the mixture of a final volume of 0.34 ml. When input DNA was 150 μg, the amount of entrapped DNA per liposome reached maximum. The mixture was dialyzed against 100 ml of Tris buffer (pH 8.0), 1 mM EDTA, 150 mM NaCl, 183.3 mM D-glucose, 23.5 mM calcein and 1 g washed SM-2 beads (93) for 24 hours. Buffers for liposomes used in animal cells did not contain glucose. Then the liposomes were extruded through a polycarbonate filter of 0.2 μm pore diameter (Nucleopore Corp. CA) to form vesicles of a narrow size distribution. Subsequently, the liposomes were separated from free calcein or DNA using a column of autoclaved Sepharose CL-2B.

Immunoliposome Preparation

Anti-H2K^k antibody, which specifically bound to H-2K^k antigen on the surface of mouse L cells and RDM-4 cells, was isolated from ascites fluid generated by the mouse hybridoma 11-4.1 (29). The ascites fluid was diluted with 0.5M potassium phosphate buffered saline (PBS) pH 7.2, making the final buffer concentration 0.1 M in potassium phosphate and was loaded onto a protein A-Sepharose (Pharmacia, N.J.) column. After washing the column with 0.1 M phosphate buffered saline pH 7.4, the antibody was eluted with 0.05 M glycine pH 4.0. The antibody was dialyzed overnight against 4 L phosphate buffered saline containing 1 mM EGTA and 0.2% NaN_3, pH 8.0. Antibody iodination was carried out

using chloramine T (29). Free iodine was separated from protein by centrifugation through a 1 ml Sephadex G-25 column.

To provide antibodies with hydrophobic anchors by which antibodies could insert into lipid bilayer, the iodinated antibody was derivatized with the N-hydroxysuccinimide ester of palmitic acid (NHSP) at molar ratio 10 to 1 (29) and was concentrated by ultrafiltration (Amicon). After concentration, the palmitoyl antibody solution was dialyzed against 2 L 0.15% deoxycholate in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2 PO_4, 1.1 mM Na_2HPO_4, pH 8.0) for 24 hours (29). The palmitoyl antibody was then incorporated into the liposomal bilayers by the detergent-dialysis method described above (20,89). The weight ratio of antibody to phospholipid was 1 to 25. In order to efficiently entrap DNA within the lipid vesicles, octylglucoside was first removed slowly by not stirring the dialysis buffer for overnight and then dialysed with stirring. After 24 hours, fresh dialysis buffer and SM-2 beads which absorbed octylglucoside were added.

Size Distribution of Liposomes

Liposomes were negatively stained with 0.5% uranyl acetate and viewed in a Hitachi 600 electron microscope. Pictures were taken and photographically enlarged. Most of the liposomes were unilamellar (Fig. 2). The size distribution of liposomes was determined by measuring more than 200 liposomes from micrographs. Results showed that DOPE/chol/OA (see abbreviations) liposomes were larger than DOPC/chol/OA liposomes. The mean diameters of DOPE/chol/OA liposomes

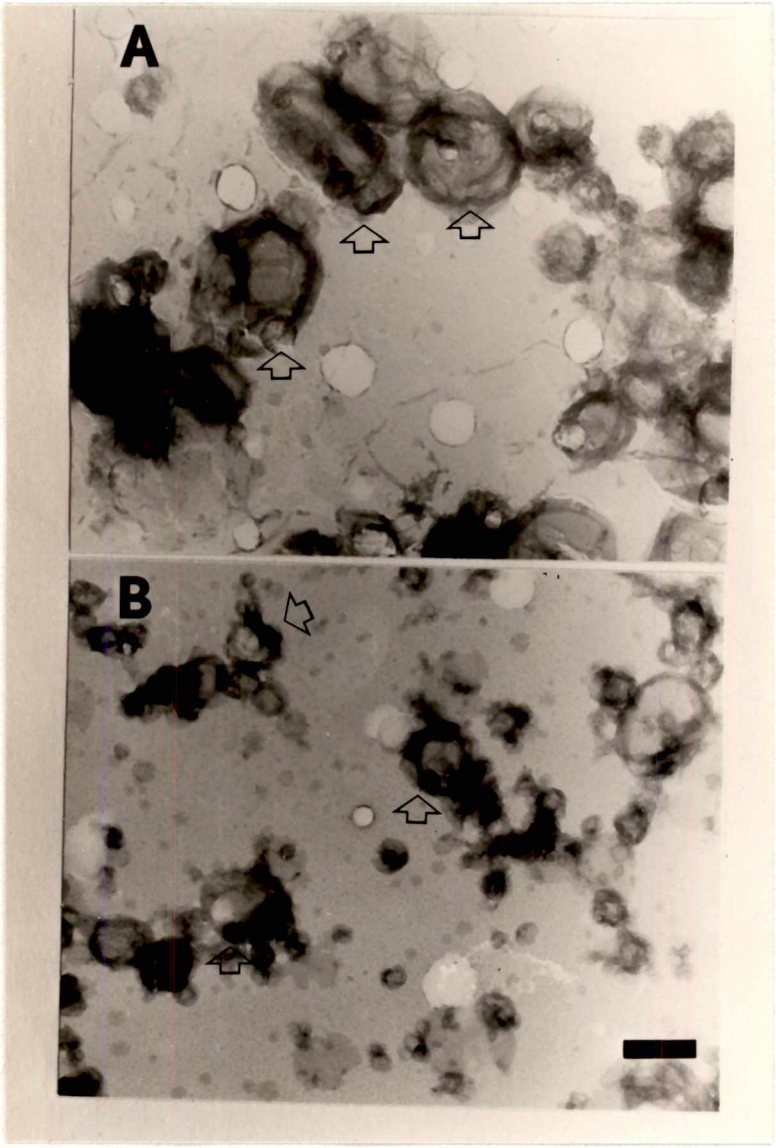


Figure 2. Electron micrography of DOPE/chol/OA liposomes (A) and DOPC/chol/OA liposomes (B). Liposomes were extruded through polycarbonate filters of 0.2 μm pore size and stained with 0.5% uranyl acetate. Bar is 0.2 μm. Arrows represent liposomes.

and DOPC/chol/OA liposomes were 0.292 ± 0.083 μm and 0.176 ± 0.055 μm, respectively (Fig. 3). Although both types of liposome were extruded through polycarbonate filters with a pore size of 0.2 μm, most DOPE/chol/OA liposomes were larger than 0.2 μm. This phenomenon has been reported by May et al. (94). They extruded vesicles through filters of 100 nm pore-size and found that size of vesicles ranged from 40 nm to 400 nm.

Incorporation of Palmitoyl Antibody into Liposomes

To determine if the palmitoyl antibody was incorporated into liposomes, the association of palmitoyl antibody with the liposomes was examined by buoyant binding in a sucrose density gradient from 5% to 65% sucrose. The peaks for liposomes and incorporated palmitoyl antibody coincided while the unassociated palmitoyl antibody appeared at the bottom of gradient (data were not shown). The number of incorporated antibody molecules per liposome was calculated on the basis of liposome size according to Enoch and Strittmatter (95). Most DOPC/chol/OA liposomes contained approximately 48 molecules of antibodies per liposome while most DOPE/Chol/OA liposomes contained 106 molecules of antibodies per liposome (Table 4). We assumed that half of the antibody molecules are on the outer surface and the other half on the inner surface of the unilamellar liposomes.

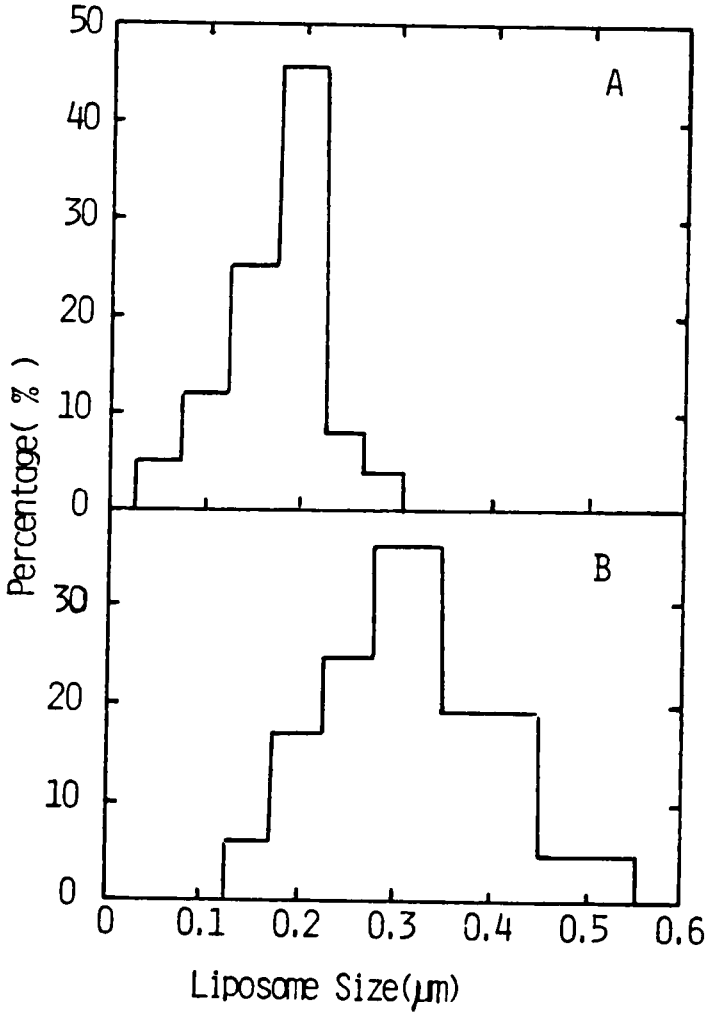


Figure 3. The size distribution of liposomes. These liposomes were extruded through polycarbonate filters of 0.2 μm pore size.

Table 4. Relationship between the size of unilamellar liposome and the number of phospholipid molecules per liposome*

liposome diameter (μm)	phospholipid molecules/liposome	antibodies/liposome
0.1	8.6×10^4	12
0.2	3.6×10^5	48
0.25	5.5×10^5	74
0.3	7.7×10^5	106
0.4	11.0×10^5	151
0.5	14.7×10^5	200

*Calculated using method of Enoch and Strittmatter (95).

pH-Sensitivity of Liposomes

To monitor the pH-sensitivity of liposomes, 23.5 mM calcein was entrapped in the liposomes. Fluorescence of calcein at this concentration is approximately 26% quenched. Fluorescence is significantly enhanced when calcein is released from the liposomes (96). Measurements were carried out in phosphate buffered saline (pH 8.0) with continuous stirring which did not affect fluorescence intensity. Fluorescence was measured using a Perkin-Elmer LS5 spectrofluorometer with λ_{ex} = 490 nm and λ_{em} = 520 nm. Serial concentrations of HCl and NaOH were prepared. After addition of the liposomes to the cuvette, the pH of the suspension was titrated to the desired value by adding 10

μl of appropriate concentration of HCl. After 5 minutes incubation the pH of the suspension was returned to the original value by adding equivalent amount of NaOH. 0.15% deoxycholate (DOC) was then added to completely release calcein from the liposomes.

The percent leakage of liposomes was calculated from the dequenching of calcein according to the following equation:

$$\% \text{ leakage} = \frac{F_f - F_o}{F_t - F_o} \times 100 \qquad (1)$$

where F_o stands for the fluorescence intensity of liposomes in phosphate buffered saline. F_f stands for the fluorescence intensity of the liposome suspension after acid treatment. F_t stands for the fluorescence intensity of liposome suspension after adding deoxycholate. Deoxycholate alone in phosphate buffered saline did not interfere with fluorescence intensity.

The compositions of liposomes used in this study are shown in Table 5.

As shown in Figure 4, all liposomes except those composed of DOPC or DOPC/stearylamine showed acid sensitivity, but DOPE/chol/OA liposomes showed the highest sensitivity in the seven compositions tested. The entrapped calcein was released dramatically when the pH of solution was dropped to 6.0 or below. The pH for half maximal dye release lies at about 6.0. The maximum dye release was about 85% of the total entrapped calcein. When oleic acid was replaced with phosphatidylserine (PS), the release of entrapped calcein decreased significantly.

Furthermore, PS containing vesicles required a more acidic pH for release. The same phenomena took place when the DOPE was replaced by DOPC.

Table 5. Liposome Compositions Used for the Study of pH-Sensitivity.

Liposome Composition	Molar Ratio	Ref.
DOPE:cholesterol:oleic acid	4:4:2	this study
DOPE:cholesterol:PS	4:4:2	"
DOPC:cholesterol:oleic acid	4:4:2	"
DOPC:oleic acid	8:2	"
DOPC		11
DOPC:Stearylamine	10:1 (w/w)	97
DOPC:dicetylphosphate:lyso PC	8:2:0.4	98

DOPC/dicetylphosphate/lysoPC and DOPC/stearylamine liposomes have been developed to deliver exogenous DNA to the cytoplasm/nucleus of plant protoplasts (97,98). However, the pH sensitivity of DOPC/ dicetylphosphate/lysoPC liposomes is much less than that of DOPE/chol/OA liposomes. The maximum dye release was only 20% of the entrapped calcein, about one-fourth of the maximum dye released from the DOPE/chol/OA liposomes. There was no evidence of entrapped calcein being released from the DOPC/stearylamine and DOPC liposomes at pH

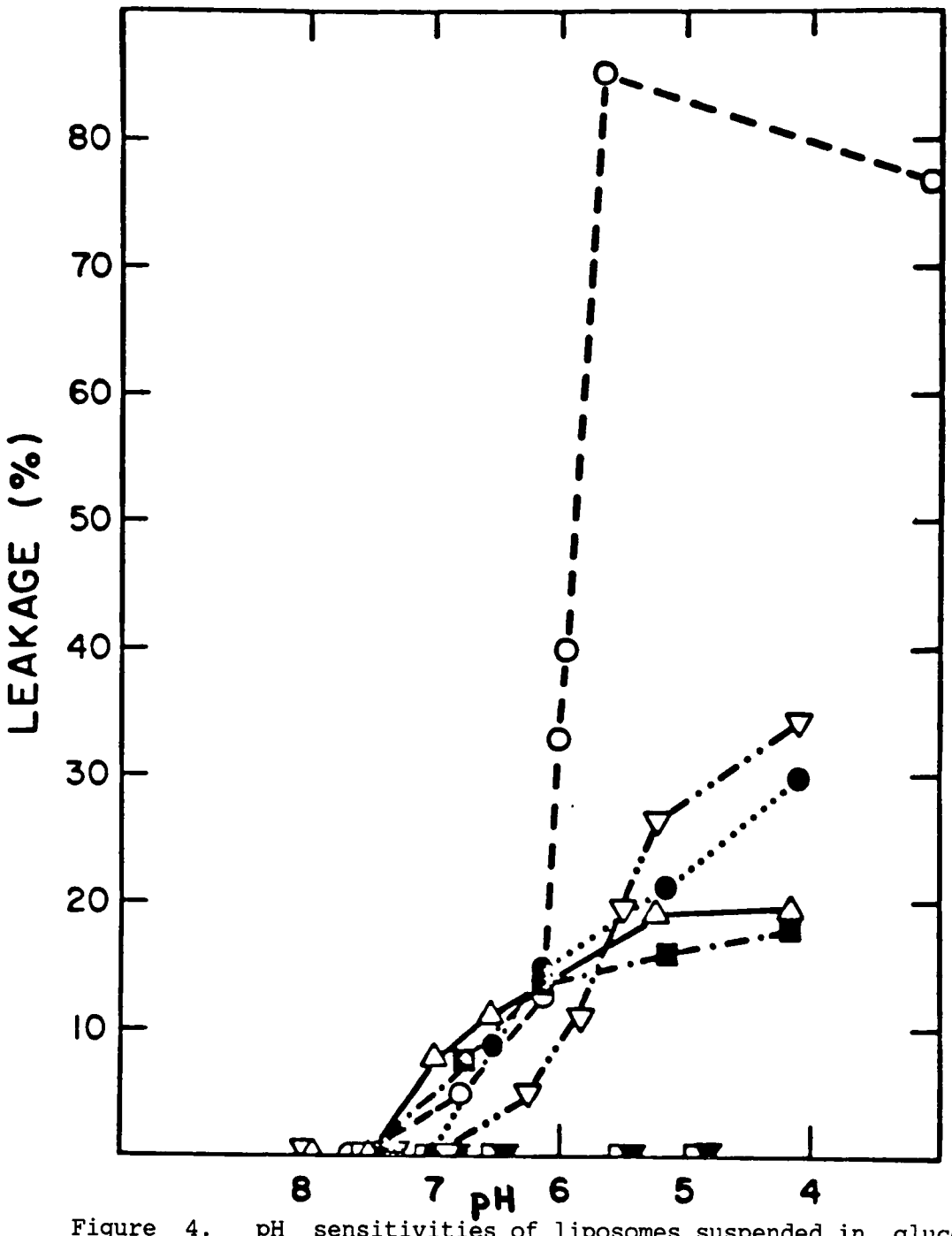


Figure 4. pH sensitivities of liposomes suspended in glucose-containing phosphate buffer as mentioned in Methods. The leakage percentage was calculated according to equation 1 and indicated the degree of calcein release from liposomes at equilibrium state at indicated pHs. DOPE/chol/OA liposomes (○-----○); DOPC/ dicetylphosphate/lysoPC (△ _____ △); DOPC/chol/OA (■ __._____ ■); DOPE/chol/PS (▽ _____.._____▽); DOPC/OA (● ---- ●); and DOPC (□ _____□) and DOPC/stearylamine (▼_____ ▼).

between 7.0 and 5.0. Although the calcein release of DOPC/OA liposomes was slightly higher than that of DOPC/chol/OA at pH 5.5, the maximum release of calcein from the DOPC/OA liposomes was only about 30% of that from the DOPE/chol/OA liposomes. It is clear that DOPE and oleic acid are both essential for maximal pH sensitivity of liposomes.

For targeted liposomal delivery of DNA to animal cells, monoclonal antibody, anti H-2K^k against antigen on the surface of mouse L and RDM-4 cells, was incorporated to the liposomes. The presence of the antibody molecules on the liposome surface did not significantly change the pH sensitivity of the liposomes (data not shown).

CHAPTER III

IMPROVED CYTOPLASMIC DELIVERY OF CALCEIN TO PLANT PROTOPLASTS VIA pH-SENSITIVE LIPOSOMES

Abstract

We demonstrated that the liposomes composed of DOPE/cholesterol/oleic acid (4:4:2) are capable of delivering their contents into the cytoplasm of carrot protoplasts. This was shown by using a soluble fluorescent dye, calcein, as the liposome-entrapped marker. We found that calcein fluorescence was evenly distributed in the cytoplasm of wild carrot protoplasts after the incubation of protoplasts with liposomes in the presence of PEG. At 0.45 μ mole phospholipid per $6X10^5$ protoplasts, uptake of pH-sensitive liposomes by protoplasts was much more efficient than the uptake of pH-insensitive liposomes.

