Title
Enhanced cytoplasmic delivery of liposome-encapsulated drugs

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Publication Date
1990

Peer reviewed|Thesis/dissertation

ENHANCED CYTOPLASMIC DELIVERY OF LIPOSOME-ENCAPSULATED DRUGS

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

To My Father and Mother

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Frank C. Szoka for his guidance on the project. I am grateful for his patience, encouragement, and advices. I have benefited both scientifically and personally from our discussions over the years working in the lab.

Special thanks to Dr. D. Eppstein and V. Marsh, Syntex Research, and Dr. M Strand, Johns Hopkins University, for their generous gifts of the viruses used in the study.

I also like to thank the members of my oral committee, Drs. L. Benet, W. Sadee, S. Hawkes, M. Shetlar, and D. Stites for their comments and helpful suggestions for this project. Gratitude is extended to the members of my thesis committee, Drs. Wolfgang Sadee and Frances Brodsky for their advices on the preparation of the thesis. I have to extend my appreciation to Dr. Svein Øie for serving as my graduate advisor and offering me his advices.

I am grateful to the members in Szoka's lab, especially, Drs. Roberta Parente, Paul Dal Monte, Aeri Kim, and Jan Dijkstra, for their friendship and support. I also like to thank the fellow graduate students in the School of Pharmacy for making my graduate student life more interesting.

Finally, I must thank my family, particularly my sister, Chun-Hui Tang, for their endless care and support and for providing me a loving shelter to rest over the years.

ENHANCED CYTOPLASMIC DELIVERY OF LIPOSOME-ENCAPSULATED DRUGS

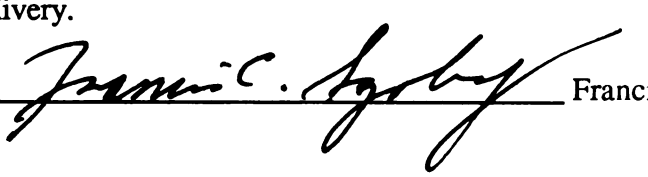
Chun-Jung Chu

Lysosomotropic delivery of liposomal contents prevents the drug from reaching the cytoplasmic compartment. To circumvent lysosomal degradation of liposomal contents, I have exploited the acidic environment of endocytic vacuoles using two delivery systems: (i) pH-sensitive liposomes, which may fuse with the endosomal membranes and release their contents into the cytoplasm; (ii) Standard liposomes encapsulating acid titratable drugs, which can be protonated in acidic compartments and permeate through the vesicular membranes into the cytoplasm. The in vivo therapeutic activity of a liposome-encapsulated acid titratable drug was investigated in a murine retroviral model.

The pH-sensitive liposomes composed of unsaturated phosphatidylethanolamine and cholesterylhemisuccinate could deliver macromolecules to the cytoplasm of cells in vitro. The efficiency of the cytoplasmic delivery estimated using fluorescent probes and a biological active macromolecule, diphtheria toxin A chain, was greater than 0.01% but less than 10% of the cell associated liposomal contents. Cytoplasmic delivery required a low pH and the presence of divalent cations.

The entry of phosphonoformate (PF) and phosphonoacetate into cells is impeded by three acid titratable groups which are ionized at pH 7.4. The relationship between the anti-Herpes simplex virus activity and the uptake of the liposomal drugs was compared to the non-encapsulated drugs in culture. Cellular uptake of liposomal but not non-encapsulated drug saturated with increasing drug concentration. This permitted a concentration of PF in liposomes to be chosen that led to a 27-fold increase in selectivity when compared to the non-encapsulated drug.

The therapeutic efficacy of PF was evaluated in vivo using a Rauscher murine leukemia viral model. The target cells of the viral infection are located in the liposome-accumulating organs (the liver, spleen, and bone marrow), but the cells did not avidly internalize liposomes. Residence time and local concentration of PF in the liver and spleen were improved 10-100 fold when delivered in the liposomal form. However liposome encapsulation did not enhance the antiviral effect of the drug. This result reflects the inability of the viral target cells to take up liposomes. Thus delivery of membrane-impermeant drugs to target cells, rather than to target organs, is essential for a success in targeted liposomal delivery.

Thesis Chairman :  Francis C. Szoka, Ph.D.

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Ab	Antibodies
AIDS	Acquired immunodeficiency syndrome
Ara-C	Cytosine arabinonucleoside
AUC	Area under the drug level-time curve
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferase
CF	Carboxyfluorescein
Ch	Cholesterol
CHEMS	Cholesterylhemisuccinate
COPE	N-succinyldioleoylphosphatidylethanolamine
CPE	Cytopathic effect
DCPA	Double-chain protonizable amphiphiles
DEPE	Dielaidoylphosphatidylethanolamine
DHPC	Diheptadecanoylphosphatidylcholine
DMEM	Dulbecco modified Eagle's medium
DMPC	Dimyristoylphosphatidylcholine
DOPC	Dioleoylphosphatidylcholine
DOPE	Dioleoylphosphatidylethanolamine
DOSG	1,2-dioleoyl-sn-3-succinylglycerol
DPPC	Dipamitoylphosphatidylcholine
DPSG	1,2-dipalmitoyl-sn-3-succinylglycerol
DPX	N,N'-*p*-xylenebis(pyridinium bromide)
DRV	Dehydration-rehydration vesicles
DRVET	Dehydration-rehydration vesicles extruded
DTA	Diphtheria A chain
EC_{50}	Concentration at 50% effect
EDTA	Ethylenediaminetetraacetic acid
Empty Lipo	Non-drug containing liposomes
EPC	Egg phosphatidylcholine
EPE	Egg phosphatidylethanolamine
EPG	Egg phosphatidylglycerol
EPA	Egg phosphatidic acid
EPS	Egg phosphatidylserine

FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FITC-poly GL	FITC labelled poly-(D-glutamic acid-D-lysine)
Fl 450/413	Ratio of fluorescence emission at excitation wavelengths of 450 & 413 nm
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H_I	Hexagonal phase
H_{II}	Inverted hexagonal phase
HIV	Human immunodeficiency virus
HPTS	8-hydroxy-1,3,6-pyrene-trisulfonate
HSV-2	Herpes simplex virus type 2
IC_{50}	Concentration at 50 % inhibition
IMI	Inverted micellar intermediate
i.v.	Intravenous(ly)
i.p.	Intraperitoneal(ly)
$L\alpha$	Lamellar liquid crystalline phase
$L\beta$	Lamellar liquid gel phase
Lipo-PAA	Phosphonoacetate containing liposomes
Lipo-PF	Phosphonoformate containing liposomes
LUV	Large unilamellar vesicles
LUVET	Large unilamellar vesicles extruded
LysoPC	Lysophosphatidylcholine
MLV	Multilamellar vesicles
MLVET	Multilamellar vesicles extruded
MTX	Methotrexate
NDC	Non-drug containing
NH_4Cl	Ammonium chloride
OA	Oleic acid
OD	Optical density
PBS	Phosphate buffer saline
PA	Phosphatidic acid
PAA	Phosphonoacetate
PAP	Pokeweed antiviral protein
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PF	Phosphonoformate
PFU	Plaque-forming units

PG	Phosphatidylglycerol
PHA	N-palmitoyl-L-homocysteine
PI	Phosphatidylinositol
PS	Phosphatidylserine
Poly-GL	Poly-(D-glutamic acid-D-lysine)
RES	Reticuloendothelial system
REV	Reversed phase evaporation vesicles
REVET	Reversed phase evaporation vesicles extruded
RMLV	Rauscher murine leukemia virus
SCD	2,3-Seco-5α-cholestan-2,3-dioic acid
SD	Standard deviation
SUV	Sonicated unilamellar vesicles
$t_{1/2}$	Half-life
TCA	Trichloroacetic acid
T_h	L_α to H_{II} phase transition temperature
TK	Thymidine kinase
TPE	Transesterified phosphatidylethanolamine from egg phosphatidylcholine

CHAPTER I: INTRODUCTION

Phospholipid vesicles are useful as a drug delivery vehicle. Numerous studies have demonstrated that liposomes are taken up by phagocytic cells of the reticuloendothelial system (RES) and accumulate in the liver and spleen when administered intravenously. Liposomes are internalized by cells via endocytosis and transported to the lysosomes where the lipids and entrapped contents are subjected to degradation by lysosomal enzymes. Attempts to direct drugs to intracellular active sites other than the lysosomes and to potentiate the therapeutic effect of drugs that act in the cytoplasm have been undertaken by the judicious selection of the drug or liposomes made with novel lipid compositions.

1. Specific Aims of the Research

The overall objectives of this research are :

(i) To evaluate new methods for cytoplasmic delivery using liposomes.

(ii) To understand the factors that influence cytoplasmic delivery of the liposome encapsulated agents.

(iii) To determine whether the inherent distribution pattern of liposomes can be used to concentrate a membrane impermeant, water soluble drug in the RES, to the extent that a therapeutic drug level can be reached in non-RES cells localized in the same organ.

2. Hypotheses of the Research

The respective hypotheses that are tested are :

(i) pH-sensitive liposome compositions can deliver macromolecules into the cytoplasm.

(ii) Drugs with acid titratable groups can show enhanced delivery when encapsulated in liposomes.

(iii) Passive targeting of liposome encapsulated non-permeant drugs can lead to increased efficacy in vivo.

In this chapter, the literature on the use of liposomes as a targeted drug carrier is reviewed. The first section is a general overview of drug delivery systems, relevant information about the anatomy/physiology of the body and how these features affect drug carriers in the body. The second section covers the relevant biochemical and pharmacological characteristics of liposomes. The third section is a review of the literature on liposomal targeted drug delivery at the cellular and subcellular level and the problems associated with the delivery. The fourth section reviews pH-sensitive liposome compositions and discusses the factors influencing the efficiency of cytoplasmic delivery via this approach. The last section will describe the scope of this thesis.

3. Drug Delivery Systems

Novel dosage forms have been developed to improve the therapeutic index of drugs and patient compliance. Two major areas of development have focused on controlled-release devices (Grass & Robinson, 1990) and site-specific delivery (Gregoriadis et al., 1983; Tomlinson & Davis, 1986). Controlled release has been applied to both conventional dosage forms, e.g. tablets/capsules, and novel dosage forms, e.g. skin-patches and implanted osmotic pumps. The sustained release of drug provides a constant plasma level which in turn predictably elicits the desired pharmacological effect without changing the therapeutic index. Targeted delivery is designed to increase the therapeutic index of the drug. This improvement occurs either by increasing the drug concentration at the target site or by decreasing the drug concentration at the toxic site. Site-specific delivery is a difficult task. This is due to the complexity of active targeting moieties and the anatomical, physiological and cellular barriers (Tomlinson & Davis, 1986). In addition to the physiology of the target site, pharmaceutical, pharmacokinetic and pharmacodynamic properties of the drug have to be considered for developing a commercially suitable targeted drug (Stella & Himmelstein, 1980; Notari, 1985; Hunt et al., 1986).

Targeted Delivery

The "magic bullet" concept of Ehrlich has been the guiding paradigm for targeted delivery. A targeting-moiety is attached to the drug and the resulting targeted "bullets" will "home" to the target site due to the specific affinity of the attached ligands to the target receptor. An example of this is immunoconjugates which are monoclonal antibodies against certain cell surface antigens which have an attached drug (Vitetta et al., 1987; Till et al., 1988). Hormones (Chaturvedi et al., 1984), growth factors, chemopeptides,

lipoproteins, and carbohydrates (Tomlinson, 1990) have all been conjugated to increase the local drug concentration at the target site. A second approach to targeted delivery is to use a prodrug which can be transformed to an active drug moiety by certain enzymatic reaction. Local high concentrations of drug are also obtainable after cleavage of an active moiety from prodrugs by site-specific enzymes present at or delivered to the target site (Stella & Himmelstein, 1985; Senter et al., 1989). The third approach is to incorporate or encapsulate the drug in a targeted carrier and form a "magic pistol". Once the carrier reaches the target site, the drug is released and a high local concentration of the drug is obtained. The last approach has the dual advantages of targeted delivery and controlled release of the drug from the carrier. Many particulate systems have been used for this purpose, e.g. liposomes, erythrocyte ghosts, and various microspheres made of chylomicrons, lipoproteins, polyamide, polycyanoacrylates, cellulose, gelatin, agarose, starch, and albumin (Tomlinson, 1990). When administered in vivo, the fate of particulate carriers is partially governed by the anatomy of the vasculature and a natural host defense mechanism - the reticuloendothelial system.