Introduction

Liposomes have been used as a vehicle to deliver biologically active molecules into animal cells (3,11); however, the use of liposomes for delivery to higher plant cells is still being developed. Recently, liposomes have been used to deliver RNA or DNA to plant protoplasts (40,41). These studies used either the Ca-EDTA chelation method or the modified reverse-phase evaporation method (REV) to produce liposomes (40,99). The frequencies of transformation with liposome-mediated nucleic acid transfer were fairly low (10^{-6}~10^{-5} transformant/treated cells) when liposomes were prepared by either Ca-

EDTA chelation or the REV method. It is probably caused by low entrapment efficiency. Philippot et al. reported that entrapment efficiency was about 40% of input DNA using detergent dialysis method (89). In this dissertation, therefore, detergent dialysis method was used to prepare DNA entrapped liposomes.

To improve the delivery efficiency of macromolecules via liposome, the mechanisms of interaction between protoplasts and liposomes have been investigated. Fusion with plasmallema (97,100) and endocytosis of liposomes by protoplasts have both been demonstrated. It is the demonstration of endocytic ability which prompted us to look into the possible use of pH-sensitive liposomes for an improved liposome-mediated delivery of calcein in higher plant protoplasts.

The characteristics of pH-sensitive liposomes (see Chapter 1) indicated that these liposomes encounter the acidic pH in endosomes and fuse with the endosomal membrane, thus releasing the entrapped contents into the cytoplasm (101). The present study is designed to test the ability of pH-sensitive liposomes to mediate cytoplasmic delivery to the higher plant protoplasts. We have used a water soluble fluorescent dye, calcein, as a convenient marker for observing cytoplasmic delivery. We have compared the delivery by pH-sensitive liposomes with that of several other pH-insensitive, conventional liposome compositions.

Materials and Methods

Materials

Cellulysin and macerase were obtained from Calbiochem, sorbitol from Sigma. Other materials were mentioned in Chapter II.

Maintenance of Carrot Cultures

Cell cultures of Dacus carota (wild carrot) were obtained from Dr. D. K. Dougall and maintained as suspension cultures in WCM-1 medium (102). Cell suspensions were subcultured by adding 5 ml of 14 day-old suspension to 20 ml of fresh WCM-1.

Isolation of Protoplasts

Preliminary experiments indicated that the best protoplast yield and viability was obtained with cell suspensions in early log phase, 4 days after subculture. Ten ml of a cell suspension from 4 day old cultures was added to a sterile 15 ml test tube and centrifuged at 100g for 5 min to pellet the cells. The medium was removed and cells resuspended in Digestion Solution (2.5% Cellulysin, 0.5% Macerase, 0.5M sorbital in Chupeau's salts plus Chupeau's minor elements (103)). The pH of the solution was adjusted to 5.8. The mixture was placed in a 15 ml petri dish, sealed with parafilm and placed on a rotary shaker at 40 RPM at room temperature in the dark for 15-17 hours. Following digestion, cells were pelleted and washed three times with Kao's K8P medium (104). Cells were resuspended in 10 ml of K8P medium and the number of protoplasts/ml were determined with a hemacytometer. Cells were resuspended in phosphate buffered saline immediately before incubation with liposomes.

Cell Incubations

Calcein-containing liposomes at a lipid concentration of 0.6 mM were incubated with 8 X 10^5 protoplasts/ml at room temperature for 1.5 hours. The above ratio of lipid to protoplast was chosen to achieve minimal structural damage of protoplasts on the basis of viability of protoplasts (see Table 6). Then PEG-6000 was added into the incubation medium to a final concentration of 11% as described (2). Incubation was carried out for an additional 1.5 hour at room temperature.

Following the second 1.5 hour incubation, the protoplasts were washed 3 times with phosphate buffered saline and then observed under a Leitz Orthoplan epiluminescence microscope equipped with an Orthomat-W camera. Photographs were taken with both phase contrast and fluorescent microscopy. All of the fluorescent pictures were taken using bandpass filter BP 470-490 with 2 min of film exposure time.

Protoplast viability

Following incubation with liposomes, cells were diluted with K8P medium to 3 X 10^5 cells/ml and cultured in sterile 48-well tissue culture clusters (Costar) with 0.2 ml of protoplasts per well. Protoplasts were cultured in the dark at 25°C for 7 days. After one week of culture, cells were stained with calcofluor white (105) and observed with a fluorescence microscope equipped with a DANS filter (BP 355-420 nm). Cell division and cell wall formation can be easily seen with this technique. Preliminary studies determined that the number of cells showing cell wall regeneration and one or more division plates do not increase after 7 days. The percent of dividing cells was recorded for each experiment at 7 days.

Table 6. Effect of liposome composition on the uptake of liposome by protoplasts, the dye release distribution and viability of protoplast.

Liposome Composition (Molar Ratio)	Percentage Protoplast Taken Up Dye	Dye Distribution	Percent Protoplasts Divided
DOPE/chol/OA (4:4:2)	89 ± 4	internal and homogeneous	79 ± 2
DOPE/chol/PS (4:4:2)	29 ± 4	surface aggregate	72 ± 1
DOPC/chol/OA (4:4:2)	84 ± 5	surface aggregate	78 ± 8
DOPC/OA (8:2)	81 ± 5	surface aggregate	73 ± 1
DOPC	77 ± 6	surface aggregate	75 ± 4
DOPC/SA* (10=1, w/w)	63 ± 2	surface aggregate	82 ± 2
DOPC/D*/lysoPC (8:2:0.4)	59 ± 4	surface aggregate and some internal	71 ± 2

Protoplast concentration was 8×10^5/ml, and lipid concentration was 0.6mM in protoplast-liposome incubation mixture. For the examination of viability, liposome incubated protoplasts (3×10^3 cells/ml) were cultured in sterile 48-well tissue culture plates in the dark at 25°C for 7 days. The cell wall formation was examined with calcofluor white stain.

*SA: Stearylamine

*D: dicetylphosphate

Incubation with chloroquine and methylamine

Chloroquine and methylamine are weak bases which can penetrate membranes of cells or organelles and raise the pH of the acidic organelles (106). In order to scrutinize the mechanism of the cytoplasmic delivery of calcein by a pH-sensitive liposome, protoplasts were preincubated with methylamine or chloroquine for 30 min before liposomes were added.

Results

Cell Incubation

We have examined the dependence of the cytoplasmic delivery of calcein on the lipid composition of the liposomes. After protoplasts were incubated with liposomes, the percentage of protoplasts which took up fluorescence was counted. There was no fluorescence in the protoplasts which had not been treated with calcein-entrapped liposomes (photograph not shown). For DOPE/chol/OA liposomes, 89% of protoplasts took up liposomes and calcein was released from liposomes into the protoplast cytoplasm. The dye distribution was homogenous within the protoplasts (Fig. 5). In some cells the fluorescence in the vacuole was brighter than out of the vacuole (Fig. 5 arrow). In contrast, there was no dye release into the protoplasts when the DOPE and OA were replaced by DOPC and PS, respectively, although liposomes aggregated on the surface showing localized bright fluorescence instead of homogenous distribution inside the protoplast (Fig. 6&7). It is worth noting that partial internal release of calcein took place when the protoplasts were incubated with DOPC/dicetylphosphate/lysoPC liposomes (Figure 8).

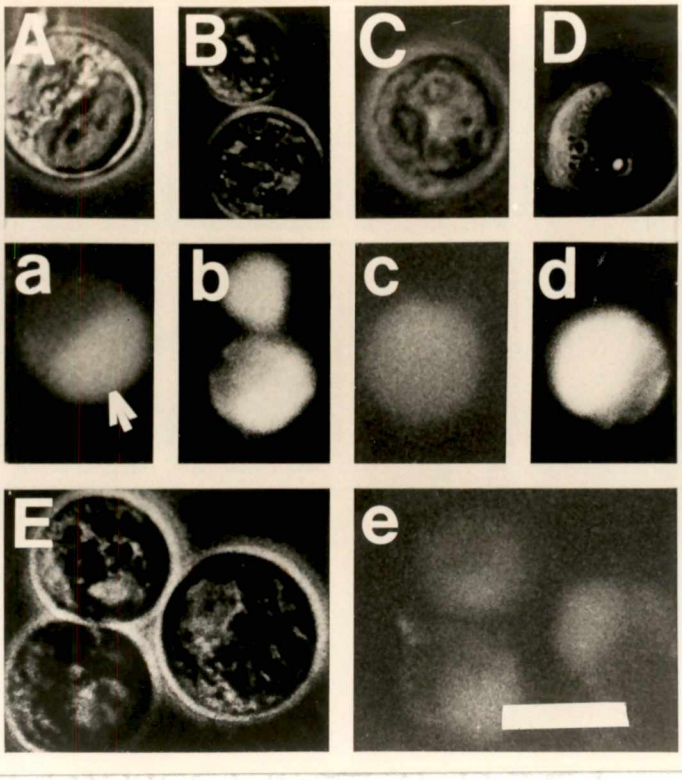


Figure 5. Fluorescence labeling of protoplasts by calcein containing pH-sensitive liposomes. Carrot protoplasts were incubated with calcein-containing liposomes composed of DOPE/chol/OA (4:4:2). A, B, C, D, E are phase contrast micrographs and a, b, c, d, e are corresponding fluorescence micrographs. Bar is 10 μm. Even distribution of fluorescence in the cytoplasm of protoplasts is evident. Arrow indicates a vacuole of the protoplast.

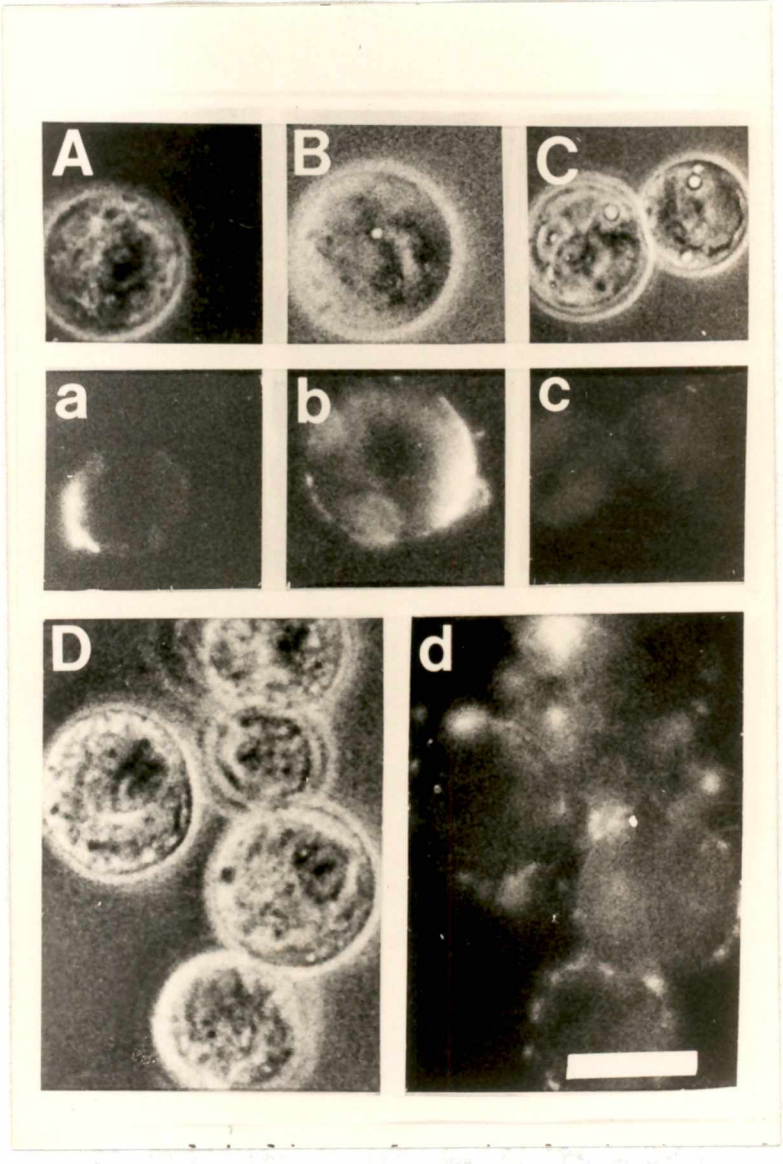


Figure 6. Fluorescence labeling of protoplasts by calcein-containing liposomes composed of DOPC/chol/OA (4:4:2) (Cc). A, B and C, are fluorescence micrographs. Bar is 10μm. Surface aggregation of fluorescence is seen.

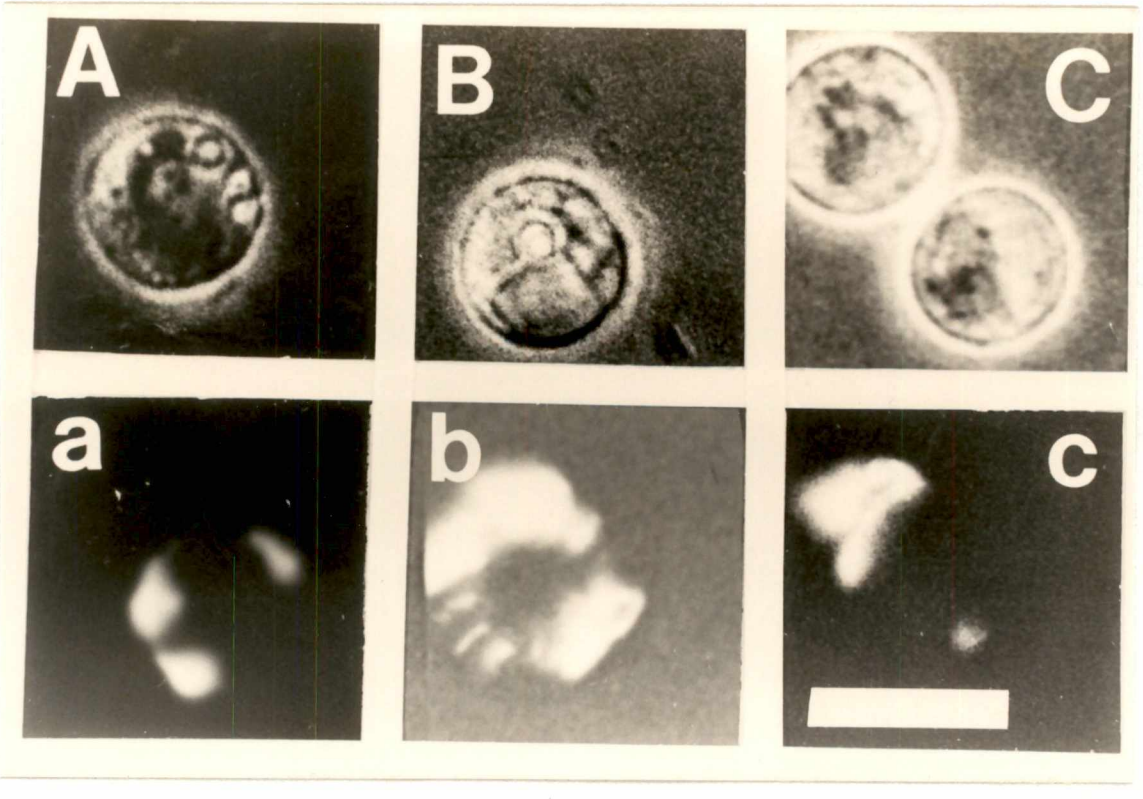


Figure 7. Fluorescence labeling of protoplasts by calcein-containing liposomes composed of DOPE/Chol/PS (4:4:2). A, B, C are phase contrast micrographs and a, b, c are fluorescence micrographs. Bar is 10 μm. Surface aggregation of fluorescence is seen.

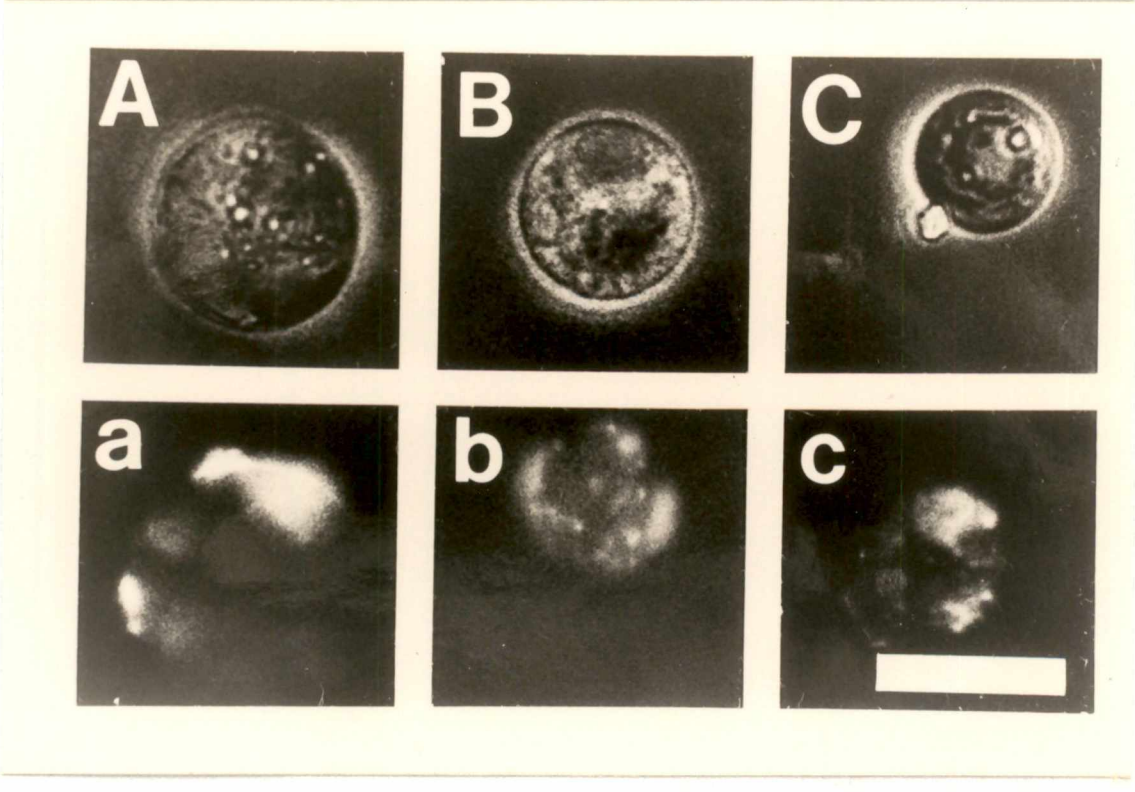


Figure 8. Fluorescence labeling of protoplasts by calcein-containing liposomes composed of DOPC/dicetylphosphate/lysoPC (8:2:0.4). The calcein was partially released inside the protoplasts. A, B. C are contrast micrographs and a, b, c are fluorescence micrographs. Bar is 10 μm.

However, the degree of internal release in this case was not nearly as high as that for DOPE/chol/OA liposomes. 77% and 84% of protoplasts incubated with other DOPC-containing liposomes took up liposomes; however, the calcein was not released from the liposomes. Fig. 9,10,11, show that liposomes aggregated on the surface of those protoplasts preincubated with DOPC/OA, DOPC or DOPC/stearylamine liposomes. The results of photographs are summarized in Table 6.

The use of PEG in the incubation protocol was essential for a high level of liposome uptake by protoplasts. In the absence of PEG only about 20% of protoplasts showed fluorescence after incubation with DOPE/chol/OA liposomes; however, the dye distribution in the protoplasts was still internal and homogeneous.

The relationship between the presence of cell wall on protoplasts and liposome uptake was examined by staining the protoplasts with 0.2% calcofluor white. Results showed that the liposome uptake by protoplasts was not correlated with the existence of undigested cell wall. 58% of the protoplasts (counted from 124 protoplasts) which had taken up liposomes had no obvious cell wall remaining (photographs not shown). This is consistent with the results by Fukunaga et al. (107) who studied the infection of protoplasts by liposome-entrapped TMV-RNA and found that the efficiency of infection was not correlated with the existence of undigested cell wall.

Viability of liposome-incubated protoplasts

The viability of protoplasts after incubation with different liposomes are shown in Table 6. The viability of liposome-incubated protoplasts was between 71 and 82%. The viability of protoplasts

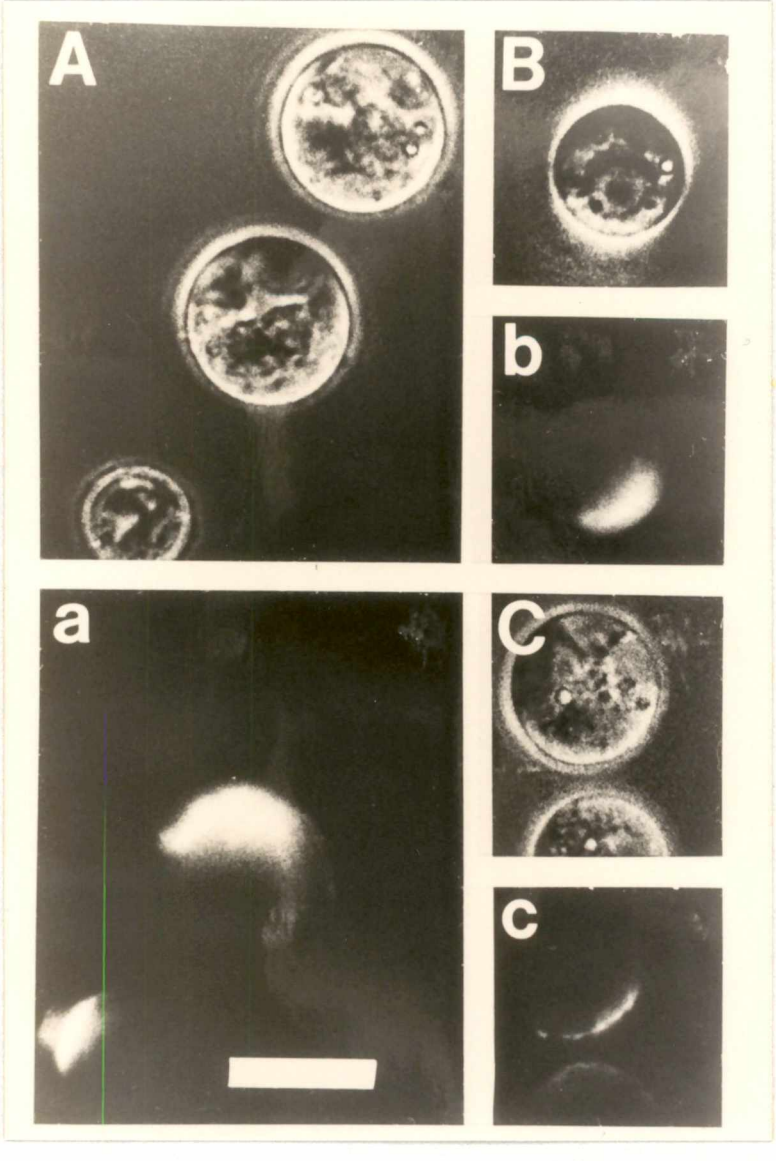


Figure 9. Fluorescence labeling of protoplasts by calcein-containing liposomes composed of DOPC/OA (8:2). Surface aggregation of fluorescence is seen. A, B, C are phase contrast micrographs and a, b, c are fluorescence micrographs. Bar is 10 μm.

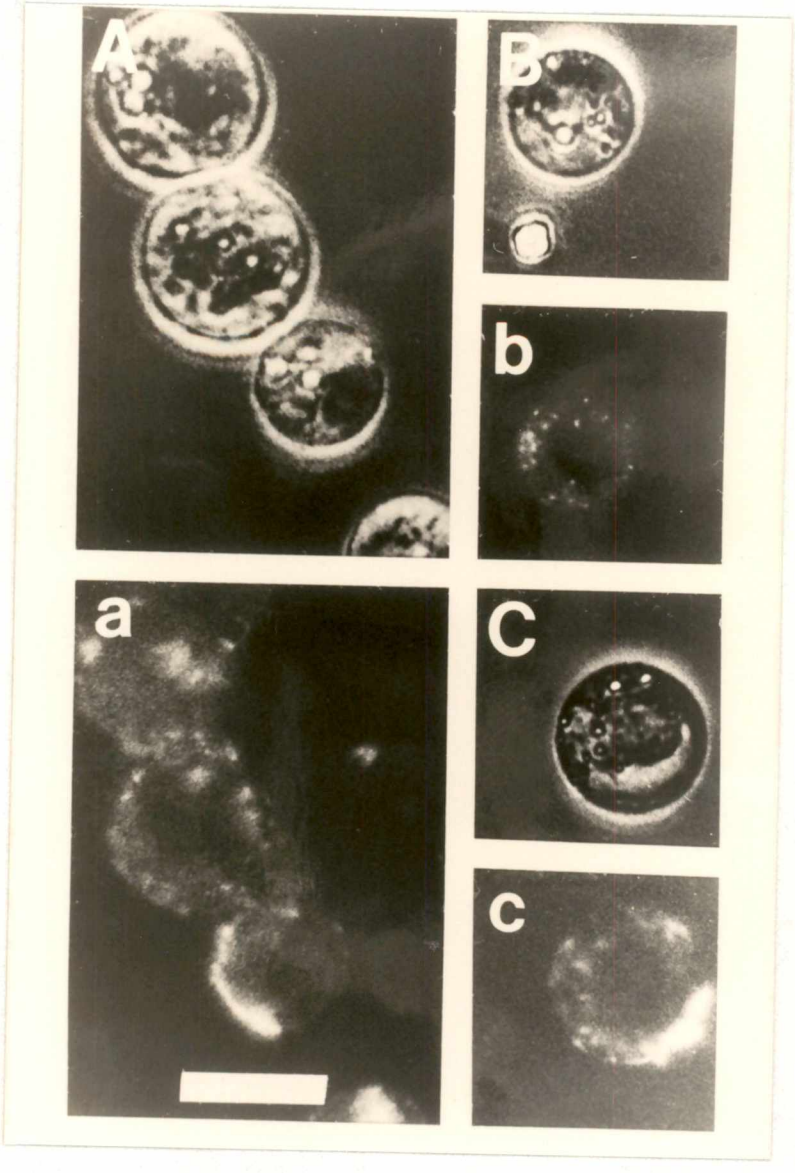


Figure 10. Fluorescence labeling of protoplasts by DOPC liposomes. The calcein-entrapped liposomes aggregated on the surface or inside of protoplasts without dye release. A, B, C are phase contrast micrographs and a, b, c are fluorescence micrographs. Bar is 10 μm.

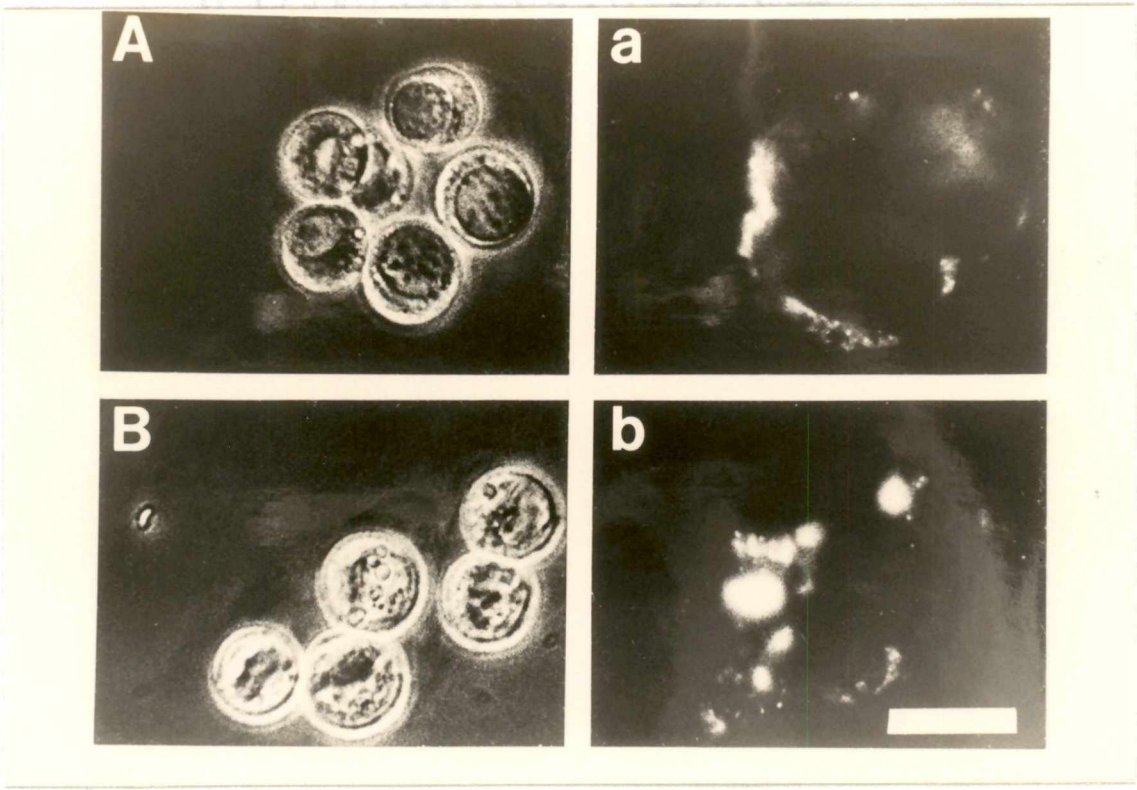


Figure 11. Fluorescence labeling of protoplasts by DOPC/stearylamine (10:1, w/w) liposomes. Surface aggregation of fluorescence is seen. A, B are phase contrast micrographs and a, b are fluorescence micrographs. Bar is 10μm.

incubated with the DOPC/stearylamine liposomes was the highest while that with DOPC/dicetylphosphate/lysoPC was the lowest. There was no obvious difference between the viability of protoplasts incubated with DOPE/chol/OA liposomes and that with DOPC/chol/OA liposomes. The viability of untreated cells is approximately 78%. Thus, the liposome treatment is mild and non-toxic.

Incubation with methylamine and Chloroquine

Preincubation of protoplasts with 30 mM of methylamine or 100 μM chloroquine for 20 min followed by removal of chemicals showed that most of the calcein-entrapped DOPE/chol/OA liposomes aggregated on the surface of protoplast, while some were endocytosed by the protoplast but the dye was not released. Figure 12 shows punctate fluorescence on the surface and inside protoplasts which had been preincubated with methylamine or chloroquine. This is to be compared with cells shown in Figure 5 which had not been preincubated with chloroquine or methylamine; only homogeneous cytoplasmic distribution of fluorescence was seen.

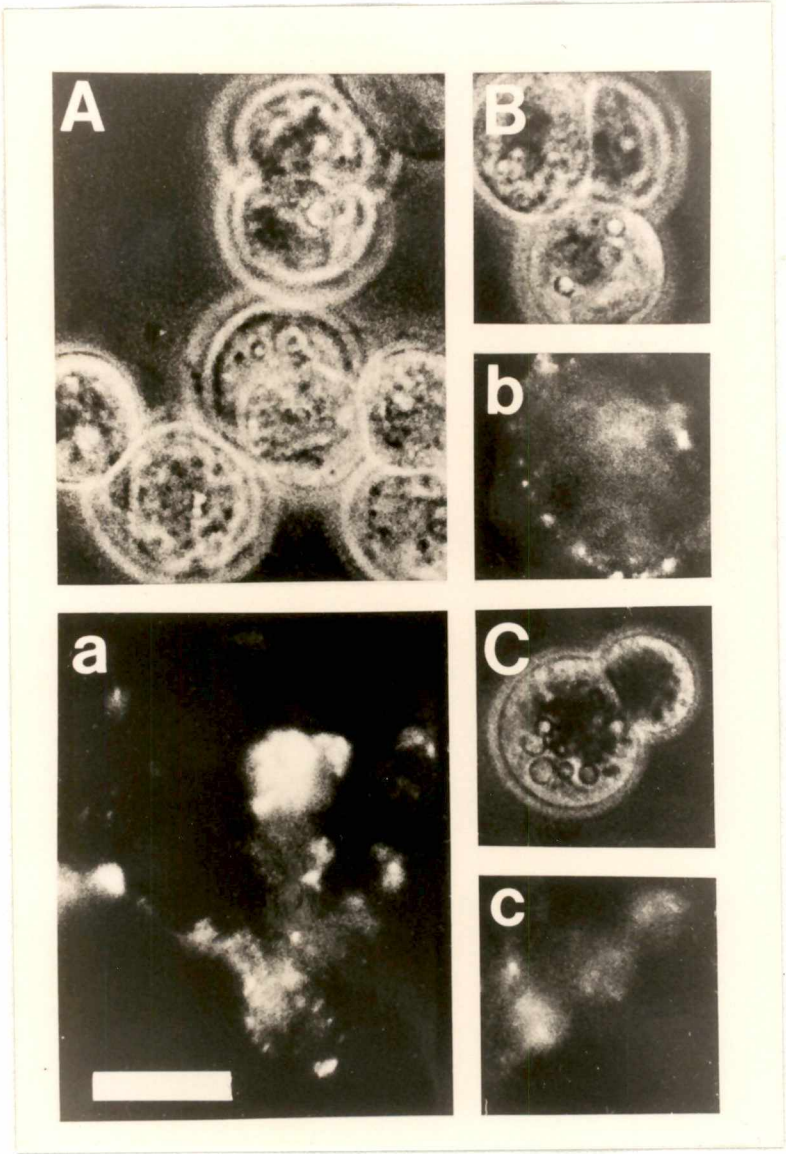


Figure 12. Effects of methylamine and chloroquine on calcein distribution. The protoplasts were preincubated with 30 mM methylamine (A,a) or 100 M chloroquine (B,b; C,c) at room temperature for 30 minutes before the addition of DOPE/chol/OA liposomes. Punctate fluorescence on the surface and inside the protoplasts is evident. A, B, C are phase contrast micrographs and a, b, c are fluorescence micrographs. Bar is 10 μm.

Discussion

pH-sensitive liposome systems have recently been developed in this (21) and other labs (19) to efficiently deliver liposomal contents to mammalian tissue culture cells. In this study we have examined the potential use of the pH-sensitive liposomes for delivery to higher plant protoplasts. In previous studies, liposomes have been used to deliver DNA and RNA into the protoplasts of higher plants (12,97,98). Fukunaga et al. (12) have prepared liposomes by Ca-EDTA chelation and used phosphatidylserine with or without cholesterol. In Lurquin's and Fraley's studies, liposomes were prepared by the reverse-phase evaporation (REV) method (11) and composed of stearylamine with phosphatidylcholine or phosphatidylserine and cholesterol. Those liposomes were either positively or negatively charged. Although 80% of protoplasts were infected by liposome-mediated TMV-RNA in Fukunaga's study, the entrapment efficiency of liposomes produced by CA-EDTA chelation method was quite low for large size DNA. The efficiency of entrapment was improved with the REV method. Particularly, the modified REV method performed at low salt condition promoted the entrapment of up to 65% of the aqueous phase (11). Nevertheless, the efficiency of transformation of protoplasts which were transformed by liposome-entrapped nucleic acids was still low (10^{-5} transformants/treated cells) (99). The low efficiency of transformation may be caused by damage to the nucleic acids during entrapment or by the incomplete release of the nucleic acids from liposomes. Liposomes used in our study were produced with a mild detergent-dialysis method in which the use of sonication and organic solvent was avoided. Our results showed

that the adsorption was high when the pH-sensitive liposomes were used in the presence of PEG. Liposomes composed of DOPE/chol/OA (4:4:2) showed the highest sensitivity to acid (Figure 4), and most efficiently delivered calcein into the cytoplasm of protoplasts (Figure 5 and Table 6). Other liposome compositions, including DOPC/dicetylphosphate/lysoPC used by Matthews et al. (98), DOPC/SA used by Lurquin and Sheehy (97), showed little or no sensitivity to acid and delivered calcein poorly to the cytoplasm of the protoplasts. Thus, the high delivery efficiency seems to correlate with the acid sensitivity of the liposomes.

The mechanisms of the interaction between liposomes and protoplasts have been studied. Using electron microscopy, Lurquin and Sheehy (97) suggested that nucleic acid transfer occurs via fusion between liposomes and the plasma membrane (97). In contrast, Fukunaga et al. (12) indicated that liposomes enter plant protoplast via endocytosis in the presence of polyethylene glycol (PEG) and the nucleic acids are released when the endocytosed liposomes fuse with the endosomal membrane. Fukunaga also suggested that PEG increases the adhesion of liposomes to the protoplasts rather than inducing liposome-protoplast fusion. Others have suggested that PEG causes intermembrane dehydration which induces fusion (100,108).

Since PEG is required for the cytoplasmic delivery of calcein by pH-sensitive liposomes, one possible mechanism of delivery of calcein was that the fusion takes place between liposomes and the plasma membrane of the protoplast. This would have result in the even distribution of fluorescence in the cytoplasm, which we observed. However,

some of the protoplasts shown in Figure 5 show stronger fluorescence in the vacuole which is the lysosome equivalent of higher plant cells (109). The vacuolar pH of protoplasts has been measured to be around 5.5 (110). Additionally, incubation of protoplasts with methylamine or chloroquine blocked the cytoplasmic release of fluorescent dye although internalization of liposomes still took place (Figure 12). These observations support a mechanism in which the fluorescent dye is delivered into the cytoplasm of protoplasts via endocytosis of liposomes. The liposomes may be internalized by protoplasts and then fuse with the endosome or vacuole membrane, resulting in the release of the fluorescent dye into the cytoplasm. The fusion between liposomes and endosome/vacuole membrane may be caused by the destabilization of liposomes when the liposomes encounter the acidic environment in these organelles. Duzgunes et al. (19) suggested that oleic acid is protonated at low pH. Thus, this mechanism appears to be similar to what we have proposed for animal cells (21,101).

The present observation is likely to find its application in the delivery of nucleic acids into protoplasts. Since the method of liposome preparation is mild and relatively rapid, entrapment of structurally intact DNA or RNA should be a relatively easy task. Philippot et al., (89) have shown that up to 45~50% of RNA can be trapped in the liposomes prepared with a method similar to ours. Furthermore the liposomal delivery is not limited to calcein. The pH-sensitive liposomes have recently been used to deliver DNA into plant protoplasts by Zhu and Hughes. The transformation efficiency was in the range of 10^{-4}~10^{-3} transformants/treated cells (unpublished result).