Anatomy of the Vasculature

Targeting to cells in the majority of organs will require extravasation of drug carriers from the circulation. The anatomic structure of the circulatory system plays a crucial role in the extravasation process. Blood capillaries are classified into three different groups according to the architecture of the endothelium and the underlying basement membrane : continuous capillaries, fenestrated capillaries, and discontinuous (sinusoidal) capillaries (Figure 1). In continuous capillaries the endothelium forms a continuous lining in which adjacent endothelial cells connect via tight junctions. In fenestrated capillaries which can be seen in the kidney glomeruli, the endothelium is interrupted by fenestrae which vary from 30 nm to 80 nm in diameter. The discontinuous capillaries are thin walled vessels found only in the liver, spleen and bone marrow. The endothelium in these vessels has large gaps (0.1-1 μm in diameter) and the basement membrane in these vessels is either interrupted or disappear, while the basement membranes in the other two capillaries are continuous. Therefore particulate drug carriers with a diameter smaller than the gap will be most likely to leave the vascular bed in the sinusoidal capillaries (Shepro & D'Amore, 1984; Simionescu & Simionescu, 1984). In contrast, large particulates cannot escape the vasculature and may arrest in the capillaries, depending upon their sizes and the diameters of vessels, by the mechanical filtration in the vessels. A comparison of linear

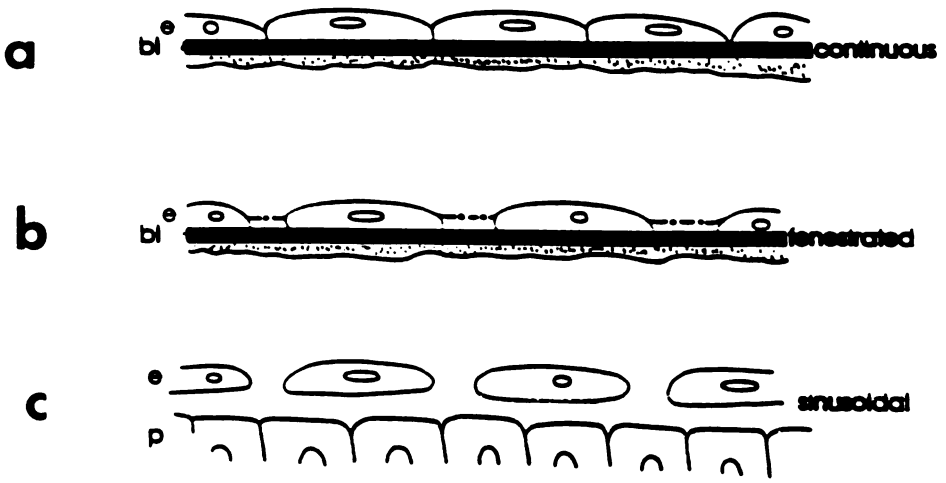


Figure 1. Schematic illustration of the structure of different classes of blood capillaries. (a) Continuous capillary. (b) Fenestrated capillary. (c) Discontinuous (sinusoidal) capillary. e: endothelial cells; bl: basement membranes; p: parenchymal cells. Permission requested from Drs. Poste & Kirsh (1983).

representation of the diameter of vessels and permeability through various pores of the vasculature is shown in Figure 2.

Administration of particulates at the extravascular site can eliminate the obstacles to extravasation. However dynamic lymph flow will bring the particles away from the administration site. The lymphatic absorption of the particles is also dependent on the particle size, since the various pores or fenestraes are present at the lymphatic vessels. Though the drainage of the lymph into the bloodstream (Figure 3) will bring the particulates into the blood circulation, these particles may pass through extravascular spaces and regional lymph nodes and encounter different kind of cells before entering the circulation (Weinstein, 1983). Depending upon the location of the target, extravascular application of drug carriers may be more advantageous than the systemic administration for targeted delivery.

Reticuloendothelial System

The clearance of endogenous senile cells, dead tissues, and foreign particles is linked with phagocytic cells of the reticuloendothelial system. The main features of the RES cells are that they are highly phagocytic, mononuclear, and present in many different organs of the body. Some cells are settled tissue components while others are wandering or migratory, all being called macrophages. The RES cells include the sinusoidal lining Kupffer cells of the liver, histiocytes of the connective tissue, alveolar macrophages of the lung, macrophages and sinusoidal lining cells of the spleen, free and fixed macrophages of the lymph nodes, macrophages and sinusoidal lining cells of the bone marrow, macrophages in the peritoneal cavity, osteoclasts of the bone tissue, and microglia of the nervous system. It is generally believed that macrophages within tissues are replenished by replacement from bone marrow, traveling in the blood as monocytes, with a limited capacity for local proliferation. Together, these RES cells are responsible for the removal of particulates from the circulatory compartment and from the extravascular spaces in the body (Bradfield, 1984). The phagocytic capacity of the RES is saturable, thus increasing doses of intravenously administered particulates leads to decreasing rates of clearance of the subsequent dose of particulates from the circulation. The saturation of the RES clearance mechanism is called RES blockade and can be employed to alter the clearance rate and biodisposition of particulate carriers (Abra et al., 1981; Bosworth & Hunt, 1982).

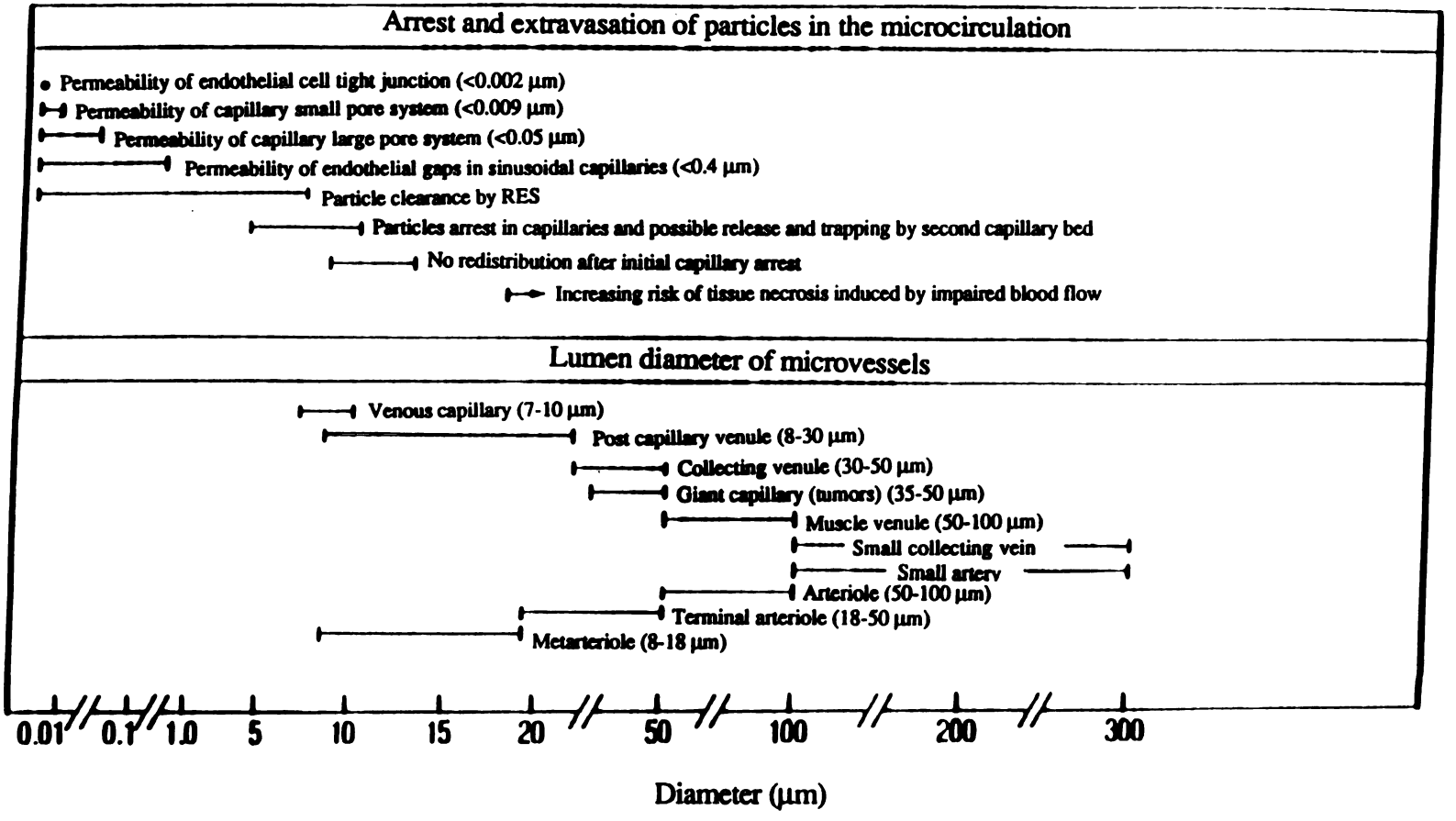


Figure 2. Sizes of pores in the vascular bed and lumen diameter of microvessels. Top panel: the effect of particles size on particle arrest in the microcirculation, clearance by the RES and the limiting sizes for particle extravasation. Bottom panel: the internal lumenal diameter of different microvessels in the venous and artial circulations which define at which level in the vascular bed arrest of particles of different sizes will occur. Permission requested from Drs. Poste & Kirsh (1983).

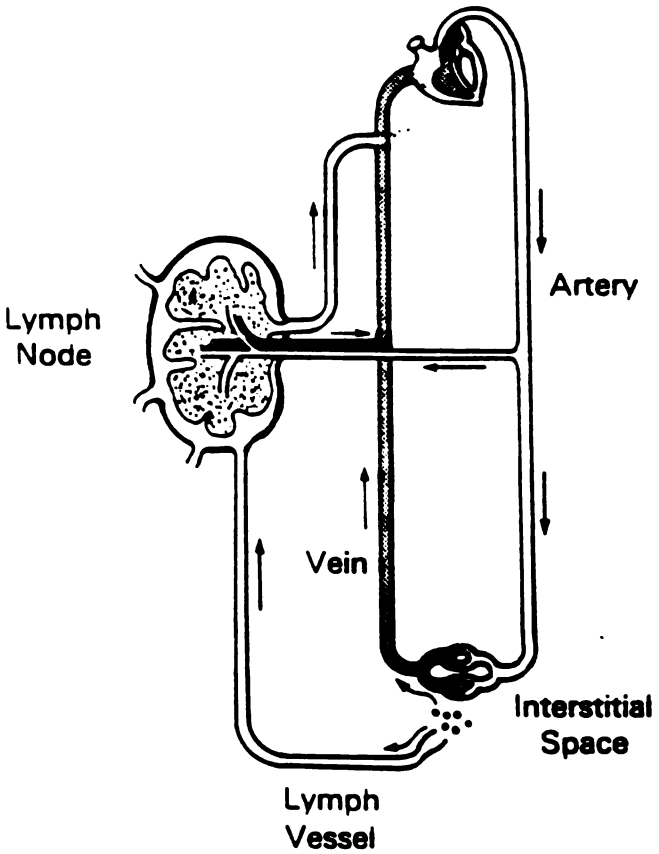


Figure 3. Schematic view of the passage of particulates from an extravascular injection site. Given the size, very few will pass into the bloodstream directly. Small-diameter particulates are mobilized from the site much more quickly than are large particulates. Permission requested from Dr. Weinstein (1983).

Biodisposition of Particulate Carriers

Due to the structure of the vasculature and the phagocytic activity of the RES, biodistribution and clearance of drug carriers are partially controlled by their size and route of administration. Particles greater than 7 μm are most likely to be entrapped in the capillary bed of the lung after intravenous (i.v.) administration. Particles with a size between 2-7 μm are easily trapped in capillaries of any organs which are close to the administered site (Tomlinson, 1990; Poste and Kirsh, 1983). Particles smaller than 2 μm in diameter are mainly cleared from the bloodstream by mononuclear phagocytes of the RES and lead to the localization of the particles in the liver, spleen, and bone marrow. Thus passive-targeting, where the distribution of the carrier is determined by the physiology and anatomy of the body, drugs to different organs can be achieved by employing large daimeter carriers. Whereas active targeting, where the homing of the carrier is controlled by the affinity of surface ligands to target receptors, may be obtainable by using small diameter carriers incorporated with active-targeting ligands on the surface. Among all the particulate systems, liposomes have been the most vigorously developed as a potential drug carrier.

4. Liposomes

Liposomes were first described by Bangham and colleagues as concentric continuous multilamellar phospholipid bilayers separating an equal number of aqueous compartments (Bangham et al. 1965). The bilayer structures are formed spontaneously upon hydration of amphiphilic phospholipids. The hydrophilic head groups of phospholipids are oriented on either side of bilayer in contact with the aqueous phase whereas the hydrophobic acyl chains form the core of the bilayer. The hydrophobic effect is the thermodynamic force which minimizes contact between the nonpolar portions of the lipids and aqueous phase. Entrapment of liposomal contents can be accomplished by passive encapsulation of a water soluble compound in the aqueous phase or by intercalation of a lipophilic substances into the lipid bilayer.

Types of Liposomes

Various methods have been developed to prepare different kinds of liposomes (Szoka & Papahadjopoulos, 1980; Deamer & Uster, 1983). Liposomes are classified according to the number of lamellar bilayers and the diameter of the vesicles, multilamellar vesicles (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) are

Table 1. Characteristics of liposomes prepared by various methods.

Procedure	Type	Diameter (μm)	Trapped volume (l/mole lipid)	Reference
Direct hydration	MLV	0.4-3.5	1-5	Bangham, et al. 1965
Sonication of MLV	SUV	0.02-0.05	0.5	Papahadjopoulos & Miller 1967
Detergent removal	LUV	0.04-0.18	0.5-10	Kagawa & Racker 1971
Reverse phase evaporation	REV	0.1-1.0	8-17	Szoka & Papahadjopoulos 1978
Serial extrusion	LUV	0.1-0.2	15	Szoka et al. 1980
Rehydration of lyophilized SUV	DRV	0.3-2.0	--	Kirby & Gregoriadis 1984
Freeze-thaw of MLV	LUV	0.5-5.0	5-10	Mayer et al. 1985

three major types of liposomes. A summary of these different types of vesicles and the methods of preparation are shown in Table 1.