Transfer of exogenous DNA into plant protoplasts has recently been carried out by electroporation (67). Although the transformation frequency achieved by this method is very high, the pores in the plasma membrane created by the electric field for DNA penetration are likely to cause the loss of intracellular macromolecules and metabolites. Although the cells can eventually recover from the initial damage, it is not clear if the method is suitable for the delivery of molecules with which the immediate, short-term effect is to be investigated. In contrast, results in this paper showed that a small molecule such as calcein was retained in the protoplasts after delivery by the pH-sensitive liposomes. It is clear that our method is mild, since the viability of liposome-treated protoplasts is quite high.

Results in this study gave evidence for the potential of pH-sensitive liposomes in the cytoplasmic delivery of macromolecules such as DNA. In the next chapter, pH-sensitive liposomes will be used to investigate the potential of pH-sensitive liposomes for DNA delivery into animal cells.

CHAPTER IV

EXPRESSION OF EXOGENOUS GENE DELIVERED BY pH-SENSITIVE IMMUNOLIPOSOMES

Abstract

pH-sensitive immunoliposomes were used to deliver the plasmid pPCTK-6A into cultured mouse cells. This 8.2Kb plasmid contains the Herpes Simplex virus thymidine kinase gene. The endogenous promoter of HSV-TK gene was replaced by the promoter of the rat phosphoenolpyruvate carboxykinase gene, which contains a cAMP regulatory region allowing cAMP control of TK expression. The plasmid was encapsulated in pH-sensitive immunoliposomes using the detergent dialysis method. Monoclonal anti-$H2K^k$ antibody was incorporated into the liposomes as a targeting antibody. The delivery and the short term expression of the exogenous gene were assayed in the liposome treated mouse Ltk^- target cells by measuring the thymidine kinase activity and by autoradiography of cells labeled with [^{3}H] thymidine. Expression was optimized with respect to the time course and dose of the liposome treatment. The optimum concentration of entrapped DNA was 2μg/10^5 cells and the optimal expression time after liposome treatment was 24 hours. We found that the existence of antibody on the liposomes was essential for the maximal level of delivery and expression of plasmid DNA. The efficiency of delivery via the immunoliposomes was more than twice as much as that obtained via the liposomes without antibody. Delivery was also dependent on the lipid composition of the liposomes. pH-sensitive

liposomes composed of dioleoyl phosphatidylethanolamine:cholesterol: oleic acid (4:4:2) gave a delivery efficiency which was seven-fold higher than that of pH-insensitive liposomes. Long-term transformation efficiency was determined by limiting dilution of cells after incubation with pH-sensitive immunoliposome containing DNA or Ca^{+2}-phosphate precipitated DNA. The long-term transformation efficiency of liposome entrapped DNA and Ca^{+2}-DNA was 9% and 1%, respectively, in terms of two divisions of cells. Southern blot analysis showed that the exogenous DNA had integrated into the host chromosome of the long-term transformants. The exogenous gene delivered by pH-sensitive immunoliposomes may have more than one integration site while the gene delivered by Ca^{+2}-phosphate showed only one integration site.

Introduction

Gene therapy has been applied to correct genetic defects in fruit flies (30), in mice (31), and in a child with severe combined immune deficiency (SCID) in 1968 (32). There are several events on which the successful genetic therapy depends. The first requirement is to develop effective techniques which permit new genes to be transferred into the target cells. The second is to eliminate the immune rejection of the transformed target cells due to expression of the foreign gene. In addition, appropriate and stable expression of the exogenous gene in the target cells is also crucial.

Autologous bone marrow cells have recently been transfected by normal genes in vitro and implanted into patients (111,112). By use of

autologous bone marrow, immune rejection is eliminated. However, transformants are still at low percentage. Thus, it is still difficult to cure the genetic disorders. Therefore, it is essential to establish a model system of gene transfer based on the criteria above. Current approaches to gene transfer include calcium phosphate precipitation, retroviral transfection, microinjection, liposome-mediated gene transfer and electroporation (for reviews see ref. 17).

The calcium phosphate precipitation method has been widely used to transfer new genes into cells in vitro. Electroporation has also been used to transfer genes into plant protoplasts (46) and mammalian cells (113). In addition, stable transfectants by microinjection were obtained at a frequency of 1 per 10^4 cells receiving DNA injection (114). However, at the present time there are still many difficulties in the application of these methods in vivo. Thus, retrovirus-mediated gene transfer is considered the best available techniques (17). When gene transfer is mediated by retrovirus, the gene in question is inserted into the retroviral genome. Although the transformation efficiency is high, the retroviral vectors are species-specific. So far, the retroviral vectors have been derived from mouse retrovirus. There is no available retrovirus vector for human gene therapy (164).

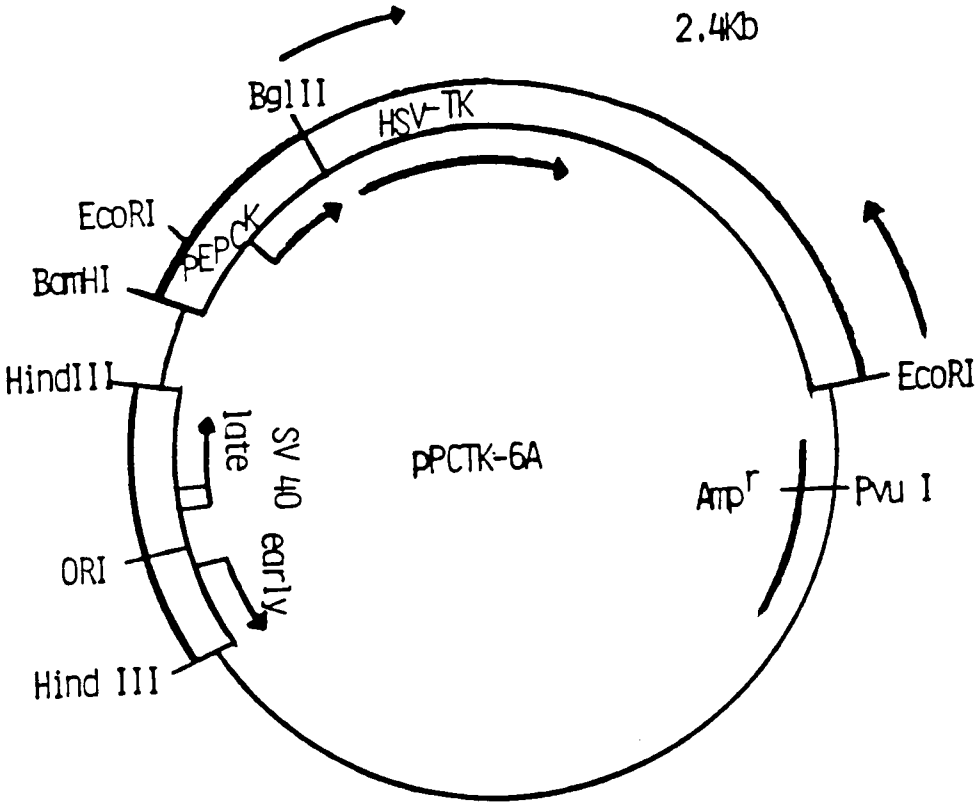
Liposomes have been used extensively as a tool to deliver biologically active molecules to cells (115,116). To enhance liposomal delivery, monoclonal antibodies specific to a cell surface antigen can be incorporated into the liposome bilayer (117). These immunoliposomes are capable of effectively delivering macromolecules into target cells

via a receptor mediated endocytosis mechanism (117). The present study is designed to test the exogenous gene delivery potential of pH-sensitive immunoliposomes for the mammalian cells. We have used the thymidine kinase gene which was ligated with a cAMP regulated promoter as a marker for gene transfer. We have also used pH-insensitive, non-targeted liposome compositions and calcium phosphate precipitation for comparison.

Materials and Methods

Materials

Dioleoylphosphatidylethanolamine (DOPE)[1] and dioleoylphosphatidylcholine (DOPC)[1] were purchased from Avanti (Birmingham, AL). Oleic acid, cholesterol, n-octylglucoside, cholera toxin, prostaglandin E1 (PGE1), 3-isobutyl-1-methyl xanthine, 8-bromo cAMP, dibutyryl cAMP and theophylline were obtained from Sigma. SM-2 beads were purchased from Bio-Rad Laboratories. Epinephrine bitartrate was obtained from Calbiochem. Protosol was purchased from NEN Research Products. HAT medium was obtained from Gibco. Plasmid pPCTK-6A, which was a gift of Drs. A. Wynshaw-Boris and R. Hanson, contains the Herpes Simplex virus thymidine kinase gene, HSV-TK (Fig. 13). The endogenous promoter gene was replaced by the promoter of the rat phosphoenolpyruvate carboxykinase (PEPCK) gene, which contains a cAMP regulatory region (118). The plasmid DNA was introduced to strain 1592 of *E. coli* by the calcium chloride procedure, amplified in the presence of 170 µg/ml



Reference: Wynshaw-Boris, A. et al. (118).

Figure 13. The map of pPCTK-6A. Plasmid pPCTK-6A was constructed on pBR 322 and contained a Herpes Simplex virus thymidine kinase gene and a promoter of rat phosphoenolpyruvate carboxykinase gene.

chloramphenicol and isolated from E. coli by alkali lysis (119). DNA was radiolabeled by nick translation with [α-^{32}P] dCTP (120).

Cell incubations

Mouse Ltk$^-$ cells were a kind gift from Dr. Leroy Hood. They were grown in DMEM medium supplemented with 10% fetal calf serum. 1 X 10^5 cells were preincubated in serum-free DMEM for 4 hours before transformation.

Liposome preparation

Immunoliposomes were prepared as described in Chapter II and were labeled with ^{3}H-CE and ^{32}P-DNA. Specific activities were 1 X 10^5 cpm/μmole phospholipid and 1 X 10^8 cpm/μg DNA for ^{3}H-CE and ^{32}P-DNA, respectively.

Transformations

Ltk$^-$ cells were transformed by liposome-entrapped plasmid DNA, free DNA or calcium phosphate-precipitated DNA. For liposome-mediated DNA transfer, liposome-encapsulated plasmid DNA was added to the serum-free medium treated cell culture. An additional incubation of 3 hours at 37°C was carried out because time course showed that thymidine kinase activity reached a plateau when the incubation time was 3 hours (data not shown). Calcium phosphate precipitation was carried out according to Wigler et al. (62). Salmon sperm DNA (Sigma) was used as a carrier.

Thymidine kinase activity

To examine the short-term expression of transferred DNA, the activity of the gene product was determined. Thymidine kinase activity was assayed according to Wigler et al. (121) with modification. After transformation, the cells were washed with culture medium and grown in serum-containing DMEM with or without cAMP (0.5 mM dibutyryl cAMP) (122) and 1 mM theophylline (118) for various periods of time. Subsequently, the cells were washed 3 times with cold PBS, scraped and suspended in PBS. After centrifugation at 15,000 xg for 10 min, the cells were resuspended in 0.5 ml of extraction buffer composed of 0.01 M Tris-HCl (pH 7.5), 0.01 M KCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 50 μM thymidine. After the KCl concentration was adjusted to 0.15 M, the cell suspension was sonicated for 15 seconds. The cell debris was removed by centrifugation at 30,000 xg for 30 min. The supernatant was collected and assayed for protein (9) and thymidine kinase activity. Thymidine kinase activity was assayed by incubating the supernatant with 100 μl of reaction mixture [0.1 M Tris-maleate (pH 6.5), 25 mM KCl, 20 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 10 mM ATP and 1.5 μl of ^3H-thymidine (specific activity 65 Ci/mmole)]. After a two hour incubation at 37°C, the reaction was stopped by spotting 50 μl of reaction mixture onto a DE-81 paper. After washing three times with 4 mM ammonium formate, the DE-81 papers were dissolved in 1 ml Protosol and the radioactivity was counted in scintillation fluor.

Transformation efficiency

To examine the transformation efficiency, limiting dilutions of treated cells were carried out. Cells were scraped from the culture dishes and seeded in 96 well plates at an average number of 0.5 cell per well. Incubation medium supplemented with HAT was used to select TK^+ cells. The growth of cells in the HAT medium containing hypoxanthine (1×10^{-2}M), aminopterin (4×10^{-3}M) and 1.6×10^{-3}M thymidine was examined on days 1, 4, and 12. In the presence of HAT, cells were forced to salvage pathway and thymidine kinase was required for survival. The transformation efficiencies were calculated by dividing the number of wells which contained ≧2, ≧4, ≧6, or ≧8 cells by the number of wells which originally contained one cell.

Autoradiography

To confirm the short-term expression of transferred DNA, incorporation of [^{3}H] thymidine into DNA in the transformed Ltk^- cells was carried out by autoradiography according to Schaeffer-Ridder et al. (9).

Effect of analogs and stimulators of cAMP

The effect of stimulators and analogs of cAMP on the transformation efficiency was examined. The stimulators and cAMP analogs used were cholera toxin (220 ng/ml), prostaglandin E1, (1×10^{-5}M), l-epinephrine bitartrate (1×10^{-5} M), 3-isobutyl-1-methylxanthine (0.31 mM), and 8-bromo cAMP (2 mM) (123,124,125,126,127). The effect of these agents on the transformed cells was investigated by examining the

thymidine kinase activity after a 24 hours exposure of transfected cells to the stimulators or cAMP analogs; however, the concentration of various stimulators was chosen as optimal condition according to literature, whereas the 24 hours incubation time was the best condition for expression of thymidine kinase gene stimulated by dibutyryl cAMP plus theophylline.

DNA blot hybridization

Transformed cells were selected in HAT medium for one month and then grown as mass cell cultures in DMEM medium. Cellular DNA was prepared by the method of Blin and Stafford (128) modified by Wynshaw-Boris et al. (118). Ten µg of DNA from transformed cells were digested with 50 units of Eco R1 or digested with Cla 1, Sal 1 and Eco R1. The DNA was separated by electrophoresis on 0.8% agarose gels and transferred to nitrocellulose by the method of Southern (129). Filters were air dried for one hour and baked at 80°C under vacuum for one hour. Prehybridization was carried out at 42° for 24 hours in prehybridization buffer (6 X SSC, 1 X Denhards, 0.5% SDS, 100 µg/ml sheared and denatured salmon DNA, 0.05% sodium pyrophosphate) (164). After the prehybridization buffer was removed, hybridization buffer (6 X SSC, 1 X Denhards, 20 µg/ml sheared and denatured salmon DNA, 0.05% sodium pyrophosphate) was added. The DNA probe (10^7 cpm) was ^{32}P-labeled by nick translation (120). 1 µg of probe (10^8 cpm/µg) was used for hybridization. The hybridization was performed at 42°C for 48 hours. Unbound probe was washed from the nitrocellulose by washing in 6 X SSC with 0.05% sodium pyrophosphate twice at room temperature and

once at 60°C. The hybridized DNA fragments were autoradiographed by using Kodak X-Omat RP-1 x-ray film.

Preparation of probe

pPCTK-6A was digested with Bgl II and Eco R1. The HSV-TK fragment adsorbed to the DEAE cellulose membrane (Schleicher & Schuell (130,131). DNA recovered from a 1% agarose gel was eluted with high salt NET buffer (1.0M NaCl, 0.1 mM EDTA, 20 mM Tris pH 8) at 68°C for 45 min. Ninety % of total DNA was recovered from the DEAE cellulose membrane.

Results

Entrapment of plasmid DNA in the liposomes

The entrapment efficiency of pPCTK-6A DNA in the pH-sensitive immunoliposomes was measured by gel filtration. As shown in Figure 14, the ^{3}H-cpm profile of liposomes was characterized by a single sharp peak at the void volume in which the liposome associated pPCTK-6A DNA was also eluted. 35 ± 5% of the total DNA associated with the liposomes (Fig. 14A). To examine the degree of DNA adsorption on liposome membranes, empty liposomes were incubated with free DNA for 24 hours. Then the unbound DNA was removed by gel filtration. The profile of incubated DNA showed that the majority of DNA was not adsorbed onto the liposomes (Figure 14B). The difference between total liposome-associated DNA and the adsorbed DNA was 20 ± 4% of input DNA, indicating that 20 ± 4% of the input DNA was entrapped in liposomes.

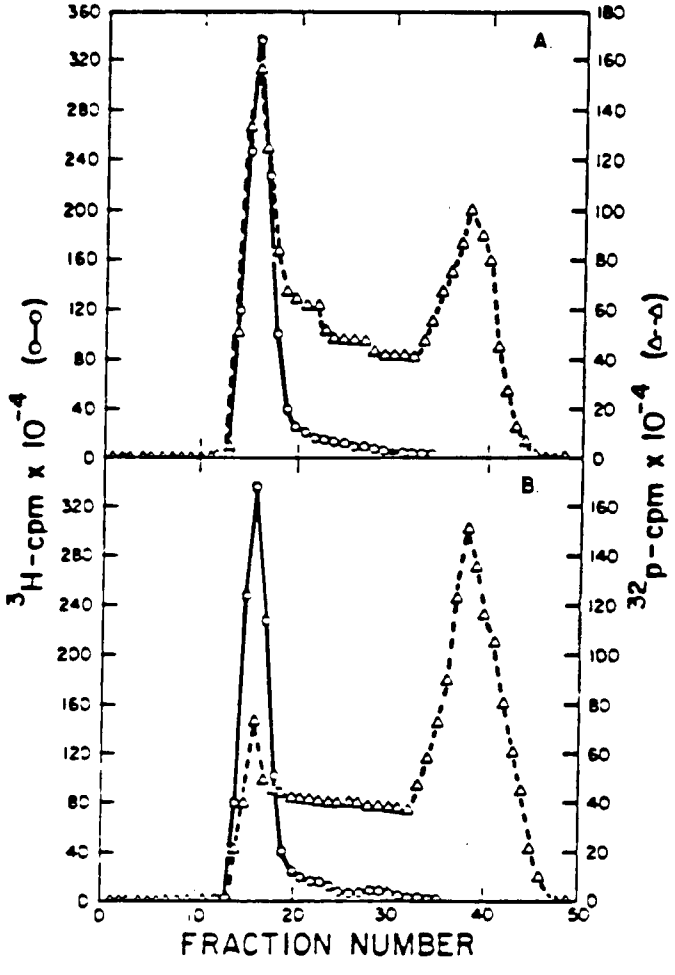


Figure 14. Gel filtration of liposomes. Large unilamellar vesicles were prepared as described in Methods. The liposome suspension was loaded on a column of autoclaved sepharose CL-2B(A). Empty liposomes (10 μmole phospholipid) and free DNA (150 μg) were mixed and incubated at 4° for 24 hours and chromatographed on the same column (B). Fractions were assayed for ^{3}H-cpm (O) and ^{32}P-cpm (Δ). Peak between 15 to 20 fractions represented entrapped DNA whereas peak between 35 to 40 fractions represented free DNA. Entrapped DNA shown in fractions 15, 16, and 17 were used in the transformation of Ltk^{-}.

Calculation based on liposome size showed that there were approximately 3~5 entrapped DNA molecules of the pPCTK-6A in each liposome.