Lipid composition determines the surface charge of phospholipid vesicles. The zwitterionic headgroups of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) confer a neutral charge on the liposome surface. Acidic phospholipids, such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI), and dicetlyphosphate provide a negative surface charge. Positive charge can be obtained by adding stearylamine to the lipid composition. The surface charge of a mixture of lipids is determined by the net charges of all components.

There are two different bilayer forms of hydrated phospholipids. The lamellar liquid crystalline phase (L_α) is a bilayer structure with the headgroups facing to the hydrophilic environment and acyl chains constituting the core part of the bilayer in a rather disordered and flexible form. The second form is the lamellar gel phase (L_β) which exists at a temperature below the transition temperature of the phospholipid where lipid molecules are packed more tightly together and acyl chains are highly ordered and rigid. Liposomes made of a single component and maintained at a temperature above the L_α to L_β phase transition temperature of that lipid are considered to be in the fluid or liquid crystalline state. When liposomes are kept at a temperature below the transition temperature, they are designated to be solid or in the gel state. Incorporation of cholesterol into a fluid bilayer can increase the surface density of the acyl chains and make the bilayer more ordered and rigid (Presti et al., 1982; De Young & Dill, 1988). Thus the fluidity of liposomes is also controlled by the lipid composition.

The diameter, surface charge, rigidity of bilayer, and lipid composition are factors controlling the behavior of liposomes both in vivo and in vitro, which in turn determines the outcome of liposomal drug delivery.

Fate of Liposomes in vivo

The stability of liposomes in the body fluid, the clearance of liposomes from the circulation, and the distribution of liposomes in the body greatly influence pharmacokinetics and biodisposition of liposome-entrapped drugs which subsequently determine the outcome of liposomal drug delivery. Extensive studies about this subject have been compiled and reviewed by Senior (1986) and Hwang (1987).

(i) Biostability of liposomes. Proteins in the body fluids may interact with the liposome bilayer or induce lipid exchange between vesicle membranes and lipid components in the fluids (Juliano, 1983). The depletion of phospholipid from the vesicles, activation of the complement pathway, and metabolism of the lipid constituents by various

enzymes can contribute to leakage of entrapped contents. The kinetics of release of encapsulated contents prior to the uptake of liposomes by cells is influenced by the stability of liposomes in the body fluid. Thus factors affecting the interaction of lipid vesicles and serum components, such as bilayer rigidity, composition and surface charges of liposomes, are crucial for pharmacokinetics of liposomal drugs.

(ii) Biodisposition and the clearance of liposomes. Numerous studies have demonstrated that the majority of liposomes administered intravenously are concentrated in the liver, spleen, and bone marrow due to the uptake of the vesicles by the RES cells lining the sinusoidal space in these organs as described earlier. The interaction of liposomes with the RES cells are governed by many factors; size of vesicles, surface charge, lipid composition, lipid fluidity, surface characteristics, opsonization of serum protein, dose, and route of administration (Senior, 1986; Hwang, 1987) . The general effect of these factors on the fate of liposomes in vivo is summarized in Table 2. However, these factors do not work independently, and liposome behavior in vivo is controlled by a complex interaction among all factors. Understanding of the intertwined relationships among the factors is critical for tailoring a liposomal carrier for specific therapeutic agents. With a judicious selection of phospholipids and a rather simple manipulation of surface characteristics, one might design a carrier that is drug specific.

Liposome-Cell Interaction

The understanding of liposome-cell interactions is crucial for optimization of the intracellular delivery of liposomal contents. There are four possible pathways (Figure 4) involved in the interaction between liposomes and cells (Pagano & Weinstein, 1978; Huang, 1983; Schroit et al., 1986).

(i) Stable adsorption of lipid vesicles to the surface of cells. Lipid vesicles may associate with cell surface nonspecifically through electrostatic/hydrophobic interactions or bind to specific receptors on the cell surface. Adsorbed vesicles do not detach from the cells when the cells are washed repeatedly and they may or may not be internalized by the cells. Leakage of encapsulated aqueous markers resulting from liposome-cell complex formation has been reported (Szoka et al., 1979; Van Renswoude & Hoekstra, 1981).

(ii) Exchange of lipid molecules (e.g. PC, PE, and cholesterol) between liposomal membrane and cell membrane. This process is believed to be carried out by the lipid transfer protein on the cell surface or in the medium. The exchange of lipid is not limited to the cell-associated vesicles.

Table 2. Factors governing the biodisposition and clearance of liposomes in vivo.

Factors	Biostability	RES uptake	non-RES distribution
Size (diameter)	LUV > SUV	> 0.1 μm	< 0.1 μm
Bilayer rigidity	solid	fluid	solid
Surface charges	neutral or positive	negative	neutral or positive
Surface hydrophilicity	favor	inhibit	promote
Surface ligand	ligand dependent	not necessary	favor
Opsonization	destabilize	promote	inhibit
Dose	favor	saturated by repeated doses	favored by repeated doses
Route of administration	route-dependent	intravenous	regional or extravascular

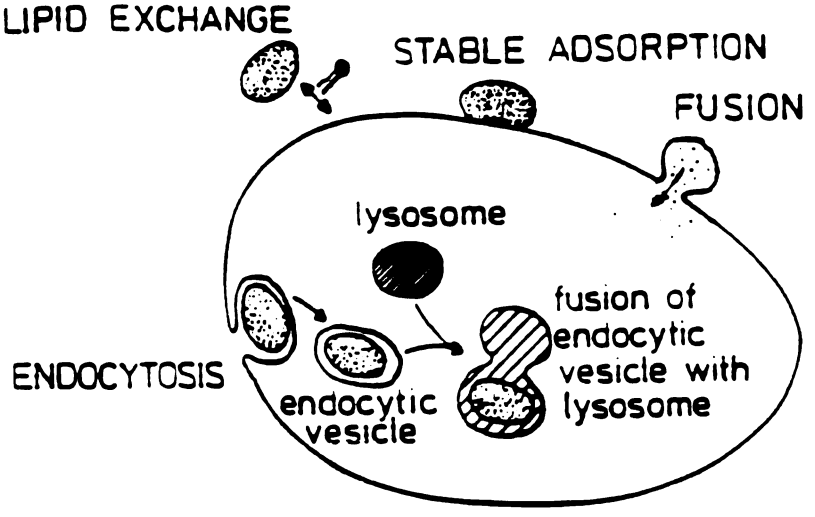


Figure 4. Major mechanisms of liposome-cell interactions. Reproduced with permission of Dr. Dijkstra (1983).

(iii) Fusion of liposomal membrane and cell membrane. Binding of liposomes to the plasma membrane brings the outer lamellar bilayer of liposomes apposed to the plasma membrane and the fusion of the two membranes may subsequently occur. Studies have clearly indicated that fusion is an extremely low frequency event (Szoka et al., 1979 & 1980a).

(iv) Endocytosis and phagocytosis of liposomes which bind to the plasma membrane. In endocytosis, liposomes are internalized in clathrin coated, plasma membrane derived vesicles which are transferred into the endosomes after clathrin is removed from the surface of the vesicles. The endosomes possesses a membrane proton pump to maintain a weakly acidic pH (5.0-6.5) inside these vesicles (Tycko & Maxfield, 1982), thus liposomes and the encapsulated compounds are exposed to a mild acidic environment in these vacuoles (Straubinger et al., 1983). The liposomes are then transferred to the lysosomes after fusion of the endosomes with the primary lysosomes (Dijkstra et al., 1984; Straubinger et al., 1983), where the pH is around 4.5-5.5. This lysosomotropic route via the endocytic pathway leads liposomes and their encapsulated compounds to the acidic, enzyme-rich lysosomes. Liposomal contents can either be digested by the lysosomal enzymes after being released from degraded liposomes, penetrate through the lysosomal membrane to the cytoplasm, or regurgitated out of cells from the lysosomes. Many studies have suggested that the endocytic route is the major mechanism of the internalization of liposomes by macrophages (Dijkstra et al., 1984) or other endocytically active cells, e.g. fibroblasts, L1210 leukemia cells, hepatocytes, lymphocytes and African green monkey kidney cells.

The difference in liposome uptake between macrophages and other cells is due to the particle size. Particles with a diameter greater than 0.1 μm are mostly phagocytosed by cells such as macrophages while smaller particles are endocytosed by nonphagocytic cells. Phagocytosis is very similar to endocytosis in terms of acidic phagosomes and lysosomotropism. Most of these studies show that liposome uptake is a temperature-sensitive and energy-dependent process. Electronmicroscopic observation of liposomes containing encapsulated cytochemical markers document the markers appearing in the compartments along the endocytic pathway (Straubinger et al., 1983; Dijkstra et al., 1984) and fluorescent microscopic demonstration of subcellular distribution of fluorescent-labelled liposomes (Szoka et al., 1980a) and their contents (Matthay et al, 1989) indicate that endocytosis is the major mechanism of liposome uptake by cells. Thus with most liposome types used to date, the majority of encapsulated compounds end up in the lysosome, not the cytoplasm. This lysosomotropic delivery is preferable for drugs used to treat lysosomal diseases but is not suitable for biolabile drugs targeted to the cytoplasmic

compartment or nucleus where many of biologically active molecules elicit their therapeutic function.

Liposomes as a Drug Carrier

Liposomes have a number of unique characteristics that make them a good choice as a drug carrier. (For reviews, Gregoriadis, 1988; Mayhew & Papahadjopoulos, 1983). (i) They are biodegradable, relatively non-toxic, and non-immunogenic in the body. (ii) The amphiphilic property of liposomes makes them a good candidate for drug delivery, since lipophilic compounds can intercalate into the lipid bilayer and hydrophilic compounds can be encapsulated in the aqueous space. (iii) Encapsulation of plasma-labile novel therapeutic agents, such as enzymes, peptides, DNAs, oligonucleotides, and ribozymes, can protect these agents from degradation or elimination in body fluids. (iv) The intriguing possible advantage of using liposomes to deliver membrane-impermeant compounds into intracellular sites of action (Heath et al., 1983 & 1985a; Szoka & Chu, 1988). (v) Liposomes can function as a depot to release the loaded drugs slowly in the body (Mayhew et al., 1982; Schäfer et al., 1987; Eppstein et al., 1989). (vi) Liposomes can deliver their contents into organs which internalize liposomes thus avoid organs which cannot take up liposomes. Therefore the therapeutic index of the encapsulated drug is augmented by this site-avoidance mechanism (Lopez-Berestein et al., 1985; Gabizon et al., 1982; Van Hoessel et al., 1984). (vii) Lastly, targeted delivery can be achieved by incorporating ligands to the surface of liposomes (Leserman & Machy, 1987; Torchilin, 1985). The most promising applications of liposome technology has been reported for anticancer, antimicrobial, and immuno therapies (Ostro, 1987).

5. Liposome-Mediated Site-Specific Drug Delivery

Liposomes have been successfully used for site-specific drug delivery. Passive targeting to the RES and ligand-mediated active targeting to the non-RES are the two most often employed strategies. The following section will focus on site-specific delivery at three levels: (i) organs/tissues, (ii) cells, and (iii) subcellular compartments.

Site-Specific Delivery to Organs or Tissues

As mentioned earlier, the majority of liposomes are taken up by macrophages in the liver, spleen, and, to a lesser extent, the lung and bone marrow following an i.v.

administration, thus liposomal contents are passively transported to these RES organs (Gregoriadis et al., 1974; Ellens et al.,1981; Allen et al., 1984; Roerdink et al., 1984).

(i) Delivery to the RES organs/tissues. Studies in which liposome encapsulated drugs are shown to potentiate drug therapy for the RES resident pathogens, such as bacterial diseases like tuberculosis, brucellosis, salmonellosis; parasitic diseases like leishmaniasis, toxoplasmosis; and fugal diseases like histoplasmosis, cryptococcosis (Juliano, 1989) are the best examples for a passively targeted drug therapy using liposomes. In these cases, the target site of liposomal delivery is the same as the colonization sites of the cellular pathogens. Suppression of microbial growth in the liver, spleen and lung contribute to the prolonged survival of the infected animals. Therefore direct delivery of drug to the site of infection; the RES, is enhanced using liposomes.

Passively targeted liposomal delivery is also successful in the the delivery of immuno modulators, e.g. muramyl dipeptide and analogues, interferon-γ, C-reactive protein (Fidler, 1989). These immuno modulators can activate macrophages and enhance their tumoricidal and antimicrobial activities in the liposome-accumulating organs. Mouse interferon encapsulated in the liposomes can control hepatitis virus infection better than nonencapsulated interferon in a murine model (La Bonnardiere, 1978). Spontaneous metastases of lung cancer can be arrested by intravenous administration of liposomal muramyl dipeptide (Fidler et al. 1981). Inhibition of liver metastases in murine colon adrenocarcinoma can be obtained by the treatment with liposomal C-reactive protein (Thombre & Deodhar, 1984). In all these successful treatments, liposomal drugs are passively targeted to the liver, spleen, or lung due to the RES uptake mechanism.

(ii) Delivery to RES organs but non-RES tissues. Passive targeting of drugs also benefits treatment for non-RES originated diseases which develop in the RES organs. The best example illustrating a successful therapy by employing the organ-targeted mechanism is achieved in using small doxorubicin-containing liposomes to control tumor metastasis in the liver and spleen (Mayhew et al.,1983; Gabizon et al., 1983; 1985). In these studies, the weight of the organs are reduced and animal survival time is extended in the group of mice treated with liposomal doxorubicin. Drug levels in these organs are elevated and the resident time of the drug in the organs is extended when delivered in the liposomal form. Although tumor cells do not extensively take up liposomes, they are exposed to elevated drug levels in the organ. The localized high drug concentration is a result of the slow release of the drug from macrophages which ingested the drug-containing liposomes (Storm et al., 1988). The success of this passively-targeted chemotherapy is partially attributed to the fact that doxorubicin is able to permeate the plasma membrane to reach the

intracellularly active site. Whether or not this scheme would work for drugs which cannot readily cross the plasma membrane is an open question.

(iii) Delivery to the non-RES tissues. When diseases do not reside in the RES organs and tissues, an active targeting strategy is needed for site-specific delivery (Leserman & Machy, 1987; Torchilin, 1985). One category of ligand is carbohydrate, e.g. galactose/lactose for targeting to hepatocytes. Incorporation of glycolipid or glycoprotein to small liposomes can increase total liver uptake mainly as a result of an increase in hepatocyte uptake mediated by an asialoglycoprotein receptors on hepatocytes (Szoka & Mayhew, 1983; Spanjer & Scherphof, 1983; Dragsten et al., 1987). Liposomes with a lactoceramide in the membrane, encapsulating plasmid containing the proinsulin gene I, was able to increase the gene expression in hepatocytes, with a simultaneous decrease of the gene expression in the Kupffer cells in rats (Soriano et al., 1983).

The more frequently used ligands have been antibodies (Ab), or their Fab' and $F(ab')_2$ fragments, specific for cell surface antigens. Such Ab-modified liposomes are designated as immunoliposomes. The proportion of immunoliposomes distributed to a low uptake target tissue may increase several to hundred-fold after i.v. injection (Wolff & Gregoriadis, 1984; Singhal et al., 1986; Debs et al., 1987; Peeters et al., 1988; Hughes et al, 1989). However, there are only a few reports describing successful targeted drug therapy mediated by immunoliposomes. Gupta and colleagues (Singhal & Gupta, 1986; Agrawal et al., 1987) attached an anti-rat RBC $F(ab')_2$ fragment to SUV and showed that these immunoliposomes had an increased binding to erythrocytes and a reduced uptake by liver in vivo. Moreover the antimalaria activity of chloroquine is enhanced in mice, though the long term survival time is not extended, when delivered via the same immunoliposomes. The success of active targeting in this model can be partially attributed to the fact that liposomes do not need to exit the vascular bed to reach the target site. Onuma and coworkers (1986) have shown that adriamycin containing SUV (0.02-0.05 μm in diameter) linked with antibodies recognizing tumor associated antigen of bovine leukemia cells were more effective in reducing the tumor volume than SUV conjugated with nonspecific antibodies in a murine model. In this case, the tumor was located at subcutaneous sites on the back or shoulder of nude mice. Another report using cytosine arabinonucleoside (Ara-C) containing immunoliposomes (0.2 μm in diameter) with antibodies recognizing an idiotype of a murine B-cell tumor shows that the antitumor activity is enhanced in inhibiting metastases in the spleen but not in a subcutaneous solid tumor (Bankert et al., 1989). The major difference in the last two trials is the size of the vesicles and the drugs used. Vesicles with a smaller diameter seem to be more efficient than ones with a larger diameter in controlling the tumor growth outside the RES organs.

The failure in achieving the goal of targeted delivery via ligand-incorporated liposomes can be attributed to (a) enhanced clearance of immunoliposomes due to opsonizing effect of Ab (Leserman et al., 1983), (b) leakage of entrapped drugs resulting from complement activation (Alving & Richards, 1983), (c) competition of binding from endogenous antigens, and (d) the difficulty of vesicles escaping from the vascular bed to reach target sites (Poste, 1983).

To increase the non-RES distribution of liposomes. RES avoidance can be achieved by using "stealth" liposomes. These are vesicles which escape RES surveillance due to a modification of the size, lipid composition and/or surface characteristics of the vesicles. For instance, liposomes composed of ganglioside GM_1 and sphingomyelin show a prolonged circulation half-life in vivo, due to reduced recognition of these lipids by macrophages (Allen & Chonn, 1987). Gabizon and Papahadjopoulos (1988) showed that small vesicles (diameter, 0.1 μm) composed of a small portion of negatively charged glycolipid, such as monosialoganglioside or PI, and a solid-phase neutral phospholipid as the bulk component (e.g. distearoyl PC) had a 60-fold increase in the fraction of injected dose remaining in the blood 24 hours after i.v. injection. Concomitantly, there was a 4-fold reduction in liposome uptake in the liver and spleen. Moreover, a 25-fold higher liposome concentration was found in a solid lymphoma tumor implanted in the leg when stealth liposomes were compared with rapidly cleared formulations. Thus avoiding RES uptake and increasing the circulation life in the blood may greatly enhance the chance of liposomes exiting from the vascular bed. This could increase the targeting efficiency of ligand-coupled liposomes

Site-Specific Delivery to Cells

Many cell culture experiments have demonstrated enhanced therapeutic effects of liposomal drugs. In these studies the physiological and anatomical factors of the in vivo situation are eliminated. Thus success of liposome delivery to target cells is dependent on the liposome-cell interaction and the drug-cell interaction.

(i) Liposomes adsorbed to the cell surface. When drugs are delivered to cells via targeted liposomal delivery, drugs may be released from surface-bound liposomes, or remain at the cell surface in the liposomes. For drugs which work at the cell surface or drugs which can readily cross the plasma membrane, adsorption of liposomes to target cells and subsequent drug leakage from the liposomes is sufficient for a targeted delivery (Ho et al., 1987, Allen et al., 1981). In contrast, for intracellularly active drugs which cannot permeate membranes readily, internalization of cell surface-bound liposomes is necessary for a therapeutic activity (Heath et al., 1983; 1985a).

(ii) Liposomes internalized. The intrinsic phagocytic or endocytic activity of cells is the major factor controlling the internalization of liposomes. Using cell lines of different origins (lymphoma, fibroblast, macrophage-like, and kidney-derived), Heath and colleagues (1985b) showed that the extent of cellular uptake of liposome encapsulated methotrexate-γ-aspartate correlates well with the cytotoxicity of this membrane-impermeant drug. Therefore, methods to increase the internalization of surface-bound vesicles can be used to enhance liposomal delivery at the cellular level. Receptor-mediated endocytosis of surface bound vesicles enhances the intracellular transport of vesicles many folds when compared to the non-modified vesicles (Heath et al, 1980, Leserman et al, 1981). Endocytic internalization of targeted liposomes is also dependent on the ligand attached (Machy and Leserman, 1987; Matthay et al., 1989). Cellular uptake and therapeutic effect of immunoliposomes has also been correlated in various cell lines (Matthay et al., 1986, 1989; Berinstein et al., 1987). Thus the increased liposome uptake accounts for enhanced activity of chemotherapeutic agents encapsulated in immunoliposomes (Machy & Leserman, 1987; Bragman et al., 1984, Matthay et al., 1984).

Ligands other than antibodies have also been used to target liposomes to specific cells. For example, low-density lipoprotein-coupled liposomes potentiate the inhibition of protein synthesis by hygromycin B in leukemic LC2 cells (Vidal et al., 1985). Epidermal growth factor bearing liposomes are taken up by rat hepatocytes in an EGF-mediated manner and might be used for targeting drugs to the EGF receptor-rich cancer cells (Ishii et al. 1989).

The size of vesicles not only dictates the distribution of liposomes in vivo, but also affects the internalization of the surface-bound vesicles. Nonphagocytic cells usually do not endocytose large particles (Steinman et al., 1983). Consequently, smaller liposomes are presumably more effective for drug delivery, because smaller vesicles are endocytosed more efficiently by these cells. Antibody-conjugated SUV were more effective than the corresponding LUV for delivery of methotrexate in anticancer therapy (Machy & Leserman, 1987). This is further supported by electronmicroscopic observations of gold-containing liposomes in African green monkey kidney cells which indicated that negatively charged REV of 0.08-0.25 μm in diameter would adsorb to cell surfaces, but only vesicles < 0.1 μm in diameter were found inside the cells (Straubinger et al., 1983).

Cellular endocytosis of lipid vesicles is a saturable process which levels off at a high lipid concentration. The saturation of cellular uptake has been shown by measurement of both lipid markers and aqueous contents. Thus, the liposome encapsulated drug uptake would be a saturable process. The therapeutic effect of drug active intracellularly is controlled by the amount of the drug transported to the active site. This is determined by

the amount of liposomes taken up by cells. The drug to lipid ratio (an indicator for drug loading in the carrier) will therefore influence the therapeutic effect of a liposome-encapsulated drug. Liposomes with a low drug to lipid ratio will result in a high lipid uptake but a low drug uptake. The drug concentration might be insufficient for a therapeutic effect. In addition, high lipid uptake may induce some lipid toxicity in the target cells (Layton et al., 1980; Heath et al., 1985b; Allen et al., 1987).

Site-Specific Delivery to Subcellular Compartments

If lysosomotropic delivery of liposomal contents after the internalization of liposomes leads to degradation of the encapsulated drugs, the purpose of intracellular delivery via liposomes is defeated. A few approaches have been taken to circumvent the lysosomal delivery of liposome encapsulated compounds and to enhance cytoplasmic delivery via liposomes. The acidic environment along the lysosomotropic pathway affords an opportunity for the delivery of reversibly acid-titratable compounds to be delivered to the cytoplasm (Leserman et al., 1981; Huang et al., 1983; Heath et al., 1983). At neutral pH, negatively charged carboxylate compounds with pKa $\cong$ 5.0 are impermeant through the plasma membrane. Protonation of the charged group at the low pH found in the endosomes or lysosomes neutralizes the compound and increases the compound permeability through membranes. Such pH-sensitive and membrane-impermeant molecules have been described as liposome-dependent drugs, of which effect can be greatly enhanced when delivered in liposome-encapsulated form (Heath et al., 1983). This approach only applies to drugs which have an acid-titratable properties (Table 3).

Designing liposomes which would mediate cytoplasmic delivery would greatly benefit subcellular site-specific delivery. Two approaches are being attempted to achieve this goal, the creation of virosomes and the development of pH-sensitive compositions. Virosomes are liposomes containing viral membrane protein so that they can mimic the entry process of certain enveloped viruses into cells (White et al., 1983; Marsh, 1984; Spear, 1987). For instance, Sendai virus binds to the cell membrane and injects its nucleocapsid into the cytoplasm of target cells by fusing with the plasma membrane. Influenza virus and vesicular stomatitis virus are internalized via receptor-mediated endocytosis. After internalization, the viral envelope fuses with the endosomal membrane, and viral genetic materials are subsequently released into the cytoplasm. The viral-cell fusion is triggered by a conformational change of viral envelope proteins which occurs in the mild acidic condition in the endocytic vacuoles. To mimic this process, the nucleocapsid-free viral envelope, which contains the spike glycoproteins responsible for binding and fusion, is reconstituted in lipid bilayer to form virosomes. Virosomes derived

Table 3. Enhanced cytoplasmic delivery of pH-sensitive drugs.

Drugs	Lipid composition	Type	Cell	Evidence	Reference
MTX-γ-aspartate	PS/cholesterol : 67/33	REVET[a] (0.1 μm)	L929, CV1-P RAW 264, Cl18	Enhanced cytotoxicity	Heath et al. 1985
5-Fluoroorotate	PG/cholesterol :67/33	SUV REV	L929, CV1-P	Enhanced cytotoxicity	Heath et al. 1985
Phosphonoformate Phosphonoacetate	EPC/EPG/Ch :9/1/8	REV (0.2 μm)	Vero	Enhanced antiviral activity	Szoka & Chu 1988

a: Preformed vesicles extruded through membranes with defined pore size.

from Sendai virus (Loyter & Volsky, 1982; Vainstein et al., 1984), vesicular stomatitis virus (Eidelman et al., 1984; Metsikkö et al., 1986; Paternostre et al., 1989), influenza virus (Stegman et al., 1987; Sizer et al., 1987), and semliki forest virus (Marsh et al., 1983) are able to fuse with cells or model membranes at the acidic pH. These virosomes may fuse with either the plasma membrane or the endosomal membranes as the parent viral particles do and deliver entrapped macromolecules to the cytoplasm of cells (Uchida et al., 1979; Nakanishi et al., 1985; Gould-Fogerite et al., 1989).

Synthetic peptides composed of amphipathic sequences of fusion proteins undergo conformational changes at low pH. Such peptides have also been shown to induce liposome fusion/destabilization (Schlegel & Wade, 1984; Murata et al., 1987; Parente et al., 1988). It is conceivable that when fusogenic peptides are covalently linked to the liposome surface, the proteoliposomes will behave like virosomes described above and deliver encapsulated contents to the cytoplasm. The possibility of an inherent toxicity and an induced host immune response recognizing the viral proteins could jeopardize the usefulness of virosomes.