Cell incubations

Transformation of Ltk^- cells with the thymidine kinase gene was performed by incubating 10^5 cells with 2 μg of liposome-entrapped DNA or Ca^{+2}-phosphate precipitated DNA at 37°C for 4 hours. Preliminary experiments showed that 4 hours of liposome treatment was the best condition based on the transformation of cells. The viability of treated cells was examined by trypan blue exclusion test (132). The liposome treated cells were 90±5% viable and the calcium phosphate treated cells were 48±7% viable. After the liposomes or Ca^{+2}/DNA were removed treated cells were grown in the DMEM supplemented with or without cAMP for 24 hours, followed by enzyme assay. The enzyme activity of transfected cells grown in the presence of dibutyryl cAMP plus theophylline was 1.9~2.5 times the enzyme activity of transfected cells grown in the absence of dibutyryl cAMP plus theophylline (Figures 15 & 16). This indicates that dibutyryl cAMP and theophylline enhanced the expression of the thymidine kinase gene in the transfected cells.

The optimal conditions of transfection were determined by measuring the time course and DNA concentration (Fig. 15 & 16). The curve of thymidine kinase activity reached a plateau when the concentration of DNA was more than 0.2 μg/ml in both the absence and presence of dibutyryl cAMP plus theophylline (Figure 15). The increase of thymidine kinase activity were different in early incubation time for the cells grown in the presence of dibutyryl cAMP plus theophylline

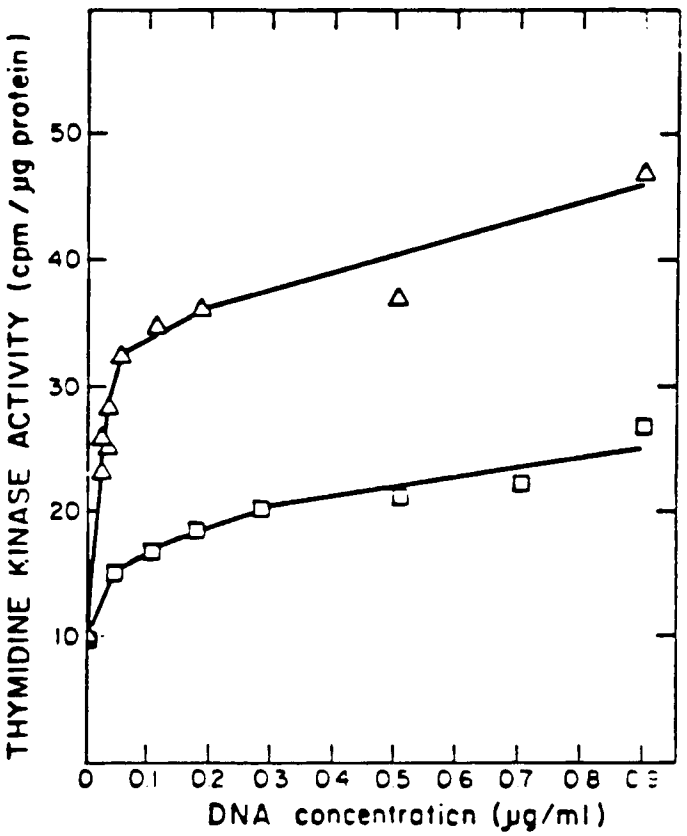


Figure 15. Effect of DNA concentration on transient gene expression. The TK activity was measured in cells transfected with DNA entrapped in PE:chol:OA liposomes containing anti-$H2K^k$ and grown in the presence (Δ) or absence (□) of 0.5 mM dibutyryl cAMP plus 1 mM theophylline for 24 hours.

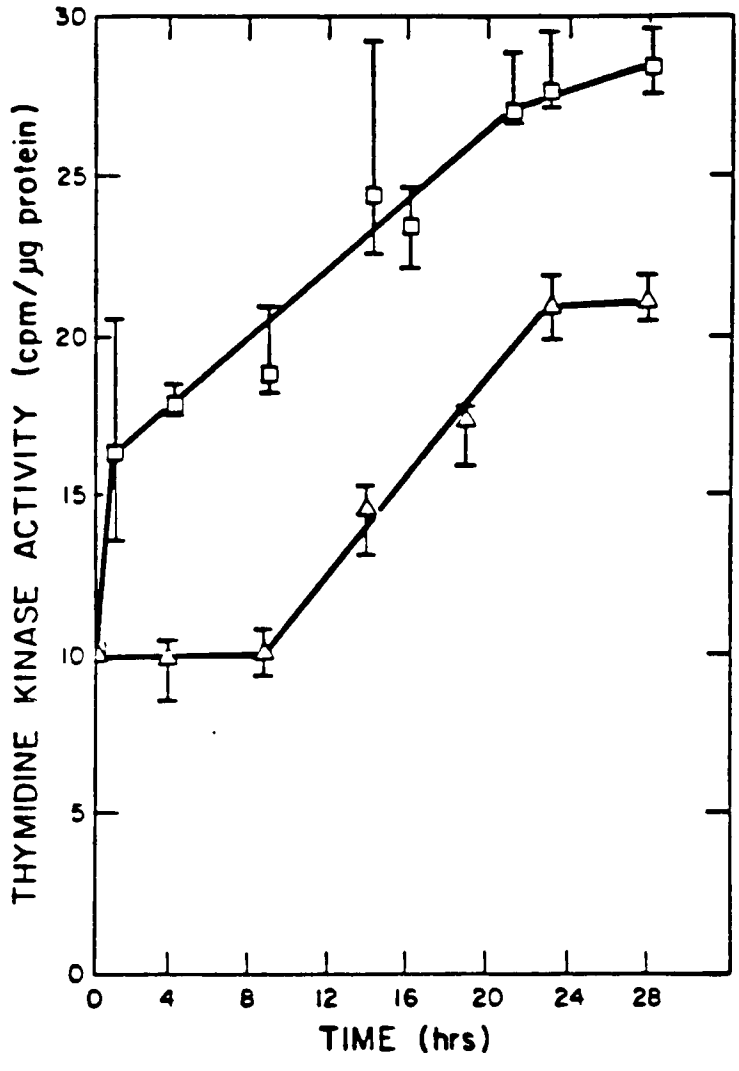


Figure 16. Effect of incubation time of transfected cells on the level of TK gene expression. Ltk^- cells transfected with 2 µg of pPCTK-6A entrapped in PE:chol:OA (4:4:2) liposomes containing anti-$H2K^k$ were grown in DMEM with (□) or without (△) Bt_2 cAMP and theophylline for varied periods as described under cell incubation section of "Methods".

from those grown in its absence (Figure 16). A 24 hour incubation is optimal since most of the effect has occurred but the cells are not yet degrading. Further experiments indicated that thymidine kinase activity declined when the incubation time was more than 30 hours (data not shown). Results here indicated that the optimal concentration of entrapped DNA was 2 $\mu g/10^5$ cells and the optimal expression time after transfection was 24 hours.

Liposome composition

Effect of liposome characteristics on the expression of plasmid DNA, was investigated using different lipid compositions. Liposomes composed of DOPE:chol:oleic acid (4:4:2 molar ratio) gave a 7.8 $\pm$ 1.2 fold more thymidine kinase activity over that delivered via the liposomes composed of DOPC:chol:oleic acid (4:4:2 molar ratio) (Table 7). It has been shown that the DOPE:chol:OA liposomes are pH-sensitive while the DOPC:chol:OA liposomes are pH-insensitive (20). Results in a previous paper (20) showed that pH-sensitive liposomes released their contents into the cytoplasm of target cells by fusing with the endosome at the acidic endosomal pH of that organelle. This result indicates that the pH-sensitive liposome is a better cytoplasmic delivery vehicle for DNA.

We also examined the effect of liposome-incorporated antibody on the delivery of DNA by examining the expression of the transfected gene. The results are shown in Table 8. The efficiency of delivery via the immunoliposomes was enhanced 5.9 $\pm$ 5.2 fold over that obtained via the liposomes without antibody. This result indicates that

Table 7. Effect of liposome composition on the thymidine kinase activity of cells.[+]

liposome composition / experiment #	DOPC[**]	DOPE[**]	DOPE/DOPC
1	12.2[*]	94.2	7.7
2	8.0	78.3	9.8
3	12.6	107.6	8.5
4	10.5	82.5	7.8
5	12.5	81.5	6.5
6	2.6	16.6	6.4
$\bar{x}$ ± S.D. (# of obs.)	9.7 ± 3.6 (30)	76.8 ± 28.7 (30)	7.8[***]

[*] thymidine kinase activity (cpm/mg protein).

[**] DOPC: liposomes composed of DOPC/cholesterol/oleic acid (molar ratio 4:4:2).
DOPE: liposomes composed of DOPE/cholesterol/oleic acid (molar ratio 4:4:2).

[***] P<0.001

[+] transformed cells were grown in MEM supplemented with 0.5 mM dibutyryl cAMP & 1 mM theophylline.

Table 8. Effect of liposome-incorporated antibody on the thymidine kinase activity of cells transformed via DOPE:Chol:OA liposomes.[+]

liposome incorporated antibody / experiment #	without antibody	with antibody	with antibody / without antibody
1	9.8[*]	81.5	8.3
2	5.8	80.5	13.9
3	26.4	64.5	2.4
4	8.9	76.8	8.6
5	8.5	14.8	1.7
6	10.8	27.4	2.5
$\bar{x}$ ± S.D. (# of obs.)	11.7 ± 6.8 (30)	57.6 ± 26.6 (30)	6.2[**]

[*] thymidine kinase activity (cpm/mg protein).
[+] transformed cells were grown in MEM supplemented with 0.5 mM dibutyryl cAMP & 1 mM theophylline.
[**] $P<0.001$.

immunoliposomes were more effective than conventional liposomes in delivering DNA to mammalian cells.

Effect of cAMP on the thymidine kinase activity of cells transformed via DOPE:chol:OA immunoliposomes was shown in Table 9. Transformed cells grown in the presence of cAMP gave a 3.2 $\pm$ 2.0 fold thymidine kinase activity over that of transformed cells grown in the absence of cAMP. Comparision of the activity of gene products transfected via pH-sensitive immunoliposomes with that via conventional Ca phosphate precipitation showed that the former gave a significantly greater delivery efficiency ($P<0.001$). This result was also confirmed by autoradiography (Table 10). Results from the controls of empty liposomes and free DNA showed that the absorbed DNA on liposomes did not enhance the thymidine kinase activity of treated cells.

Transformation efficiency

In order to examine the transformation efficiency of the Ltk^- cells by the PEPCK-TK gene, the transformed Ltk^- cells were incubated in growth medium supplemented with HAT. Limiting dilutions of transfected cells were carried out in 96 well plates. The results are shown in Table 11. For the cells transfected via pH-sensitive immunoliposomes and grown in the presence of dibutyryl cAMP plus theophylline, 17.8 percent of the wells contained more than one cells and 5.6% contained more than 4 cells after four days of incubation in HAT medium. These two percentages increased after 12 days of incubation. In contrast, the survival rate of transfected Ltk^- cells in HAT medium without dibutyryl cAMP and theophylline decreased from 2.8 to

Table 9. Effect of cAMP on the thymidine kinase activity of cells transformed via DOPE:Chol:OA immunoliposomes.

cell treatment / experiment #	without cAMP	with cAMP	with cAMP / without cAMP
1	86.1	94.1	1.1
2	36.2	78.2	2.2
3	20.2	106.8	5.3
4	14.5	81.5	5.6
5	18.5	80.5	4.4
6	6.6	15.7	2.4
7	5.2	24.7	4.8
8	5.1	26.0	5.1
$\bar{x}$ ± S.D. (# of obs.)	24.0 ± 25.4 (40)	63.4 ± 33.2 (40)	3.9**

*thymidine kinase activity (cpm/mg protein).
**P = 0.02.

Table 10. Comparison of pH-sensitive immunoliposome-mediated transformation and calcium phosphate method.

assays / method / experiment #	Thymidine Kinase Activity			% Cell Incorporated ^{3}H-TdR		
			Liposome			Liposome
	Ca^{+2}-phosphate	Liposome	Ca^{+2}-phosphate	Ca^{+2}-phosphate	Liposome	Ca^{+2}-phosphate
1	69.1*	94.1*	1.4	37.4	58.0	1.5
2	34.8	78.2	2.2	34.8	49.0	1.4
3	38.2	106.8	2.8	33.0	43.0	1.3
4	29.6	81.5	2.8	35.2	46.9	1.3
5	30.0	80.5	2.7	36.2	45.7	1.3
6	6.7	15.7	2.3	27.6	43.1	1.6
7	31.8	76.5	2.4	29.0	49.6	1.7
8	36.0	76.8	2.1	43.0	49.8	1.2
9	36.2	79.1	2.2	32.2	43.8	1.4
$\bar{x}$ ± S.D.	34.7 ± 15.1	76.6 ± 23.5	2.3**	34.3 ± 4.3	47.7 ± 4.4	1.4**

*thymidine kinase activity (cpm/mg protein).
+transformed cells were grown in MEM supplemented with cAMP.
**P<0.001.

Table 11. Growth of differently treated Ltk$^-$ cells in medium supplemented with HAT.

No. of wells which contain 1 cell	DNA	Dibutyryl cAMP plus Theophylline	≥ 2 cells				≥ 4 cells				≥ 6 cells				≥ 8 cells			
			Day 4		Day 12		Day 4		Day 12		Day 4		Day 12		Day 4		Day 12	
			well no.	%	well no.	%	well no.	%	well no.	%	well no.	%	well no.	%	well no.	%	well no.	%
1404	entrapped[1]	+	250	17.8	265	18.9	79	5.6	126	9.0	14	1.0	77	5.5	8	0.6	27	1.9
936	entrapped[1]	-	26	2.8	18	2.0	8	0.8	4	0.4	4	0.4	3	0.3	2	0.2	0	0
1672	free[2]	+	38	2.3	34	2.5	4	0.2	17	1.0	2	0.1	0	0	2	0.1	0	0
1464	free[2]	-	23	1.6	9	0.6	6	0.4	0	0	0	0	0	0	0	0	0	0
953	None	-	22	2.3	6	0.6	2	0.2	0	0	0	0	0	0	0	0	0	0

[1]DNA was entrapped in pH-sensitive immunoliposomes.

[2]DNA was delivered by calcium phosphate precipitation.

0.6 percent. These results indicate that the long-term expression of the TK gene was cAMP-dependent. It also confirmed Wynshaw-Boris' report (118) that the expression of the TK gene was under cAMP control. We have arbitrarily chosen the definition of "long-term transformation" as at least two cell divisions in 12 days. Therefore, the long-term transformation efficiency was 9% for pH-sensitive immunoliposomes mediated transfection, and 1% for calcium phosphate precipitation method.

Effect of cAMP on cell growth

Results from previous experiments provided adequate evidence of the effect of cAMP on enzymatic activity of gene products. To understand more clearly the effect of cAMP on the growth of Ltk^- cells, the growth of untreated Ltk^- cells and transfected cells in the presence or absence of cAMP or cAMP stimulators was examined (Figure 17). Transfected cells were incubated for 24 hours in the DMEM medium with or without dibutyryl cAMP plus theophylline and then transferred into DMEM supplemented with HAT. Cell number in the cAMP stimulated plates showed a small increase for four days and then declined. In contrast, cell number in cAMP-free plates gradually decreased from the beginning. The growth rates of both types of cells in HAT medium were enhanced again by the addition (arrows in Fig. 17) of dibutyryl cAMP plus theophylline until these stimulators were depleted in the medium. It indicated that cell growth was cAMP dependent. This phenomenon in transformed cells differed from that in untransformed cells. The growth of untransformed cells in the presence and absence of dibutyryl

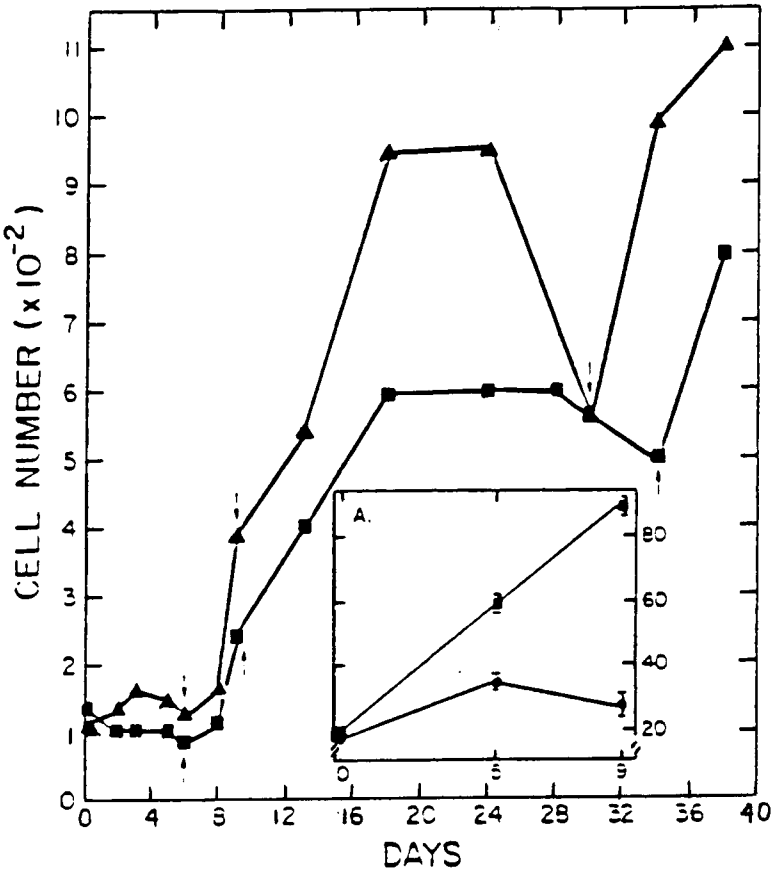


Figure 17. Effect of cAMP on growth of cells. Ltk$^-$ cells were transfected by PEPCK-TK gene via pH-sensitive immunoliposomes and incubated 24 hrs in DMEM supplemented with HAT medium in the presence of (▲) or in the absence (■) of 0.5 mM Bt_2 cAMP and 1 mM theophylline. After 24 hrs the media were changed to HAT medium. The day 0 represented the day cells were grown in the HAT medium.Arrows represent the addition of Bt_2 cAMP and theophylline. Panel A represents growth of Ltk- cells in DMEM medium supplemented with 10% FCS in the presence (●) or absence (■) of Bt_2 cAMP plus theophylline.

cAMP plus theophylline was shown in panel A of Fig. 17. In the presence of cAMP Ltk$^-$ cells grew more slowly than those cells in the absence of cAMP. This growth behavior is similar to other fibroblastic cells in culture (133). Thus, although the growth of the original Ltk$^-$ cells was inhibited by cAMP, the growth of the transformed cells in the HAT medium completely depended on the presence of cAMP.