An alternative approach to increase cytoplasmic delivery is to modify the liposome composition so that it becomes more fusogenic under weakly acidic condition. These pH-sensitive liposomes must become fusogenic at the weakly acidic conditions (pH 5-6.5) in the endosome. Liposomes which respond to extremely acidic pH (pH < 4), but not to the physiological acidic pH, e.g. phosphatidylserine vesicles, are not appropriate for cytoplasmic delivery. pH-sensitive liposomes generally consist of phosphatidyl-ethanolamine stabilized by a weakly acidic hydrophobic, organic acid In the next section, pH-sensitive liposomes will be discussed in detail.

6. pH-sensitive liposomes

Biophysical Aspect of pH-Sensitive Liposomes

pH-sensitive liposomes depend upon acid-induced destabilization of the vesicle bilayer structure. Therefore an understanding of the formation of different lipid structures is necessary for designing pH-sensitive liposomes. Hydrated lipids exhibit polymorphic structures which depend upon the molecular shape of the lipid, lipid concentration, temperature, pH, ionic strength and pressure (Gennis, 1989). The major organized forms are schematically depicted in Figure 5. (a) Lamellar liquid crystalline phase (L_α): This is a bilayer structure with the headgroups facing the hydrophilic environment and acyl chains forming the core of the bilayer in a rather disordered alignment. (b) Lamellar gel phase

(L_β): This lamellar form exists at lower temperatures where lipid molecules are packed more tightly together and the acyl chains are highly ordered. (c) Hexagonal I phase (H_I): In this form, the lipids are organized in the cylindrical form packed in a hexagonal array. In each cylinder, lipids arrange in a micellar form with the headgroups of lipids on the outside in contact with water and acyl chains are inside. (d) Inverted hexagonal phase (H_{II}): The lipids are in cylindrical form, but with the acyl chains on the outside and the polar headgroups facing inside and surrounding a column of water. Again, the cylinders are packed in a hexagonal pattern.

In lipid systems that can undergo a lamellar to hexagonal phase transition, the inverted hexagonal phase (H_{II}) has been proposed to be involved in the membrane fusion (Siegel, 1987). During membrane fusion a proportion of bilayer membrane is proposed to undergo a transient structure, which is termed an inverted micellar intermediate (IMI). Aggregation of IMI forms an H_{II} phase of elongated cylindrical inverted micelle which form a bridge between adjacent bilayers. The last step involves a final rearrangement of the inverted micellar lipid and results in membrane fusion. The fusion process is schematically depicted in Figure 6.

The packing of headgroups and acyl chains determines the shape of lipid molecules which in turn affects the morphology of the hydrated lipids (Cullis et al., 1983). Most phospholipids have a bulky headgroup area compared with the cross section area of acyl chains. The comparable area of the headgroup and acyl chain allows lipid molecules adopt a cylindrical shape compatible with lamellar bilayers (Figure 7). PE has a compact and rigid headgroup network due to the strong intermolecular interaction between -OH and -NH_2 of the headgroup (Hauser et al., 1981; Papahadjopoulos & Miller, 1967). At high temperature, the expanded unsaturated acyl chain matrix and the inflexible headgroup transform PE into a conical shape molecule (Figure 7) that permits PE to adopt the H_{II} structure. Therefore as the temperature increases, PE can convert from the L_α to the H_{II} phase. The L_α to H_{II} phase transition temperature (T_h) of various PE is dependent on the configuration of the acyl chains, pH, and ionic strength of the environment. Any factors which can reduce the ratio of cross section area of acyl chain to area of headgroup, e.g. factors decreasing intermolecular interaction of headgroups, can shift the T_h to a higher temperature and stabilize the bilayer phase. In contrast, factors which increase the ratio will lower the T_h and promote H_{II} phase formation.

At temperatures below the T_h, PE cannot form a stable bilayer vesicle at physiological pH. However, stabilization of bilayer vesicle structure of PE can be achieved by several approaches. (i) Addition of another lipid, e.g. PC, PG and PS at greater than 30% can decrease the intermolecular interaction of the headgroups of PE and increase the

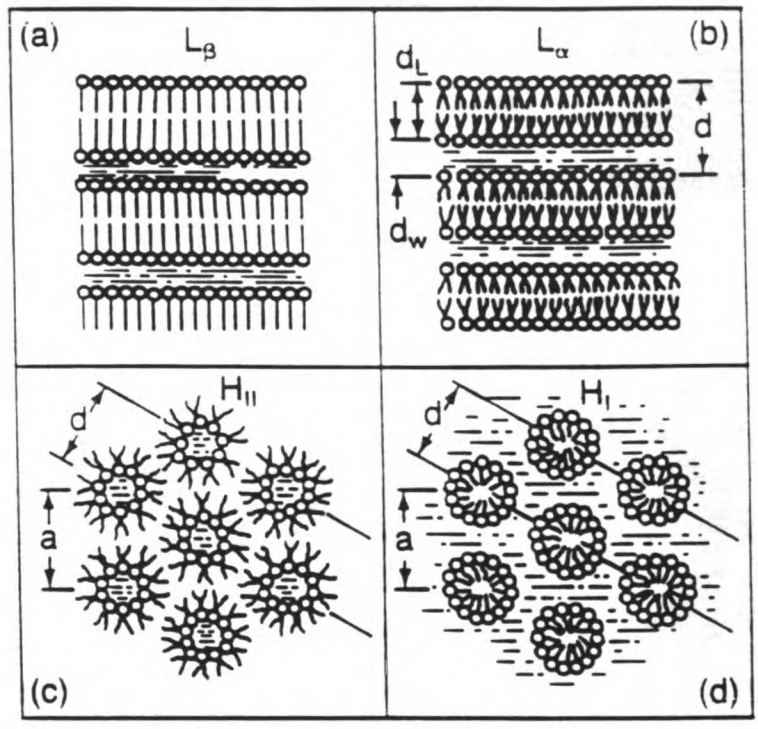


Figure 5. Schematic representations of lipid-water phases: (a) lamellar gel; (b) lamellar liquid crystalline; (c) hexagonal type II; (d) hexagonal type I. Permission requested from Dr. Gennis (1989).

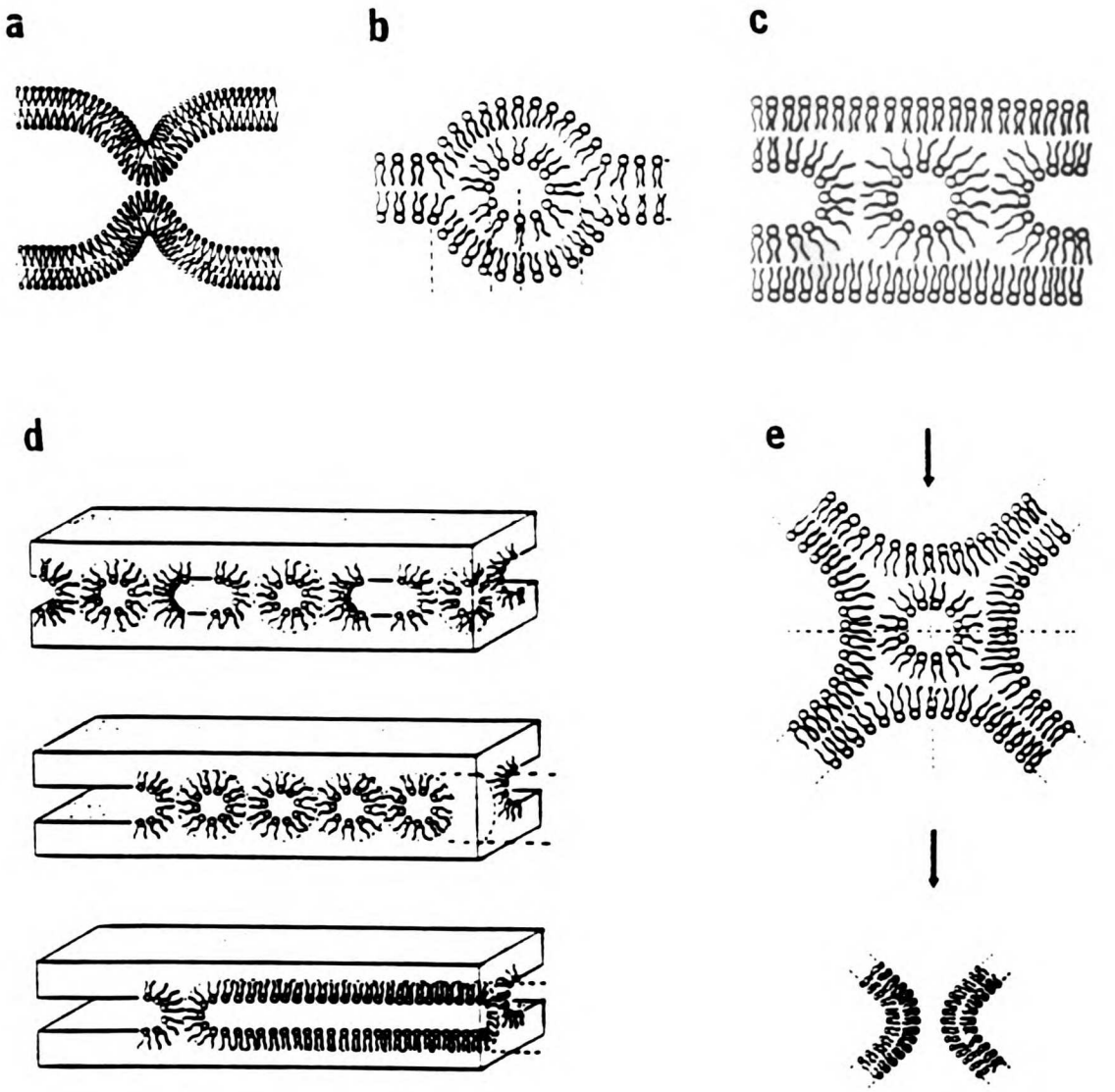


Figure 6. Schematic diagram of (a) membrane attached site; (b) inverted micelle sandwiched in monolayer; (c) inverted micellar intermediate (IMI); (d) aggregation of IMI to form H_{II} phase: many IMI are present between apposed lamella (top), IMI aggregates form strings of inverted micelles (middle), aggregated micelles form an elongated cylindrical micelle (bottom); (e) rearrangement of micellar lipid results in membrane fusion. Reproduced with permission from Dr. Lai (1984).

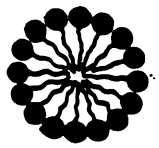
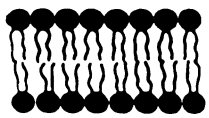
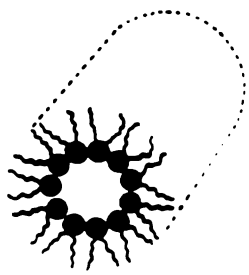
LIPID	PHASE	MOLECULAR SHAPE
Lysophospholipids Detergents	 Micellar	 Inverted Cone
Phosphatidylcholine Sphingomyelin Phosphatidylserine Phosphatidylinositol Phosphatidylglycerol Phosphatidic Acid Cardiolipin Digalactosyldiglyceride	 Bilayer	 Cylindrical
Phosphatidylethanolamine (Unsaturated) Cardiolipin - Ca^{2+} Phosphatidic Acid - Ca^{2+} (pH < 6.0) Phosphatidic Acid (pH < 3.0) Phosphatidylserine (pH < 4.0) Monogalactosyldiglyceride	 Hexagonal (H_{II})	 Cone

Figure 7. Polymorphic phases and molecular shapes of membrane lipids. Permission requested from Dr. Cullis and colleagues (1983).

surface hydration (Hope et al., 1983). (ii) Deprotonation of amine groups at pH ≅ 9.0 enables PE to assume a net negative charge. The net negative charge results in much less intermolecular interaction of PE headgroups and results in higher hydration around the headgroups. Thus PE can form a bilayer configuration of liposomes at elevated pH, but the lipid vesicle structure collapses to lamellar sheets when the pH is reduced to neutral and headgroups become neutralized (Hauser et al., 1981). (iii) When acid-titratable charged amphiphilic molecules are incorporated to PE, the resulting electrostatic repulsion of charged groups can reduce the intermolecular interaction of the PE headgroups, a stable liposome structure at physiological pH can be constructed (Lai et al., 1985b; Düzgünes, et al. 1985). The bilayer configuration of these liposomes is destabilized at a reduced pH when the titratable groups are protonated. Thus pH-sensitive liposomes can be made of PE and a charged amphiphilic molecule containing an acid-titratable group.

pH-Sensitive Liposomes in Model Systems

Various pH-sensitive liposomes have been developed in the past decade. The composition of these liposomes, size, fusion/destabilization assay are summarized in Table 4 & 5. The chemical structure of each component used to prepare pH-sensitive liposomes is shown in Table 6.

The initial report of pH-sensitive liposomes (Yatvin, et al., 1980) used an acylated amino acid, N-palmitoyl-L-homocysteine (PHC), as the acid-sensitive component (12-16%) together with diheptadecanoylphosphatidylcholine (DHPC) and dipalmitoyl-phosphatidylcholine (DPPC) to construct the pH-sensitive liposomes. These liposomes were designed to destabilize in the weakly acidic environment surrounding tumors, infection and inflammation and release entrapped drug at these areas. At neutral pH, PHC resembles a free fatty acid with a negative charge and is incorporated into bilayers. At weakly acidic pH (< 6.8), PHC prefers a neutral thiolactone structure and destabilizes the bilayer by lateral phase separation of protonated PHC molecules in the bilayer so that encapsulated carboxyfluorescein (CF) is released. The amount of CF released at 37 °C was approximately threefold higher at pH 6.5 than at 7.4 and fivefold greater at pH 6.0 than at 7.4. Regional targeted delivery was the intent of this pH-sensitive composition, not a fusogenic liposome.

Stable vesicles of PE can be made when other phospholipids are included as mentioned earlier. Hope and colleagues (1983) showed that vesicles made of PS/PE or PA/PE fused at pH 3. The extremely acidic pH neutralized the negative charges of PS or PA and neutralized vesicles collapsed to the H_{II} phase which facilitated fusion. When this vesicle composition was aggregated by binding of a lectin, ricin, to glycolipids included in

Table 4. pH-sensitive liposomes in model membrane systems.

Composition	Type (diameter)	pH threshold	Evidence	Reference
Acylated amino acids				
PHC/DHPC/DPPC: 16/74/10	SUV	< 6.8	Leakage of contents	Yatvin et al. 1980
PHC/DOPE: 2/8	SUV	< 6.5	Lipid mixing Electron micrograph Leakage of contents	Connor et al. 1984
Phospholipids				
EPS/soya PE: 1/4; EPA/soya PE: 1/4	SUV	3.0	Electron micrograph	Hope et al.1983
Glycolipid/PA/PE, Glycolipid/PS/PE: 10/30/60	REV	< 4.5	Lipid mixing Leakage of contents	Bondeson et al. 1984
Cholesterol derivatives				
CHEMS/TPE: 3/7	REVET[a] (0.1μm)	< 5.0	Leakage of contents Lipid mixing	Ellens et al. 1984 Ellens et al. 1985
CHEMS/TPE: 25/75	MLV	4.5	Electron micrograph Calorimetry thermogram	Lai et al. 1985

Table 4. (continued)

SCD/DEPE: 98/2, w/w	MLVET[a] (0.2μm)	< 6.0	Lipid mixing Contents mixing Calorimetry thermogram	Epand et al. 1988
Fatty acids				
OA/TPE: 3/7	MLVET[a] (0.2μm)	< 6.5	Lipid mixing Contents mixing Electron micrograph Irreversible increase in turbidity	Düzgünes et al. 1985
OA/DOPE/Ab[b]: 2/8/0.005	DRVET[a] (0.1μm)	< 6.5	Leakage of contents	Collins & Huang 1987
Miscellaneous				
COPE/DOPE:3/7	MLVET[a] (0.1μm)	< 5.0	Leakage of contents	Nayar & Schroit 1985
DCPA/DEPE:75/25	REVET[a] (0.1μm)	< 6.5	Leakage of contents Contents mixing Lipid mixing	Leventis et al. 1987 Collins et al. 1990

a: Preformed vesicles extruded through membranes with defined pore size.
b: Anti-H-2K^k antibodies. Lipid to antibody ratio, 10/1 (w/w).

Table 5. Cytoplasmic delivery mediated by pH-sensitive liposomes in cultures.

Lipid composition	Type (diameter)	Target cells	Evidence	Reference
PHC/DOPE/Ab[a]: 2/8/0.005[b]	REV	L-929	Cytoplasmic calcein	Connor & Huang 1985
OA/TPE: 3/7	MLVET[c] (0.2 μm)	CV-1	Cytoplasmic calcein & FITC-dextran	Straubinger et al. 1985
OA/TPE: 3/7	MLVET[c] (0.2 μm)	CV-1	Cell growth inhibition by pokeweed antiviral protein	Baldwin et al. 1986
OA/DOPE/Ab[a]: 2/8/0.005[b]	REV	L-929	Cell growth inhibition by Ara-C	Connor & Huang 1986
OA/DOPE/Ab[a]: 2/8/0.0025	DRVET[c] (0.1 μm)	L-929	Leakage of calcein Protein synthesis inhibition by DTA	Collins & Huang 1987
OA/DOPE/Ch/Ab[a]: 2/4/4/0.002[d]	LUVET[c] (0.2 μm)	RDM-4	Expression of CAT gene	Wang & Huang 1987
OA/DOPE/Ch/Ab[a]: 2/4/4/0.002[d]	LUVET[c] (0.2 μm)	Ltk$^-$	Expression of TK gene	Wang & Huang 1990
DPSG/DOPE/Ab[a]: 2/8/0.005[b]	LUVET[c] (0.1 μm)	L-929	Lipid mixing Protein synthesis inhibition by DTA	Collins et al. 1989

a: Anti-H-2K^k antibodies.
b: Lipid to antibody ratio, 10/1 (w/w).
c: Preformed vesicles extruded through membranes with defined pore size.
d: Lipid to antibody ratio, 25/1 (w/w).

Table 6. Chemical structure of components of various pH-sensitive liposomes

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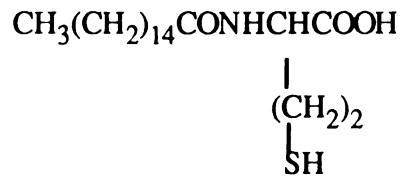
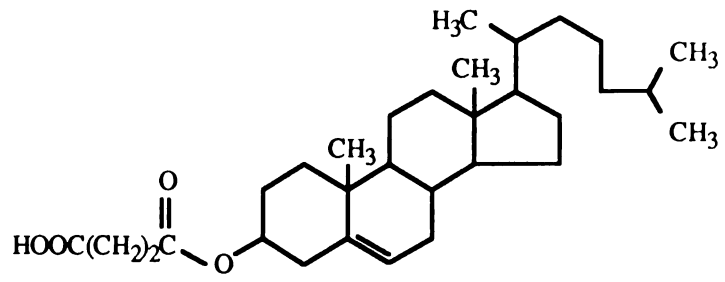
N-palmitoyl homocysteine (PHC)



Cholesteryl hemisuccinate (CHEMS)



2,3-Seco-5α-cholestan-2,3,-dioic acid (SCD)

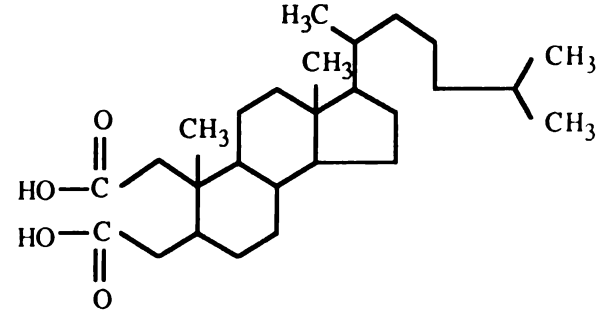
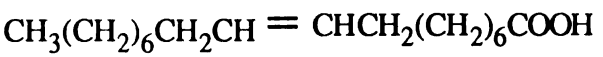
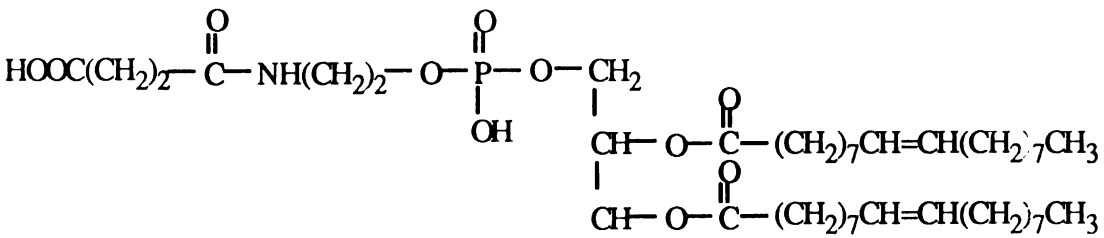


Table 6. (continued)

Oleic acid (OA)

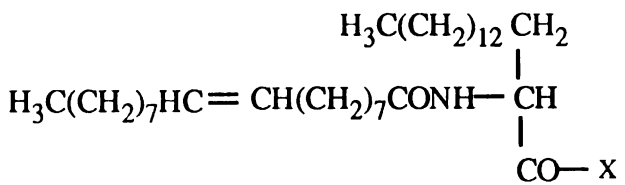


N-succinyldioleoyl-phosphatidylethanolamine (COPE)



Double-chain protonable amphiphiles (DCPA)

(1) N-oleoyl-2-aminopalmitic acid



(2) 2-(cis-9-octadecenoyloxy)hexadecanoic acid

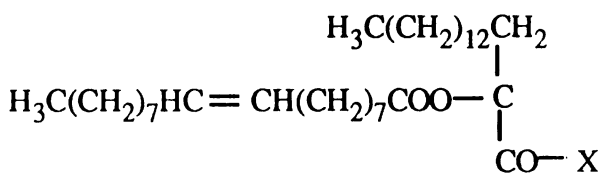
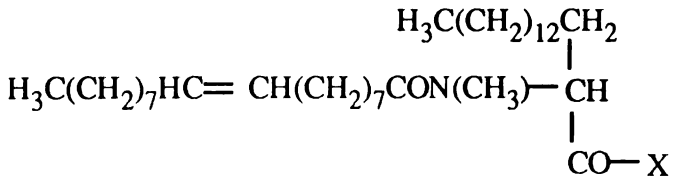
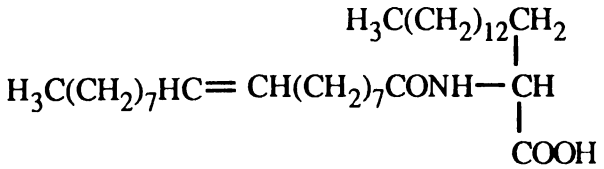


Table 6. (continued)

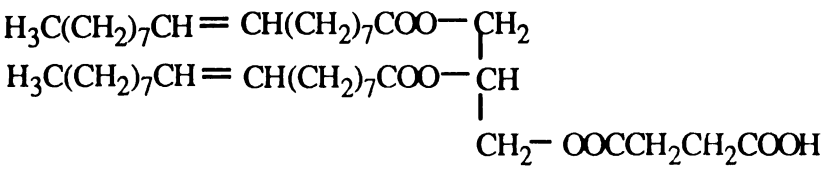
(3) N-oleoly-2-methylaminopalmitic acid



(4) N-elaidoyl-2-aminopamitic acid



(5) 1,2-dioleoyl-3-succinylglycerol (DOSG)



(6) 1,2-dipalmitoyl-3-succinylglycerol (DPSG)

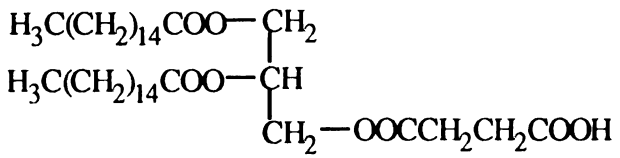
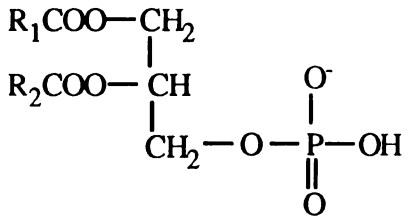
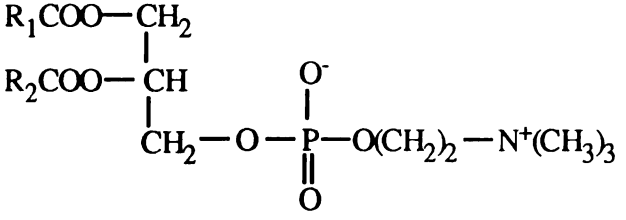


Table 6. (continued)

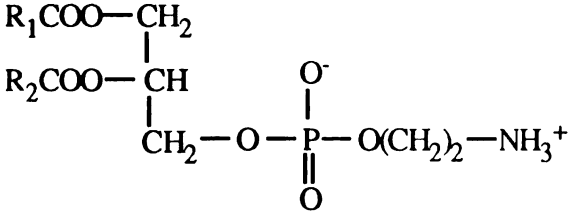
Phosphatidic acid (PA)



Phosphatidylcholine (PC)



Phosphatidylethanolamine (PE)



Phosphatidylglycerol (PG)

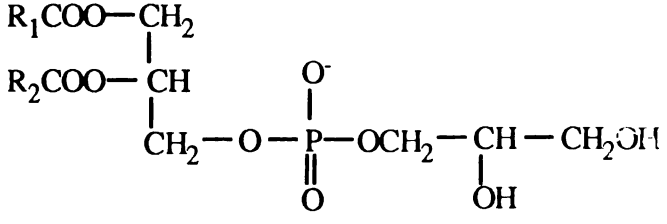
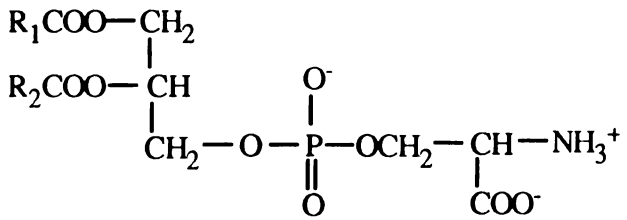


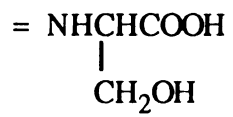
Table 6. (continued)

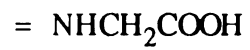
Phosphatidylserine (PS)

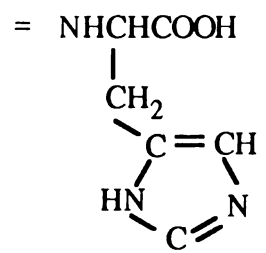


R_1 & R_2 = Acyl chains

X = OH







the vesicles, the pH-dependent fusion was enhanced (Bondeson et al., 1984). This result suggests that the proton-triggered fusion can be promoted by a bridging agent to facilitate bilayer contact. However, this kind of pH-sensitive liposome cannot be used for cytoplasmic delivery due to their extremely low pH sensitivity which is not readily obtainable under physiological condition.