Effect of the cAMP stimulators

In order to further characterize the cells transformed via pH-sensitive immunoliposomes, the influence of different cAMP stimulators on the expression of the transferred gene were studied. The effects were determined by quantitating the enzymatic activity of gene products 24 hours after 3 hours incubation with DNA-containing liposomes. Results shown in Figure 18 were not the optimal thymidine kinase activity of the six effectors tested (Figure 18). However, stimulation of the gene expression with cholera toxin, PGE1, 3-isobutyl-1-methyl xanthine, 8-bromo cAMP, or dibutyryl cAMP plus theophylline showed approximately 1.5-1.6 fold enhancement of thymidine kinase activity over the basal activity. All of P values of the difference between epinephrine, PGE or BT and control were <0.001, where the P values for CT, Xan and 8BrC were > 0.1. Since all six effectors are well established as agents which either increase cellular cAMP levels or mimic its actions, this result confirmed that the enhanced thymidine kinase activity was mediated with a cAMP regulatory mechanism.

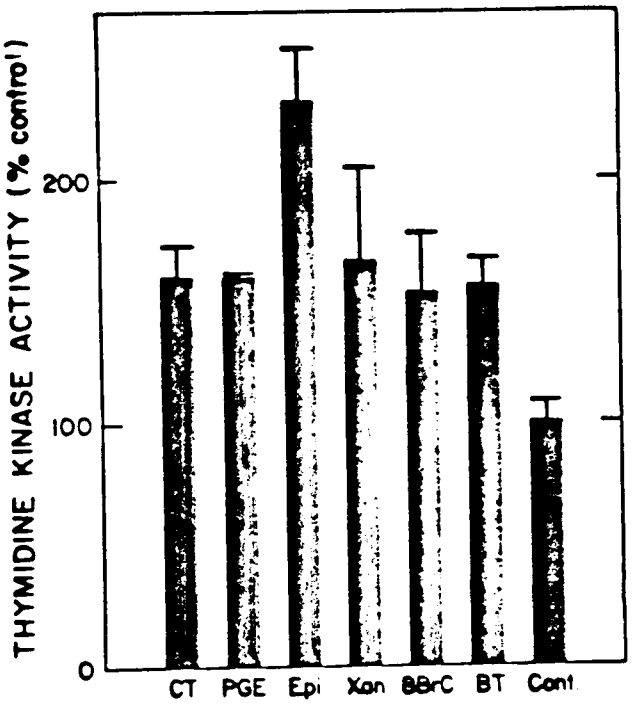


Figure 18. The inducibility of TK in transferred cells with PEPCK-TK. Cells were incubated for 24 h with cholera toxin [CT] (200 ng/ml), PGE1 (1 X 10^{-5} M), Epinephrine (1 X 10^{-5} M), 3-isobutyl-1-methylxanthine [xan] (0.31 mM), 8-BromocAMP (2 mM) or Bt_2 cAMP (0.5 mM) plus theophylline (1 mM). Cells were extracted and TK activities were determined as described in Methods. Number of observations for each treatment is two.

TK genes are integrated into Ltk$^-$ cells transfected with pPCTK-6A

Total cellular DNA was isolated from various Ltk$^-$ clones transformed with pPCTK-6A for DNA blot analysis. Figure 19 shows EcoR1, Cla I and Sal I digestion of DNA from clones. A 2.4-kb EcoR1 and Bg1 II fragment of pPCTK-6A was used as a probe. Ltk$^-$ cells did not contain any DNA sequence which hybridized to this fragment (lane 4). However, all the transfected cell clones contained one or more EcoR1 fragments homologous to this probe. The pattern of bands in the cells transfected by calcium phosphate precipitated DNA (lane 1,2,3) was different from the pattern in the cells transfected with liposome entrapped DNA (lane 5,6,7). All three independent clones transfected by calcium phosphate precipitated DNA showed one band of 7.6 kb, whereas the clones derived from liposome mediated transfection showed more than one band of greater than 4.8 kb. This result suggests that the plasmid DNA may integrate into the cellular genome at different sites, depending on the mode of DNA delivery.

Discussion

It has been shown that the conventional pH-insensitive immunoliposomes are taken up by cells through a receptor-mediated endocytosis and are delivered to the lysosome and degraded (3). pH-sensitive immunoliposomes, on the other hand, have been shown to mediate cytoplasmic delivery of fluorescent dye (20,21), anticancer drugs (86), and diphtheria A toxin fragment (88). The results of the present study show that DNA can also be efficiently delivered to target cells by

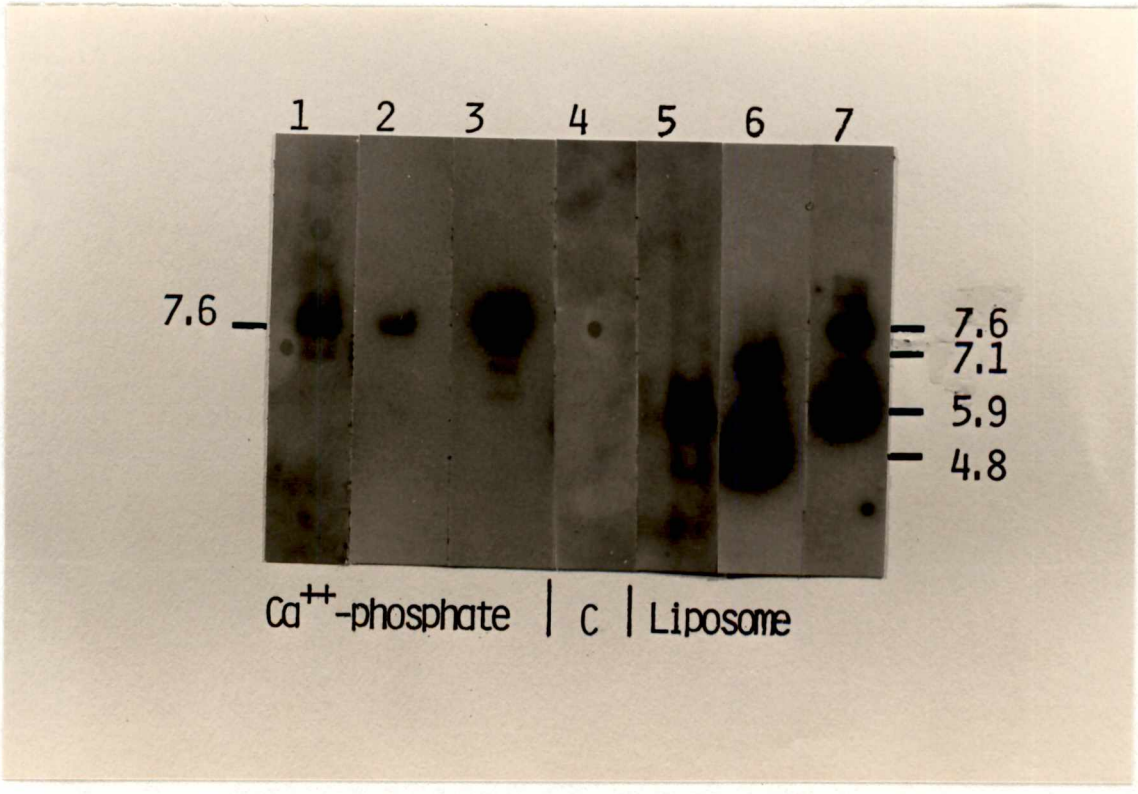


Figure 19. Integrated genes in transformants. DNA was isolated from cells transformed with DOPE:Chol:OA liposome entrapped pPCTK-6A (lane 5,6,7), with calcium precipitated DNA (lane 1,2,3) or from parent untransformed cells (lane 4). All of DNA was digested with restriction enzymes Cla I, Sal I and Eco R1 and hybridized as described in "Methods". The 2.4 Kb Eco R1-Bgl II fragment of pPCTK-6A was used as a probe for the entire-TK gene.

pH-sensitive immunoliposomes. Based on previous work in our laboratory (20,21,86), we suggest that transfection of target cells by DNA-containing pH-sensitive immunoliposomes occurs after receptor-mediated endocytosis of the immunoliposome and liposome-endosome fusion. In addition, results in enzymatic assay showed that liposomes which had been extruded through 0.2 μM filter gave about 2.5 fold enhancement of thymidine kinase activity over liposomes which were not extruded (data not shown). This is likely due to the fact that the smaller liposomes are more efficiently endocytosed by the cells (134,135,136). A possible scheme of the interaction of immunoliposomes with Ltk^- cells is presented in Fig. 20. Cytoplasmic delivery by pH-sensitive immunoliposomes probably occurs via an acid induced fusion of the liposome with the endosome membrane (86,137) similar to the pathway taken by enveloped viruses such as Semliki Forest Virus (138,139), Influenza Virus and Vesicular Stomatitis Virus (139). The DNA delivered into the cytoplasm may be transported into the nucleus by "nuclear membrane binding protein(s)" or "DNA-binding protein(s)." (140). The exogenous DNA in the nucleus is eventually integrated to chromosome which resulted in the expression of gene product(s). The exogenous DNA transfected by calcium phosphate precipitation method may be delivered into the lysosomes so that the majority of transfected DNA was degraded. The undegraded DNA may be released from the lysosomes and then expressed, resulting in a low efficiency of transformation.

The results of both short and long term gene expression experiments indicated that thymidine kinase activity in transfected cells is

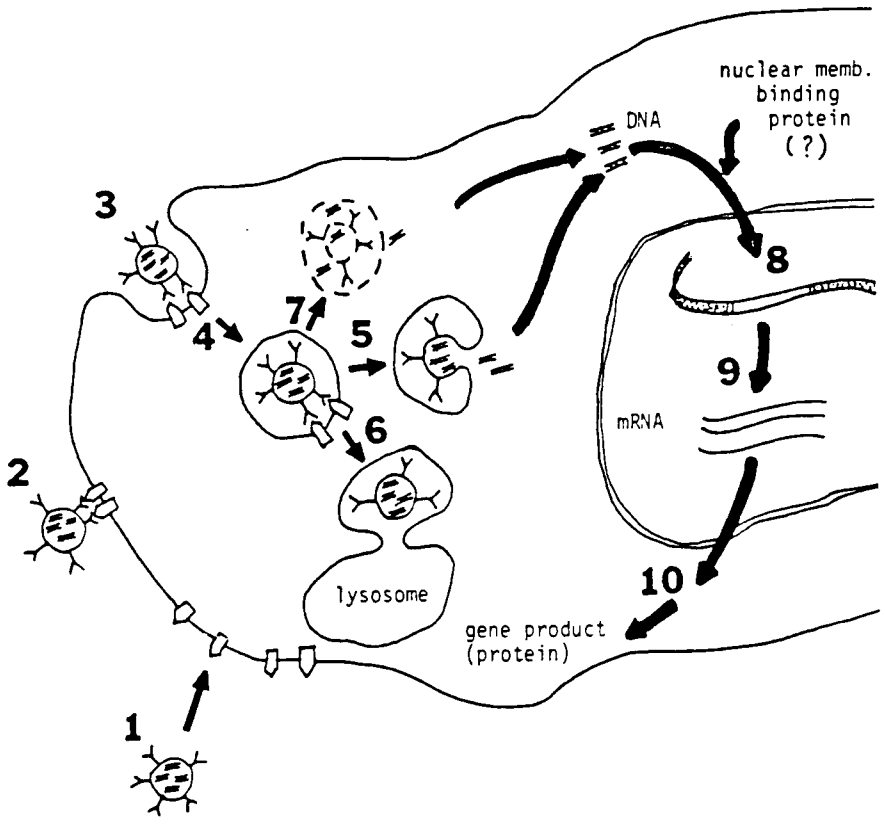


Figure 20. Schematic presentation of the presumed interaction of immunoliposomes with Ltk$^-$ cells and RDM-4 cells. The liposomes bind to cell membrane by interaction between antigens (⬠) and antibodies (Y) (1,2). First, liposomes appear in the endosomes (4) via endocytosis pathway. Some of the endocytosed liposomes may be transported into lysosomes (6) which resulted in degradation of the liposomal DNA. Most of the pH-sensitive liposomes in the acidic endosomal compartments may either fuse with endosomes that resulted in the delivery of DNA into cytoplasm 95). Alternatively, disruption of the endosomal membrane may also deliver the DNA into cytoplasm (7). The DNA delivered into cytoplasm may be transported into nucleus by the "nuclear membrane binding protein(s)" or the "DNA-binding protein(s)." The exogenous DNA in the nucleus is eventually integrated to chromosome (8) that resulted in the expression of gene products, (9, 10).

due to expression of the transferred viral gene rather than reversion of Ltk^- cells because the expression of the enzyme activity is under the control of cAMP. P values of difference between with and without cAMP is 0.02 (degree of freedom = 14). These results agreed with those presented by Wynshaw-Boris et al. (118). It is well known that in prokaryotes, cAMP directly regulates gene transcription by binding to a cAMP receptor protein (141). In eukaryotes, however, mechanisms by which cAMP regulates gene expression are not well understood. It has been recently shown by Liu et al. (personal communication) that mouse L cells, the wild type parent of Ltk^- cells, contain a cAMP control mechanism by which the transfected mouse L cells show cAMP regulation of an expression plasmid containing a 621 bp fragment of the phosphoenolpyruvate carboxykinase gene. Furthermore, Miller et al. (141) have shown that protein extracts of cAMP-treated L cells bind specifically with the PEPCK promoter sequence using a nitrocellulose filter binding assay. These studies, together with the results described in this chapter, clearly demonstrated that mouse L cell and their mutants are excel lent systems for studying cAMP regulation of gene expression in eukaryotic cells.

Short term expression of the transferred gene was examined by monitoring enzymatic activity and [^{3}H]-thymidine uptake while long term expression was determined by monitoring survival of transfected cells in HAT containing medium. Approximately 48% of cells transfected via pH-sensitive immunoliposomes and grown in the presence of cAMP stimulators took up [^{3}H] thymidine (Table 10). It indicated that 48% of

treated cells transiently expressed thymidine kinase. The difference between the degree of increase in thymidine kinase activity on the % of cells that showed increased incorporation of ^{3}H-TdR indicates that pH-sensitive immunoliposomes not only increase the absorption of DNA on the target cells but also increase to an even greater extent the enhanced expression of DNA injected. Southern blots show a clear difference in sites of integration and size of hybridized DNA. It also indicates that clear difference exists in the nature of the DNA introduced to the nucleus. The long term transformation as measured as the survival of cells in the HAT medium containing cAMP stimulators was approximately 9% (Table 11). This indicated that 9% of transformed cells produced enough thymidine kinase to survive in the selection medium. In both experiments, the transformation efficiency of the cells via pH-sensitive immunoliposomes was still much higher than that via the conventional calcium phosphate precipitation method (Table 10 and 11). The P value for both thymidine kinase activity and % cell incorporated ^{3}H-TdR was <0.001 (d.f. = 16).

Genetic materials have previously been introduced into mammalian cells by calcium phosphate precipitation (62,121), microinjection (142,143), retroviral vectors (17) and electroporation (67,144). However, the efficiency of transformation is usually in the range of 10^{-3} to 10^{-5} (145). The low efficiency of transformation may be caused by inefficient delivery, extracellular DNA degradation, or DNA degradation intracellularly. pH-sensitive liposomes increase the efficiency of delivery by (1) maintenance of intact DNA in the extracellular

environment by protecting the DNA from nucleases, (2) specific and enhanced uptake of liposomes by the target cells via antibody and antigen binding and (3) efficient cytoplasmic delivery of DNA via endosome-liposome fusion. Furthermore, the viability of Ltk^- cells after liposome-mediated transfection was much better than that of cells transfected via the calcium phosphate precipitation method. Therefore, it is now possible to study the short-term gene expression immediately after the transfection protocol without the need to wait for cell recovery as is always necessary for other protocols such as calcium phosphate precipitation and electroporation (62,67).

Results from DNA blot hybridization showed that the transfered exogenous DNA was integrated into the host chromosome. Cells which were transformed by calcium phosphate precipitated DNA had one DNA fragment hybridized with the radiolabelled thymidine kinase probe on the Southern blot while cells transformed by pH-sensitive immunoliposomes had two hybridizable DNA fragments. In Wynshaw-Boris' study, transformants of FTO-2B rat hepatoma cells showed one or more than one band on a Southern blot (118) when they were transformed by the calcium phosphate method; however, in our experiments, three independent clones of calcium phosphate transformed Ltk^- cells showed the same size of hybridized DNA fragment. The source of the discrepancy is not clear. Nevertheless, chromosomes of various clones which were transformed via pH-sensitive immunoliposomes contained more than one integration site. The sizes of major fragments on the Southern blots varied from clone to clone. It indicates that liposome delivered DNA

integrated into the chromosome of Ltk^- cells randomly. In addition, the size of hybridized fragment was larger than the size of the probe, PEPCK-HSVTK (see abbreviation). One possibility is that one of the EcoR1 sites on the plasmid was lost when the plasmid integrated into the chromosome of target cells.

Since retroviruses are able to transfer their genetic information with high efficiency into eukaryotic cells, they have been regarded as excellent vectors for gene insertion. Recently, retroviruses have been successfully used to deliver exogenous genes into various hematopoietic cell types in vitro and into murine bone marrow stem cells. However, application of vectors in human gene therapy requires safety assurance. Although the viral oncogene can be eliminated by the removal of encapsulation signal of viruses, the possibility of recombination between vectors and endogenous viral sequences in human cells is still an open possibility and a potential hazard (83). Compared with retroviruses, liposomes which are made of natural phospholipid are nontoxic and degradable in the living cells. The method of liposome preparation is relatively simple and rapid. Therefore, it can be an excellent vector in gene therapy. This is particularly the case if the gene is to be delivered to specific target cells, because the immunoliposomes are target specific via the antibody specificity. In experiments described in the next chapter, the potential of the pH-sensitive immunoliposome for in vivo gene therapy is evaluated in a mouse model.

CHAPTER V

pH-SENSITIVE IMMUNOLIPOSOMES MEDIATE A TARGET SPECIFIC DELIVERY AND CONTROLLED EXPRESSION OF A FOREIGN GENE IN MOUSE

Abstract

We have entrapped a plasmid DNA containing the E. coli chloramphenicol acetyltransferase (CAT) gene in the pH-sensitive immunoliposomes. This bacterial gene was placed under the control of a cAMP-sensitive promoter. The interaction of the monoclonal antibody-coated liposomes (immunoliposomes) with target RDM-4 lymphoma cells grown in immunodeficient nude mice was investigated. About 20% of the injected immunoliposomes were taken up by target cells grown in the peritoneal cavity of nude mice. The uptake was much less if the liposomes did not contain the antibody, or if the mouse did not bear the tumor cells. The results also indicated that the monoclonal antibody on the surface of liposomes provided specific binding to the tumor cells and decreased the uptake of immunoliposomes by spleen. Expression of the CAT activity was only found in target cells treated with pH-sensitive immunoliposomes. Furthermore, exogenous DNA expressed in RDM 4 cells was under the control of cAMP, as only the cells in mice injected with 8-bromo-cAMP and isobutyl methylxanthine showed CAT activity. These results are discussed in terms of the DNA carrier potential of the immunoliposomes in cancer chemotherapy and genetic therapy.