The second generation of pH-sensitive liposomes were developed to mimic the behavior of viral particles. They are based on the propensity of PE to form an H_{II} structure, which as mentioned above is thought to be involved with membrane fusion. Connor and colleagues (1984) demonstrated that SUV composed of PHC/dioleoyl-phosphatidylethanolamine (DOPE) (2/8) were fusogenic at weakly acidic condition (pH 4.8-7.0), while control liposomes composed of PHC/dioleoylphosphatidylcholine (DOPC), PHC/PS, PS/DOPE, DOPC/DOPE, or DOPC/PS, showed limited fusion under the same acidic condition. Mixing of lipids between different populations of fluorescently labeled vesicles indicated fusion had occurred. Negative-stain electron micrographs showed that about two-thirds of the vesicles became larger, from about 0.05 μm to 0.3 μm in diameter, after acid treatment (pH 4.8). All of an entrapped quenched fluorophore was released during the fusion reaction and the mixing of entrapped contents was not confirmed in this study.

pH-sensitive liposomes were also constructed by including cholesteryl hemisuccinate (CHEMS) in the PE bilayer. CHEMS has two function in this composition; first, it can be protonated at weakly acidic pH and cause destabilization/fusion of lipid bilayer membranes (Ellens et al., 1984), and second, cholesterol stabilizes the bilayer structure at neutral pH (Lai et al., 1985a). Ellens and coworkers showed that liposomes made of PE and CHEMS, which are negatively charged at neutral pH and becomes protonated at pH < 5.5, become aggregated and leak at pH less than 5.5. Lipid mixing between vesicles, measured by the energy transfer assay, was observed at pH < 5.0 (Ellens et al., 1985). Contents mixing was not observed at low pH but was observed in the presence of calcium. This suggests that acid induced bilayer contact of the CHEMS/PE vesicles causes lipid mixing and contents leakage, but not fusion.

Lai and coworkers (1985a) demonstrated that the effect of CHEMS on the thermotropic behavior of DPPC and dimyristoylphosphatidylcholine (DMPC) is very similar to that of cholesterol. At neutral pH, addition of CHEMS to transesterfied egg PE (TPE) raised the lamellar-hexagonal transition temperature of PE. Increasing the molar ratio of CHEMS in the CHEMS/PE mixture shifted the order-fluid transition temperature to a lower range while the lamellar-H_{II} transition temperature was elevated at pH 7.4 (Lai et al., 1985b). At low pH the lamellar-H_{II} transition in the CHEMS/PE underwent a shift to a

lower temperature. These results indicate that incorporation of CHEMS to PE can stabilize PE bilayer structures at physiological pH and promote formation of the H_{II} phase at the acidic pH.

Another cholesterol derivative employed to prepare pH-sensitive liposomes is 2,3-seco-5α-cholestan-2,3-dioic acid (SCD) (Epand et al. 1988). Mixing of SCD with PE raises the lamellar-hexagonal transition temperature of dielaidoylphosphatidylethanolamine (DEPE) at pH greater than 6.6, and lowers the transition temperature at pH less than 6.6. Below pH 6, vesicles containing SCD fuse as measured by mixing of aqueous contents and by a lipid mixing assay. Since SCD promotes changes in the intrinsic curvature of vesicles, a substantial fraction of the vesicles burst instantaneously when acidified. This may not favor the pH-dependent fusogenic activity of the vesicles.

Different fatty acid/PE compositions also have been employed to form pH-sensitive liposomes. Oleic acid (OA) is the most widely used fatty acid because the acyl chain has a low melting temperature and accommodates well to the acyl chain configuration of unsaturated PE. Mixing of OA/PE (molar ratio < 0.3) forms vesicles at neutral pH (Düzgünes et al., 1985). Liposome composed of OA/PE leak their contents and fuse at pH < 6.5. Freeze-fracture electron micrographs showed that the vesicle size increased several-fold after acid treatment. Control liposomes made of OA/PC or PS/PE do not fuse as the pH is lowered to pH 4.0, which indicates both PE and an acid-titratable compound with an appropriate pKa are necessary to form pH-sensitive liposomes suitable for cytoplasmic delivery in vivo. Other fatty acids, such as arachidonic acid, elaidic acid, or stearic acid also were used to prepare pH-sensitive liposomes. The pH-dependent fusion kinetics of the various fatty acid containing liposomes indicates that the fatty acid not only provides a titratable moiety, but also affects the phase behavior of the PE (Düzgünes et al., 1987).

N-succinyldioleoylphosphatidylethanolamine (COPE), a carboxylated derivative of PE, when incorporated into DOPE permits the formation of a relative stable bilayer structure at neutral pH, and pH-sensitive destabilization of the vesicles is observed at acidic pH (Nayer & Schroit, 1985). However, fusion was not observed under the same conditions where release of the contents occurs. Whether or not this kind of pH-sensitive liposome will be useful for cytoplasmic delivery is unclear.

Leventis and coworkers (1987) have prepared a series of novel double-chain protonizable amphiphiles (DCPA), including acylated palmitic acids, amino acid conjugates of these species and 1,2-diacyl-3-succinylglycerol. These amphiphiles can be combined with PE to form stable vesicles at neutral pH. The vesicles become leaky at the weakly acidic pH (< 6.5) and fused, as indicated by an extensive lipid mixing and limited contents mixing, at low pH. In addition, the authors demonstrate that DCPA/PE vesicles are more

stable than fatty acid (OA) incorporated vesicles in the presence of serum, albumin or other phospholipid vesicles. This is because the single chain fatty acids are rapidly transferred to proteins or other lipids. Thus DCPA/PE vesicles may retain their entrapped contents when exposed to the serum and may be useful for in vivo drug delivery.

pH-Sensitive Liposomes in Cell Lines

Cytoplasmic delivery of encapsulated contents is the ultimate goal of developing pH-sensitive liposomes. Thus further evidence of cytoplasmic delivery in cell culture (Table 5) is essential for this concept to advance. PHC/DOPE vesicles incorporated with the targeting ligands of anti-H-2K^k antibody (immunoliposome) were incubated with H-2K^k expressing L929 cells and resulted in a diffuse fluorescence from encapsulated calcein delivered to the cytoplasm of the cells (Connor & Huang, 1985). A punctate (vacuolar) fluorescence pattern was observed with immunoliposomes composed of DOPC, low incubation temperature (< 20 ^oC), or treatment with chloroquine, an inhibitor of acidification of endosome and lysosomes. Connor and Huang estimated that about one thousand of the pH-sensitive immunoliposomes released their encapsulated calcein into the cytoplasm of each cell. This work suggests efficient cytoplasmic delivery involves fusion/destabilization of the acidic organelles in the endocytic pathway when PHC/DOPE immunoliposomes are used. Since the uptake efficiency of the vesicles by the target cells was not quantitated, the overall efficiency of cytoplasmic delivery via the PHC/DOPE immunoliposomes is not known.

When calcein or FITC labeled dextran containing OA/PE vesicles were incubated with green monkey kidney cells (CV-1 cells), a diffuse cytoplasmic fluorescence was observed, though intense cytoplasmic fluorescence indicative of high level of cytoplasmic delivery could only be seen after exposure to hypertonic glycerol. A vacuolar fluorescence was obtained with the corresponding vesicles of PS/PE or OA/PC (Straubinger et al. 1985). Cytoplasmic delivery was reduced in the presence of chloroquine or monesin; two compounds which increase the pH in the endosomes/lysosomes. Pokeweed antiviral protein (PAP) encapsulated in OA/PE vesicles was about 2500- and 4-fold more potent than nonencapsulated PAP and PAP entrapped in the non-pH-sensitive liposomes (PS/PE), respectively, in inhibiting 50% of CV-1 cell growth in vitro (Baldwin et al., 1986). Although fusion of vesicles composed of OA/PE occurs in biophysical studies, this composition does not induce extensive cytoplasmic delivery in cell cultures.

Antibodies have been attached to the OA/PE composition to enhance the cytoplasmic delivery to target cells (Connor and Huang, 1986). Vesicles composed of OA/DOPE with anti-H-2K^k antibodies on their surface retained pH-sensitivity as

demonstrated by the acid-induced leakage of encapsulated calcein. The vesicles delivered the encapsulated cytotoxic agents, 1-ß-D-arabinofuranosylcytosine (Ara-C), to the cytoplasm of H-2K^k-bearing L929 cells. Inhibition of cell proliferation is significantly enhanced by delivery in the pH-sensitive composition when compared with that of the drugs delivered in a nonencapsulated form, entrapped in the non-pH-sensitive immunoliposomes, or encapsulated in non-immunoglobulin containing pH-sensitive liposomes. The cytotoxicity is specific for antigen-bearing cells, and no inhibitory effect is observed with antigen-free cells. As a comparison, methotrexate (MTX) was also delivered in the pH-sensitive and non-pH-sensitive compositions but no increase in its cytotoxicity was observed with pH-sensitive composition. This is because MTX is able to diffuse from the acidic lysosomes, due to the increased permeability of protonated MTX, to the cytoplasm and exhibits its cytotoxicity. This suggests that drugs which do not readily permeate from the lysosomes to the cytoplasm will benefit the most from pH-sensitive liposome delivery.

Diphtheria toxin A chain (DTA) is a very potent protein synthesis inhibitor if it can reach the cytoplasm (Yamaizumi et al., 1978). However it is unable to cross the cell membrane due to the absence of a membrane-binding domain (Sandvig & Olsnes, 1980). By measuring cellular protein synthesis, one obtains an indicator for cytoplasmic delivery of DTA. The DTA encapsulated in OA/DOPE/anti-H-2K^k composition caused an inhibition of protein synthesis in the target cell (Collins and Huang, 1987). The delivery is pH-dependent and is specific for target cells only. In addition to the pH-sensitivity, the antibody moiety is necessary for the efficient delivery of DTA to inhibit protein synthesis in the target cells.

Due to the instability of OA/PE vesicles in the presence of serum (Liu and Huang, 1989), cholesterol was added to the formulation to construct a serum-stable preparation. The resultant pH-sensitive immunoliposomes composed of OA/cholesterol/DOPE in 2/4/4 molar ratio were able to deliver plasmid DNA, containing Herpes simplex virus thymidine kinase (TK) gene, to target cells in culture (Wang and Huang, 1989). In this case also, the incorporation of antibody is necessary to obtain DNA delivery. Interestingly, the pH-sensitive lipid composition is only 8-fold more efficient in transfecting the target cells than that of the corresponding non-pH-sensitive composition. Thus the antibody seemed to be more important for gene transfer than the pH sensitivity per se. When all liposome and incubation conditions are optimized, the target cells showed a 47% efficiency for short-term expression of the gene and 2% for long-term transformation, while the conventional calcium phosphate precipitation method yielded 34% and 0.12% for short-term and long-term transformation, respectively. The authors indicated that the pH-sensitive

immunoliposomes are 100 to 1000-fold better than other non-pH-sensitive compositions, reported to date, in inducing long term transfection in mammalian cells in vitro. This remarkable efficiency in gene transfection using the pH-sensitive immunoliposomes provides the most convincing evidence to date for future use of pH-sensitive liposomes as a cytoplasmic delivery vector for bioactive macromolecules, though the efficiency of the cytoplasmic delivery is still unknown.

Taking advantage of the biostability of DCPA incorporated pH-sensitive liposomes in the presence of serum (Leventis et al., 1987), Collins and coworkers (1990) used 1,2-dipamitoyl-sn-3-succinylglycerol (DPSG) and 1,2-dioleoyl-sn-3-succinylglycerol (DOSG) to incorporate to DOPE to form pH-sensitive liposomes. These vesicles exhibit acid-induced leakage of entrapped contents and lipid mixing at pH < 6 in model systems. Delivery of DTA entrapped in the pH-sensitive immunoliposomes of this composition can inhibit protein synthesis in the target cells. However here again the efficiency of cytoplasmic delivery was not quantitated.

pH-Sensitive Liposomes in vivo

There are a limited number of reports concerning pH-sensitive liposome behavior in vivo. The most thoroughly investigated are the pH-sensitive liposomes composed of OA/DOPE with or without H-2K^k antibody (Connor et al. 1986). These pH-sensitive liposomes are rapidly cleared from blood circulation and accumulate in the liver and spleen as indicated by distribution of radioactivity of ^{3}H-cholesterol ether, which is used as a lipid marker for liposomes. The pH-sensitive liposomes tend to aggregate upon exposure to the plasma and therefore show a higher trapping in the lung. Attachment of antibody (H-2K^k) to the liposome surface does not significantly alter the overall tissue distribution after i.v. injection in antigen-negative mice and causes a slight acceleration in the liposome clearance from the blood stream in H-2K^k bearing mice. The entrapped contents leaked from the pH-sensitive immunoliposomes at a faster rate and to a greater extent in the antigen bearing mice than in the control mice. The instability of the composition suggests that the composition is not suitable for in vivo use.