Introduction

The rapid development of the recombinant DNA technology has brought up many new classes of highly effective therapeutic agents. Correction of genetic disorders is now a key aim in the recombinant DNA field. The exogenously introduced normal gene may replace or coexist with the defective gene and produce a normal gene product. This type of work is encouraged by the observation that stem cells of bone marrow can be transformed with a normal gene *ex vivo* and implanted back into patients (111,112). A successful therapy of genetic disorder requires better knowledge of the structure, function, delivery and expression of the new gene that plays an important role in the defective cell. Subsequent studies of recombinant DNA technology have uncovered new information about how the exogenous DNA is organized in cells and how it functions. The growth hormone genes have also been successfully introduced into several kinds of agriculturally important animals to make the animals grow faster or larger (158). Although gene therapy has a great potential in biotechnology, it has not been widely applied. This is due in part to the poor efficiency of DNA delivery. Current methods of delivery of new genetic information into cells *in vitro* include cell fusion (69), chromosome-mediated insertion (70,71), microcell-mediated gene transfer (72,73), liposome carrier of DNA (9), spheroplast fusion (74,75), DNA-mediated gene transfer (76,77), micro-injection (78), infection with recombinant RNA viruses (64,79,80) and infection with recombinant DNA viruses (81,82,83). However, most of these techniques are not applicable for use in animals or humans

because of low efficiency, instability of introduced genes, introduction of extraneous or undesirable genetic information, and the lack of target specificity.

In previous studies, we have used a water soluble fluorescent dye, calcein, as a convenient marker for observing cytoplasmic delivery (20) and used HSV-TK gene as a selection marker for gene transfer (Chapter IV). Our results strongly support a notion that liposomal contents are released into the cytoplasm and the transferred DNA can be expressed in target cells with a high efficiency.

To increase specific binding of liposomes to target cells, anti-$H2K^k$ antibodies were incorporated into the lipid bilayer of the liposome. The present study is designed to test the DNA delivery potential of the pH-sensitive immunoliposomes in an animal model. We have used the chloramphenicol acetyltransferase coding gene as a convenient marker for observing gene transfer and gene expression which is under the control of cyclic AMP. We have also used for comparison a pH-insensitive liposome composition and antibody-free liposomes.

Materials

Acetyl coenzyme A, n-octylglucoside, 8-bromo-cAMP and 3-isobutyl-1-methyl-xanthine (MIX) were obtained from Sigma. SM-2 beads were purchased from Bio-Rad Laboratories. Plasmid pBBO.6 CAT was a gift from Dr. W. D. Wicks. The plasmid DNA was prepared and purified by standard techniques (119). The map of pBBO.6 CAT is shown in Figure 21. The PEPCK gene was the same fragment as in Hanson's plasmid pPCTK-

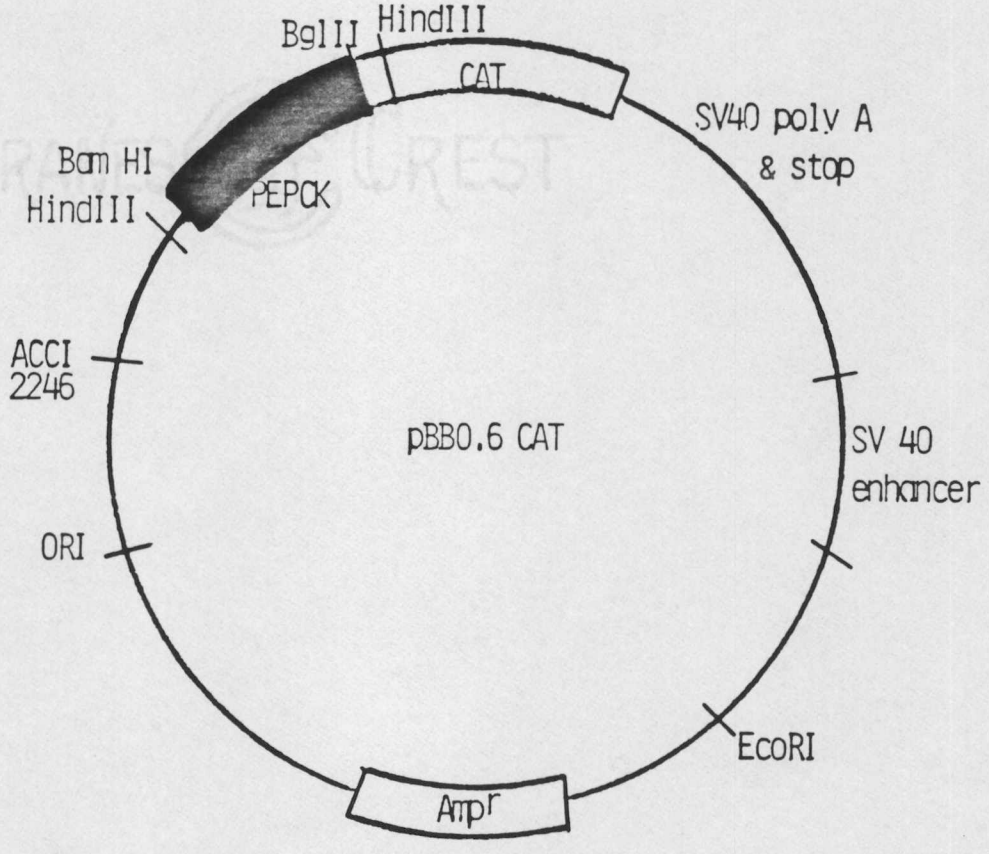


Figure 21. The map of pBBO.6 CAT. Plasmid pBBO.6 was constructed on pBR 322 and contained a bacterial chloramphenicol acetyltransferase gene and a promoter of rat phosphoenolpyruvate carboxykinase gene.

6A in which the promoter, in a 621bp upstream sequence of rat PEPCK gene, was responsible for the sensitivity to cyclic AMP (118). Dichloroacetyl-1, 2-^{14}C-chloramphenicol was purchased from New England Nuclear. Balb/c nude mice were purchased from Life Sciences (St. Petersburg, FL).

Liposome Preparation

Large unilamellar vesicles were prepared as described previously (20) except that pBBO.6 plasmid DNA and palmitoyl antibodies were added into the lipid suspension. The liposomes were separated from free DNA using a column of autoclaved Sepharose CL-2B. ^{3}H-CE (1 X 10^{5} cpm/10 µmole phospholipid) and ^{32}P-DNA (10^{8} cpm/µg DNA) were used to label liposomes and DNA, respectively.

Cell Culture

RDM-4 cells were grown in RPMI 1640 medium supplemented with 10% Fetal Bovine serum and 0.01% sodium pyruvate.

Cell Transformation

1 X 10^{7} RDM-4 cells in 0.5 ml PBS (pH 7.4) were inoculated intraperitoneally (I.P.) in 6 week old nude mice. After 7 days, the mice were also intraperitoneally injected with liposome-entrapped DNA at a dose of 20µg DNA per mouse. One mg per 100 g body weight of 8-Bromo cyclic AMP and 0.5 mg per 100 g body weight of 3-isobutyl-1-methylxanthine were injected intraperitoneally 24 hours later. After 5

hours ascites fluid was harvested from mouse peritoneal by injections of 2 ml phosphate buffer saline (pH 7.4) five times. The ascites fluid which contained RDM 4 cells and macrophages was incubated in a glass petri dish for 6 hrs. The non-adherent RDM-4 cells were harvested from the supernatant and adherent macrophages were harvested from the glass with a cell scraper. After centrifugation, cell pellets were resuspended in 0.25 mM Tris buffer for CAT activity assay. The blood, heart, spleen, lung, stomach, kidney and liver of the mice were also collected and homogenized. An aliquot of the homogenized organs was dissolved in Protosol and counted for ^{3}H and ^{32}P. Two mice per group were used. After centrifugation, the extract of organs was assayed for CAT activity.

CAT assay

CAT activity was determined by a modified method of Gorman (159). Cells were suspended in 125 µl 0.25 mM Tris-HCl, pH 7.4 (Sigma) and frozen at -20°C. The cells were thawed and homogenized by sonication for 15 seconds. The resulting mixture was centrifuged at 12,000 xg for 15 min and the supernatant carefully removed. Protein concentration in the supernatant was measured by Lowry assay (160). Dichloroacetyl-1, 2-^{14}C chloramphenicol (0.5 µCi) and 20 µl acetyl coenzyme A (4 mM) were added into a 125 µl cell extract (1 mg protein) in 0.25 mM Tris-HCl, pH 7.5. The reaction was performed at 37°C for 90 minutes and terminated with the addition of 1 ml of ethyl acetate. Chloramphenicol was extracted with ethyl acetate, evaporated to dryness under a vacuum,

redissolved in 30 µl ethyl acetate and spotted on glass-backed silica gel thin layer chromatography plates (Sigma, T-6895). Separation was achieved in a chloroform:methanol solvent mixture (95:5). The air-dried plates were then exposed to x-ray film for autoradiography with overnight exposure. Regions of the chromatogram containing the acetylated or non-acetylated chloramphenicol were then scraped from the plates and ^{14}C-cpm radioactivity determined by liquid scintillation counting.

RESULTS

Entrapment of Plasmid DNA in the Liposomes

DNA-containing immunoliposomes were prepared by the dialysis method and extruded through polycarbonate filters of 0.2 µm pore size. The pH-sensitive immunoliposomes composed of DOPE:chol:OA (4:4:2 mole ratio) entrapped 16.5% of input DNA. The pH-insensitive immunoliposomes composed of DOPC:chol:OA (4:4:2) entrapped 14.4% of input DNA as determined by gel filtration (chromatograms not shown).

Distribution of Liposomes in Balb/c Nude Mice

The distribution of liposomes was monitored by the appearance of radioactivity from ^{3}H-CE in several organs. This radiolabeled lipid has been shown to be a faithful marker for liposomes and it is neither exchangeable with cellular lipids nor metabolizable by cells (90). Results in Table 12 showed that the presence of antibodies and specific target cells affected the distribution of liposomes among the organs.

Table 12. Biodistribution of liposomes in Balb/c nude mice.[a]

Organ	Liposome PIIL[d] ext.	PSIL unext.	PSIL ext.	PSIL ext. Non-tumored mouse	PSL ext.
Liver	3.6[a]±0.0 (4.5±0.0)	2.5±0.3 (2.5±0.5)	1.6±0.7 (2.1±0.9)	4.5±1.4 (4.5±0.4)	2.2±0.9 (1.5±0.3)
Heart	33±1.9 (3.1±0.3)	40.0±7.9 (4.1±0.2)	30.7±12.2 (3.3±1.3)	7.4±2.7 (0.8±0.1)	11.5±3.2 (1.5±0.7)
Spleen	40.0±5.9 (8.1±0.2)	43.8±7.6 (4.0±0.2)	30.5±4.2 (5.1±1.5)	145.0±27.4 (22.2±3.8)	144.4±24.8 (29.5±4.5)
Lung	13.5±0.9 (1.5±0.3)	8.8±1.1 (1.0±0.1)	13.0±2.8 (2.0±0.8)	6.5±1.3 (0.5±0.2)	6.2±1.7 (1.1±0.6)
Stomach	13.0±3.1 (11.0±4.4)	17.8±2.3 (13.4±6.8)	24.4±3.3 (14.8±6.5)	20.7±1.3 (6.4±1.9)	12.1±4.2 (9.8±6.6)
Kidney	3.9±1.1 (1.3±0.0)	9.6±1.3 (2.5±0.7)	7.0±0.7 (2.4±0.4)	2.1±1.1 (1.1±0.5)	3.0±0.7 (1.5±0.9)
Blood[b]	1.0±0.7 (1.2±0.3)	0.7±0.3 (0.1±0.0)	0.6±0.1 (0.1±0.0)	0.6±0.2 (0.1±0.0)	0.4±0.0 (0.2±0.1)
Ascites Cells[c]					
Adherent	45.0±3.9 (13.3±0.6)	35.7±11.4 (9.8+4.2)	33.8±7.5 (9.6±1.9)	45.0±3.9 (14.4±0.9)	35.5±4.5 (10.5±0.2)
Non-adherent	49.5±11.0 (16.6±2.3)	37.1±12.3 (18.9±2.5)	57.3±14.1 (20.5±3.1)	-	24.5±2.9 (10.1±0.8)

[a]Data expressed as % of liposomes recovered/g organ wt. (mean ± variation of two mice); numbers in parenthesis are the % of total injected liposomes.

[b]% Liposome Bound/ml.

[c]% Liposome Bound/mg protein.

[d]PIIL: pH-insensitive immunoliposome
PSIL: pH-sensitive immunoliposome
PSL: pH-sensitive liposome
ext: extruded through 0.2 μm filter
unext.: unextruded

The recovery of ^{3}H-CE in these organs and cells was in the range of 60% to 70% of input ^{3}H counts. Five different treatments were performed: (1) mice bearing RDM-4 cells and injected with pH-insensitive immunoliposomes extruded through 0.2 μm filter, (2) mice bearing with RDM-4 cells and injected with unextruded pH-sensitive immunoliposomes, (3) as in (2) except that the liposomes were extruded, (4) mice not bearing RDM-4 cells but injected with extruded pH-sensitive immunoliposomes, and (5) mice bearing RDM-4 cells and injected with antibody-free, pH-sensitive liposomes.

There were significantly different counts in the spleens from differently treated mice. The non-tumor bearing mice injected with immunoliposomes or mice bearing RDM-4 cells but injected with antibody-free liposomes showed a very high degree of ^{3}H-CE accumulation in the spleen. Mice bearing RDM-4 cells showed a 3-4 fold decrease in the spleen accumulation of ^{3}H-CE no matter if the immunoliposomes were pH-sensitive or not, or whether they were extruded or not. However the accumulation of ^{3}H-CE in the heart increased 3-5 fold in the latter cases. ^{3}H-CE in non-adherent cells, the majority of which were RDM-4 cells, increased about 2-fold when compared with the mice treated with antibody-free, pH-sensitive liposomes. There was no significant change of ^{3}H-CE accumulations in livers, lungs, stomachs, kidneys, blood and adherent cells of the ascites fluid.

Expression of Plasmid DNA in Ascites Cells

The short-term expression of exogeneous plasmid DNA was monitored by enzyme activity assays. The gene product, chloramphenicol acetyltransferase, catalyzes the transfer of one or two acetyl groups from coenzyme A to chloramphenicol. Eukaryotic cells do not contain this activity (159). When the RDM-4 cells (non-adherent cells in ascites) were transformed with this CAT gene, they expressed and produced chloramphenicol acetyltransferase (Fig. 22). Results here also showed that the expression of the exogenous gene was dependent on cyclic AMP stimulation and the presence of antibody on the liposomes. CAT activity was not found in extracts of the non-adherent cells without stimulation by 8-bromocyclic AMP plus 3-isobutyl-1-methylxanthine (MIX) (Fig.22). To enhance specific binding of liposomes onto target cells, anti-$H2K^k$ antibodies were used. RDM-4 cells express the $H2K^k$ antigen on cell surface while cells of the Balb/c nude mice do not. CAT activity (443 pmol/mg/hr) extracted from non-adherent cells transformed by pH-sensitive immunoliposomes was much higher than that (73 pmol/mg/hr) generated by antibody-free liposomes (Fig. 22). Mice treated with free DNA showed no CAT activity in the cells. There was no detectable CAT activity in the adherent cells (containing macrophage) no matter what type of liposome was used. RDM-4 cells grown in tissue culture also showed no activity.

The expression of CAT activity in the ascites cells seemed dependent on the type of liposomes used. Table 13 shows the data of an experiment in which three different kinds of liposomes were used. It

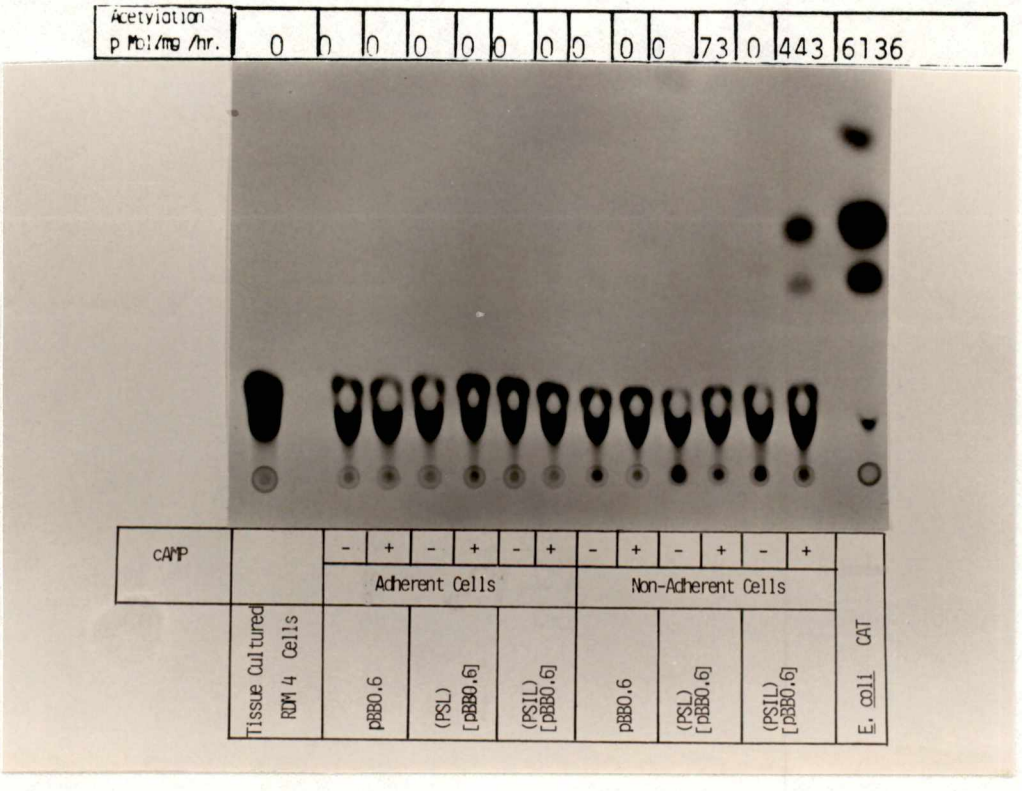


Figure 22. Chloramphenicol acetyltransferase (CAT) activity in Balb/C nude mice. The CAT assay was performed as described in Methods. [pBBO.6] stands for entrapped DNA while pBBO.6 stands for naked DNA. PSL represents pH-sensitive liposomes and PSIL represents pH-sensitive immunoliposomes. Number of observations was one.

is clear that pH-sensitive immunoliposomes were more effective than the pH-insensitive immunoliposomes for gene expression in the non-adherent cells (RDM-4 cells). Liposomes not extruded through a 0.2 μm filter, and thus larger in size, seemed more efficient than those extruded. In all cases, gene expression in the adherent macrophage cells was fairly low.

Table 13. CAT activity in ascites cells of Balb/c nude mice.[a]

	Acetylation (pmol/mg/hr)	
Liposomes[b] Used	Non-Adherent Cells	Adherent Cells
PSIL extruded	502[c]	88
PSIL unextruded	3119	114
PIIL extruded	122	220

[a]All mice were injected with 8-Br-cAMP plus MIX.

[b]See table of abbreviations. PIIL: pH-insensitive immunoliposomes; extruded: liposomes extruded through a 0.2 μM filter.

[c]Number of observations was one.

Gene Expression in the Organs of Nude Mice

To examine if the transferred DNA expressed in the organs of nude mice, CAT activity in the tissue extracts was assayed (Figs. 23, 24 and 25. Extracts from kidney, liver, heart, spleen and lung tissue

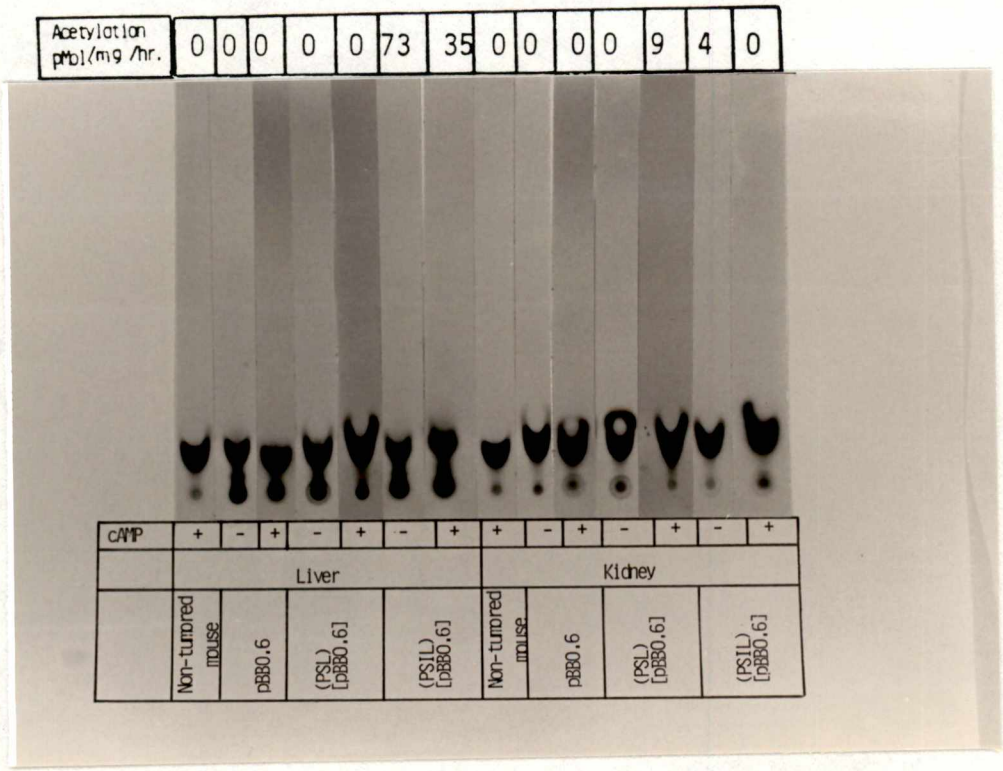


Figure 23. Chloramphenicol acetyltransferase (CAT) activity in livers and kidneys of Balb/C nude mice. CAT assay was performed as described in Methods. [pBBO.6] stands for entrapped DNA while pBBO.6 stands for naked DNA. PSL represents pH-sensitive liposomes and PSIL represents pH-sensitive immunoliposomes.

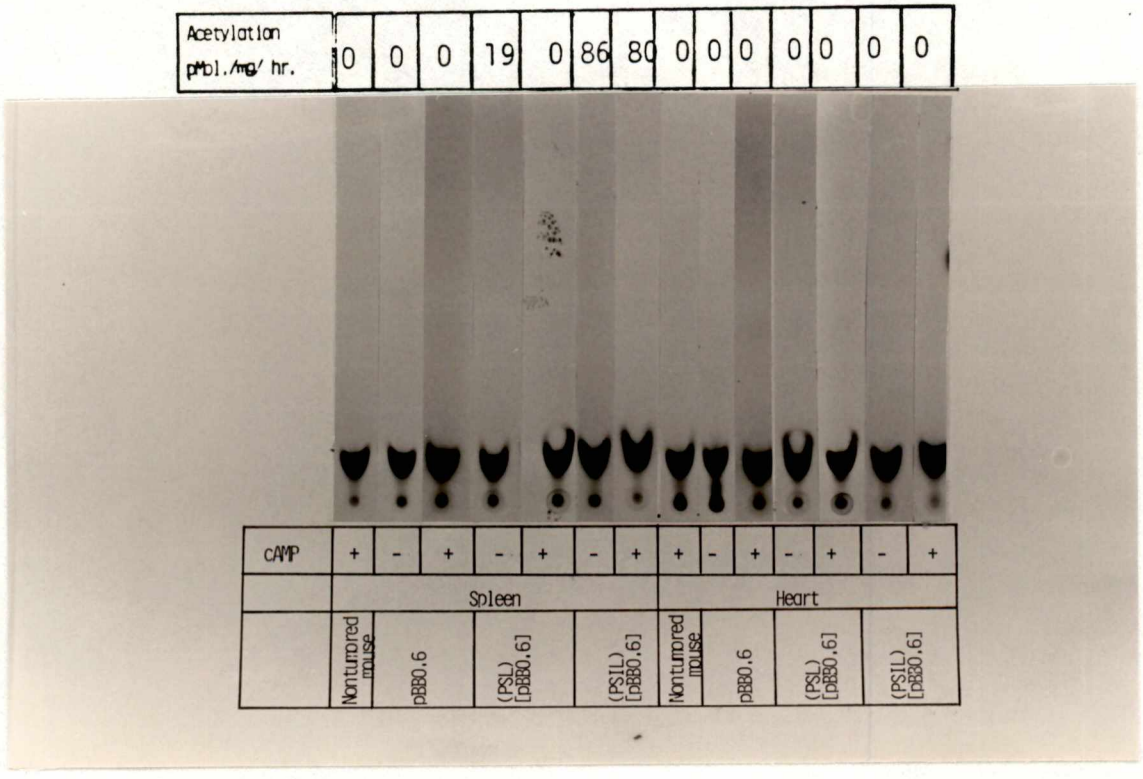


Figure 24. Chloramphenicol acetyltransferase (CAT) activity in spleens and hearts of Balb/C nude mice. CAT assay was performed as described in Methods. [pBBO.6] stands for entrapped DNA while pBBO.6] stands for naked DNA. PSL represents pH-sensitive liposomes and PSIL represents pH-sensitive immunoliposomes.

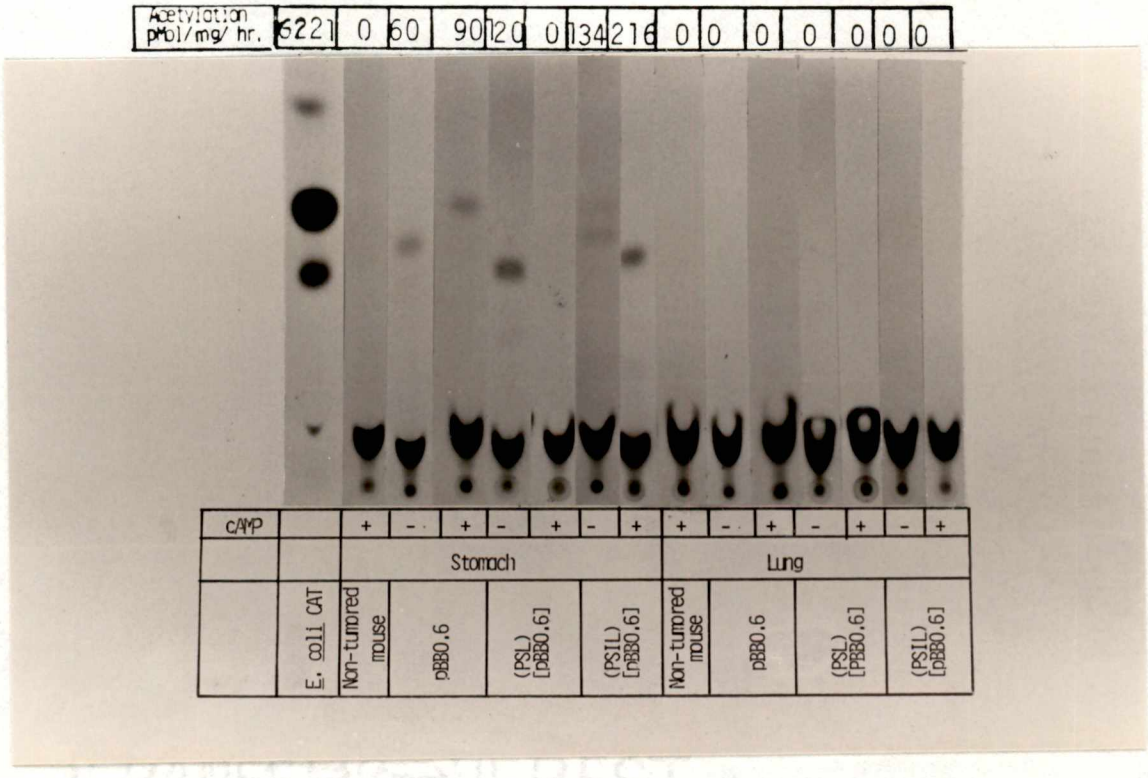


Figure 25. Chloramphenicol acetyltransferase (CAT) activity in stomachs and lungs of Balb/C nude mice. CAT assay was performed as described in Methods. [pBBO.6] stands for entrapped DNA while pBBO.6 stands for naked DNA. PSL represents pH-sensitive liposomes and PSIL represents pH-sensitive immunoliposomes.

showed no detectable CAT activity. However, spleens and livers of mice which were treated with pH-sensitive immunoliposomes had low levels of CAT activity (80/86 and 35/73 pmol/mg/hr for with/without cAMP stimulation, respectively). Extracts from stomachs of the treated mice showed variable CAT activities. A separate examination of an untreated normal nude mouse also showed CAT activity in the stomach. Since the food residues in the stomach were not removed before the extract was prepared, the CAT activity in the stomach extract was probably due to the microorganisms in the food residues. The acetylated chloramphenicol catalyzed by the stomach extracts showed irregular migration on TLC plates. In addition, the intensities of the acetylated chloramphenicol was not related to the presence of cAMP stimulation.

DISCUSSION

This study provides evidence that pH-sensitive immunoliposomes can mediate target-specific DNA delivery in an animal model. In the current study, mice bearing no tumors but treated with immunoliposomes or the tumor-bearing mice treated with antibody-free liposomes showed a high degree of accumulation of liposome in the spleen, whereas the tumor-bearing mice treated with immunoliposomes had a much lower liposome accumulation in the spleen. Although the accumulation in the RDM-4 cells was only modestly (about 2 fold) increased in the latter case, it is clear that the biodistribution of the immunoliposomes is significantly different from that of the antibody-free liposomes. Nonspecific uptake by spleen cells is likely due to the

reticuloendothelial cells (macrophages) which are also abundant in liver. However, accumulation in liver is fairly low. This observation may be unique to the nude mice since IP injected liposomes accumulate in the liver of normal mice (163). Further studies are required to clarify this point.

Although the target RDM-4 cells were not the only cells in the mouse to which pH-sensitive immunoliposomes bound, these cells were the only ones which expressed a significant level of CAT activity. It is interesting to note that the adherent macrophage cells in the same peritoneal cavity did not express any enzyme activity despite the fact that they accumulated some liposomes. Furthermore, the spleen cells in the non-tumored mice also took up a large amount of the pH-sensitive immunoliposomes, but showed only a very low level of gene expression. Several possibilities exist for the explanation of this observation. It is possible that the liposomes were unstable in the mouse peritoneal cavity resulting in separation of DNA from liposomes, and then the liposome remnants entered the circulation via the thoracic duct and were taken up by the macrophages in the spleen. Other possibilities were that DNA was degraded in the macrophages, or the intact DNA was delivered but could not be efficiently expressed in macrophages because of the lack of cAMP regulatory factor(s) (141). Wynshaw-Boris et al. (118) have reported that such a factor(s) may be tissue specific. When liposomes containing ^{125}I-tyraminyl-inulin were intravenously injected into mice, the DOPE:oleic acid liposomes were very leaky in the presence of plasma (162). However, the presence of cholesterol

significantly increased the stability of DOPE/oleic acid containing liposomes (Tsusaki and Huang, unpublished observation). When ^{14}C-inulin was entrapped in DOPE/cholesterol/oleic acid (4:4:2 molar ratio) and injected i.p. into the peritoneal cavity of nude mice, its distribution profile was similar to that of ^{3}H-CE (data not shown). Thus, cholesterol containing pH-sensitive liposomes appear to be stable in vivo. This result suggests that intact liposomes were delivered to the macrophages, but the DNA was either degraded or not expressed. Further studies are required to distinguish between these possibilities.

Data from the CAT assays (Table 13) indicated that replacing the DOPE of pH-sensitive immunoliposomes (PSIL) with DOPC (PIIL) to generate the pH-insensitive immunoliposomes decreased the ability of immunoliposomes to deliver DNA to RDM-4 cells. These results indicate that the pH-sensitive immunoliposome may release its contents into the cytoplasm of a target cell after it is endocytosed; whereas the pH-insensitive immunoliposome delivers its contents to the lysosomes where they are degraded. This is in agreement with our previous conclusion (20,21). The same experiment also showed that larger unextruded liposomes were more effective delivery vesicles than the smaller extruded ones (Table 13). This result is not consistent with that of the in vitro experiments in which the extruded liposomes were more efficient in delivering the HSV-TK genes to the mouse Ltk^- cells (Chapter IV). Whether it is due to a difference in cell types or growth conditions (in vitro vs. in vivo) is not clear at the present time.

Nicolau et al. (14,15) have shown gene expression in the livers of rats which received intravenous injections of the preproinsulin I gene encapsulated in antibody-free, conventional pH-insensitive liposomes. Although this work clearly demonstrated the gene transfer potential of liposomes *in vivo*, it has several important differences from our work. Our previous work (Chapter IV) and the work described here have indicated that pH-sensitive immunoliposomes gave a transformation efficiency which was much higher than that of the pH-insensitive immunoliposomes. The current study also utilized an antibody to the $H2K^k$ antigen to enhance the DNA delivery potential of liposomes to the target tumor cells. Although the uptake of liposomes by reticuloendothelial cell-rich tissues such as spleen could not be avoided, the immunoliposome system still indicated its superior potential in gene delivery. Nicolau's work showed gene delivery only to liver presumably because of the natural homing tendency of the untargeted liposomes.

The implications of our results in cancer therapy cannot be overlooked. DNA coding for cytotoxins such as the A fragment of diphtheria toxin (165) could be delivered to the target tumor cells for killing. This novel type of chemotherapy is potentially more superior than the immunoliposome mediated delivery of conventional cytotoxic drugs such as methotrexate in several ways. First, the action of the gene should be much more potent than the action of drugs due to a large degree of amplication of the gene through transcription and translation. Next, if the DNA leaks out of the liposomes it should not be toxic because of rapid extracellular nuclease digestion; whereas the free cytotoxic

drugs are toxic to normal tissues. Our results also indicated that although the host organs took up liposomes, we did not find a significant CAT activity from spleen, liver, kidney and heart. This added level of specificity in cancer chemotherapy may be very important, because by selecting appropriate control mechanisms, the delivered toxic gene may be expressed only in the tumor cells. This new modality of cancer therapy is currently under investigation.

The current work also has implications in gene therapy. Two potential advantages of the antibody-coated liposomes in delivering DNA are their high transfer efficiency and safety. Successful human gene therapy will require efficient gene transfer and proper expression of the delivered gene in appropriate target cells (17). Retroviral vectors have been used as successful gene carriers (146-157). In many cases, the transferred genes were able to express in several hematopoietic cell types in vitro and in murine bone marrow stem cells. Although the retrovirus system has a much higher transformation efficiency than the conventional liposomes do, the viral vectors are species-specific. So far, there is no available retrovirus vector for human gene threapy. The pH-sensitive immunoliposomes may be a good compromise in this regard. Furthermore, the fact that expression of the liposome delivered gene can be properly controlled by an external signal, such as the cAMP in the present study, is also an important advantage in human gene therapy.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Perhaps the most significant novel contribution of this dissertation work is the establishment of the pH-sensitive lipid composition, dioleoyl phosphatidylethanolamine (DOPE): cholesterol:oleic acid (OA) (4:4:2) as the best liposome composition for cytoplasmic delivery (shown in Chapter II and ref. 18). The inclusion of cholesterol is very important as it significantly stabilizes the structure of the liposome, particularly in serum. The superior delivery properties of this liposome composition, for both plant and animal cells, correlate well with its sensitivity to acid (Chapter II). Thus, these results further substantiate our notion that the pH-sensitive liposomes deliver their contents by fusing with the endosome membrane once endocytosed by the cell (137). This very mild method is likely to find its application in the delivery of many different kinds of biomolecules to target cells.

The emphasis of the present work is to deliver one special class of biomolecule, i.e. DNA. A very mild method was first developed for efficient entrapment of plasmid DNA in liposomes. This method completely avoids the use of organic solvents and sonication and gives reasonably high trapping efficiency for DNA. Acylated monoclonal antibody can be easily incorporated into the surface of the liposomes using this method.

The pH-sensitive liposome was first tested in higher plant

protoplasts. Because specific antibody was not available, we have used a relatively low level of PEG to promote liposome-protoplast interaction. Our results clearly demonstrated that the pH-sensitive liposomes are more superior to other conventional liposomes for cytoplasmic delivery. Although the initial work was done by using a fluorescent dye, calcein, subsequent work in another laboratory (Zhu and Hughes, unpublished observations) showed that these liposomes were also efficient in delivering DNA to produce transgenic plants.

Major emphasis was placed on the delivery of plasmid DNA to animal cells, both *in vitro* and *in vivo*. These studies took advantage of a known cAMP regulatory sequence isolated from the 5' flanking region of the rat PEPCK gene. This control element which included the promoter was placed upstream to the foreign genes for a controlled expression by cAMP. HSV thymidine kinase gene was delivered to the Ltk^- cells *in vitro* and the *E. coli* chloramphenicol acetyltransferase (CAT) gene was delivered *in vivo* to mouse RDM-4 lymphoma cells grown in Balb/c nude mice. Again, the results showed that the pH-sensitive liposomes performed better than the pH-insensitive ones, and the presence of the targeting antibody was essential for optimal delivery. For the mouse Ltk^- cells, pH-sensitive immunoliposomes mediated a 9% long-term transformation efficiency which was much higher than the conventional calcium phosphate precipitation method.

A surprising result was obtained with the *in vivo* delivery of the CAT gene. Although the pH-sensitive immunoliposomes did not exclusively accumulate in the target RDM-4 cells, these cells were the

only ones which showed significant expression of the CAT gene. The result may be fortuitous in that the lymphoma cells may be the only cells which are capable of expression of this foreign gene. Nevertheless, the results demonstrated for the first time that a foreign gene could be delivered and expressed under the control of an external signal, i.e. cAMP, in the target cells *in vivo*. The enormous implications of this result in cancer chemotherapy and genetic therapy are discussed in Chapter V.

The potential of using pH-sensitive immunoliposome for target-specific DNA delivery is demonstrated in this work. This is only the beginning of a new era which is likely to blossom in many important areas of biology, agriculture, and medicine.

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