Wang and Huang (1987) showed that a plasmid containing the Escherichia coli chloramphenicol acetyltransferase (CAT) gene entrapped in pH-sensitive immunoliposomes could be transferred to the H-2K^k bearing tumor cells in a mouse model, into which both target tumor cells and liposomes were injected intraperitoneally. CAT activity could be detected in the target tumor cells, and only relatively low activity was detected in nontargeted macrophage cells collected from the ascites fluid. Interestingly, the non-pH-sensitive immunoliposomes (OA/DOPC/anti-H-2K^k) could also transfect target cells, albeit

at a lower efficiency. The pH-sensitivity (OA/DOPE versus OA/DOPC) did not influence the distribution of the immunoliposomes as indicated by distribution of a radio-tracer as the lipid marker. Incorporation of antibody to the pH-sensitive liposomes (OA/PE/anti-H-2K^k versus OA/PE) enhanced gene expression in the target cells collected from ascites fluid. The enhancement may be due to the higher uptake of targeted liposomes than nontargeted liposomes by the target cells, since the distribution study showed a two-fold uptake of pH-sensitive immunoliposomes by target tumor cells when compared with that of non-targeted pH-sensitive liposomes. These results imply that the efficiency of the cytoplasmic delivery depends both on the efficiency of liposome uptake by the target cells and the pH-sensitivity of liposomes. Since both target cells and liposomes were administered into the peritoneal cavity, one cannot evaluate the potential usefulness of this composition in vivo.

Another important factor affecting the efficiency of liposome delivery in vivo is the stability of liposomes in the presence of body fluid/plasma. Liu and Huang (1989) have shown that pH-sensitive liposomes devoid of cholesterol are extremely unstable in the presence of plasma. Almost all of the liposomal contents are released from OA/DOPE vesicles within one hour during incubation with plasma. As mentioned above the biostability of liposomes can be modulated by the incorporation of cholesterol into the lipid formulation. The extent of contents leakage from the OA/DOPE vesicles decreases as the amount of cholesterol increases in the total lipid composition from 10 to 50%. However, the acid-sensitivity of the cholesterol-containing pH-sensitive liposomes decreases as the cholesterol contents increases. Therefore optimal cytoplasmic delivery in vivo mediated by fatty acid containing pH-sensitive liposomes may require a compromise between pH-sensitivity and biostability of the lipid vesicles.

DCPA incorporated pH-sensitive liposomes are quite stable in the presence of serum (Leventis et al., 1987; Collins et al., 1990). Only a small fraction (< 20%) of entrapped contents were released after three hours incubation in the presence of 90% of plasma. Recently, Liu and Huang (1990) included 5% of ganglioside (GM_1) in pH-sensitive liposomed containing DOPG/DOPE or DPSG/DOPE to increase the circulation half-life of the liposomes. The authors showed that inclusion of GM_1 in the lipid composition progressively decreased the acid-sensitivity of the vesicles, but only slightly increased the plasma-induced leakage of contents. Inclusion of up to 5% GM_1 retained partial acid-sensitivity (40-50 % release of contents at pH 4) and showed a transit (< 5 hr) increase in the blood level and a concomitant decrease of the liver and spleen uptake of entrapped inulin in vivo. It is conceivable that the efficiency of cytoplasmic delivery can be greatly enhanced using these kind of biostable pH-sensitive liposomes.

Finally the cholesterol derivative, CHEMS, has a number of advantages for in vivo use. This is because it behaves like cholesterol in its interaction with phospholipids (Lai et al., 1985a, 1985b) and therefore should confer biostability to pH-sensitive liposomes.

Factors Affecting Efficiency of pH-Sensitive Liposomes

Four factors influence the efficiency of cytoplasmic delivery from pH-sensitive liposomes: (i) cellular uptake of vesicles, (ii) inherent fusogenicity, (iii) acid-sensitivity, and (iv) biostability of liposomes at neutral pH.

(i) Cellular uptake of vesicles. Targeted pH-sensitive vesicles are much more efficient than nontargeted ones for cytoplasmic delivery (Connor & Huang, 1985, 1986; Collins & Huang 1987; Wang & Huang, 1989). This is because that cellular uptake of targeted liposomes via receptor-mediated endocytosis is more efficient than uptake of nonmodified liposomes via nonspecific endocytosis (Heath et al., 1983; Matthay et al., 1984). Therefore the efficiency of pH-sensitive liposomes is dependent upon the cellular uptake process of vesicles.

Cytoplasmic delivery mediated by non-pH-sensitive control liposomes is less efficient (or nondetectable) than that from the corresponding pH-sensitive liposomes. The less efficient cytoplasmic delivery may be a consequence of the lower cellular uptake of non-PE containing vesicles. Cellular uptake of PE-containing vesicles is much higher than of corresponding PC vesicles (Schroit et al., 1986) due to the higher affinity of a less hydrated surface of PE to cell surface. When a targeting ligand is attached to the surface (PC versus PE), a comparable uptake of both liposome compositions by the target cells occurs (Ho et al., 1986). The non-pH-sensitive immunoliposomes delivered DNA to the cytoplasm with 20% of the efficiency of the corresponding pH-sensitive immunoliposomes (Wang & Huang, 1989). This suggests that acid-induced fusion or leakage is the most important factor for optimal cytoplasmic delivery.

(ii) Inherent fusogenicity. Cytoplasmic delivery does not appear to be correlated with inherent fusogenicity, as opposed to enhanced leakage. In biophysical studies, OA/PE compositions showed a strong fusogenic activity among vesicles at acidic pH. When applied to cell culture, these liposomes do not yield high levels of cytoplasmic delivery (Straubinger et al., 1985; Baldwin et al. 1986). Substantial delivery is only obtained after incorporation of a targeting ligand (Connor & Huang, 1986; Collins & Huang 1987). In contrast, CHEMS/PE vesicles aggregate and release their contents, but do not fuse, at pH 5 in model systems (Ellens et al., 1984, 1985). However, the CHEMS/PE composition results in a much more efficient cytoplasmic delivery than OA/PE composition in cell culture (Chu et al., 1990, Chapter 2).

(iii) Acid-sensitivity. The pH-sensitivity of liposomes prepared from various lipid composition are listed in Table 4. Since the physiological pH in the endosomes varies from cell to cell, ranging from 5 to 6.5 (Tycko & Maxfield, 1982; Tycko et al., 1983), a few pH-sensitive compositions (Hope et al., 1983; Bondeson et al. 1984) are not appropriate or practical for cytoplasmic delivery due to the extreme acidic pH needed to trigger their destabilization or fusion .

The effect of the acid-sensitivity of the liposome composition on the cytoplasmic delivery was investigated by Collins and coworkers (1989). Immunoliposomes composed of DOPE with OA, PHC, or DSPG showed different delivery kinetics but a similar final extent of delivery, using DTA as a marker for cytoplasmic delivery. The liposome diameter and stabilizer/PE ratio were similar among various preparations. Acid sensitivity was represented as the pH value (pH_{50}), at which 50% of the vesicles underwent lipid mixing in model systems. The final extent of delivery was measured by the IC_{50}, which is the concentration at which 50% the cellular protein synthesis is inhibited, of the DTA assay (Collins & Huang 1987; Collins et al., 1989), and did not correlate with the pH_{50} of the three different pH-sensitive liposomes. However, the absence of a correlation may be due to the limitation of DTA assay, which cannot exactly quantitate the number of DTA molecules in the cytoplasm.

The kinetics of cytoplasmic delivery was monitored by measuring cellular protein synthesis. In this assay, NH_4Cl was added to the culture medium to raise the intra-endosomal/lysosomal pH at the requisite time after the start of liposome internalization. This treatment should have stopped the fusion of the pH-sensitive liposomes with the endosome/lysosome and blocked further delivery of DTA. From the kinetics of delivery, the more acid-sensitive (higher pH_{50}) composition can elicit DTA inhibition ($t_{1/2} \cong 5$–15 min after internalization) from compartments which correspond to early endosomes in the endocytotic pathway. The less acid-sensitive vesicles require a longer lag time ($t_{1/2} \cong 25$ min after internalization) to obtain an inhibitory effect with DTA. Control non-pH sensitive liposomes did not cause inhibition of protein synthesis. Thus acid-sensitivity is required for cytoplasmic delivery and may influence the rate of delivery but does not seem to affect the overall efficiency.

(iv) Biostability. Leakage of liposome-encapsulated contents can be induced by disruption of vesicles resulting from plasma protein adsorption and liposome-cell contact as mentioned in section 2. It is well known that cholesterol can increase rigidity and biostability of bilayer membrane and results in reduced leakage of contents. Incorporation of cholesterol into pH-sensitive composition renders the composition more stable in the presence of plasma, but the acid-sensitivity of the cholesterol-containing pH-sensitive

liposomes decreases as cholesterol content increases (Liu & Huang, 1989). Cholesterol-like amphiphiles may be a better choice since they behave similarly as cholesterol in stabilizing PE bilayers and retain their acid-sensitivity (Lai et al., 1985a; Epand et al., 1988). pH-sensitive liposomes composed of PE and DPSG or DOSG of the DCPA group are stable in the presence of plasma as well (Collins et al., 1990). The acid-sensitivity of liposomes formed from these cholesterol derivatives and PE was not compromised by the increasing stability of the vesicles. These pH-sensitive liposomes with higher biostability should retain their contents before internalization by target cells in vivo and thus deliver more drugs to the cells and subsequently into the cytoplasm.

Quantitation of Cytoplasmic Delivery via pH-Sensitive Liposomes

The fraction of liposomal contents delivered to the cytoplasm versus the fraction delivered to the lysosomes is an index of the efficiency of cytoplasmic delivery via pH-sensitive liposomes. To estimate the efficiency, cellular uptake of liposomes is first quantitated. Then either the fraction of liposomal contents that go to the lysosomes or the fraction that is delivered into the cytoplasm must be measured.

There is only one report where the amount of encapsulated contents delivered to the cytoplasm has been estimated (Connor & Huang, 1985). A calibration curve of calcein concentration in MLV versus fluorescence on a microscopic photometer was constructed. From this curve, the cytoplasmic concentration of calcein in cells was derived. The authors concluded that about one thousand liposomes had released their entrapped dye into the cytoplasm of a target cell. Due to the complications associated with this assay, such as, the difference in thickness of MLV and the cytoplasm, uneven distribution of dye in the cytoplasm, interaction of cellular components with the dye, self-quenching of fluorescence, and leakage of liposomal dye during the incubation period, the assay is neither accurate nor reliable. Moreover, the amount of liposomes which become cell-associated under the incubation condition was not measured, thus the fraction of the cell-associated dye delivered into the cytoplasm, instead of the lysosomes, remains unanswered. The complications mentioned above also apply to other fluorescent markers. Such complications make the quantitation of intracellular concentration of fluorescent dyes difficult.

Liposomal cytotoxic drugs have been used in a qualitative manner and show that toxins in pH-sensitive liposomes can inhibit cell growth more efficiently than toxins delivered in non-pH-sensitive liposomes (Connor & Huang, 1986; Baldwin et al., 1986). The lack of a relationship between the intracellular concentration of the cytotoxic agent and

the cytotoxicity makes it impossible to determine how many molecules have been delivered into the cytoplasm.

Assays using potent bioactive molecules, such as DTA, RNA or DNA, pose another problem; these molecules are extremely potent or their effect can be amplified. Thus discrimination of one molecule from a large number of molecules is difficult.

Mechanism of Cytoplasmic Delivery via pH-Sensitive Liposomes

pH-sensitive liposomes are designed to mimic the entry through the endocytic pathway of certain envelope viruses. The low pH in the endocytic vacuoles, endosomes or the lysosomes, is the trigger for the destabilization/fusion of pH-sensitive liposomes. Incubation of calcein-entrapped immunoliposomes (PHC/DOPE) at low temperature (< 20 °C) does not result in diffuse cytoplasmic fluorescence, but incubation at 37 °C does (Connor & Huang, 1985). Moreover the metabolic inhibitors, azide and 2-deoxy glucose, inhibit the development of the diffuse cytoplasmic fluorescence from calcein containing OA/PE vesicles (Straubinger et al., 1985). These results imply that efficient cytoplasmic delivery via liposomes involves the energy-dependent endocytotic process.

Cytoplasmic delivery is also abolished by compounds which raise the pH in the acidic vacuoles, such as NH_4Cl, chloroquine, or monesin. For instance, diffuse cytoplasmic fluorescence disappeared (Connor & Huang, 1985; Straubinger et al., 1985) and the inhibitory activity of entrapped Ara-C or DTA on cell growth or protein synthesis decreased (Connor & Huang, 1986; Collins & Huang, 1987) when NH_4Cl or chloroquine were present during the incubation of cells with pH sensitive liposomes. These results strongly suggest that cytoplasmic delivery is triggered by the low pH in the endosomes or lysosomes.

In model systems, divalent cations are involved with fusion or bilayer destabilization and may be responsible for the cytoplasmic delivery. Divalent cations, Ca^{2+} or Mg^{2+}, induced membrane aggregation, destabilization, or fusion among liposomes composed of acidic phospholipid has been widely studied (Düzgünes et al., 1987). Both Ca^{2+} and Mg^{2+} are synergistic with protons to induce membrane fusion or destabilization among pH-sensitive liposomes (Connor & Huang, 1985). The divalent cations can neutralize the negative charge of the stabilizing component of pH-sensitive liposomes and bring PE molecules on apposed bilayer into close contact which facilitates the fusion. Therefore addition of Ca^{2+} to CHEMS/TPE vesicles causes lipid mixing and release of the contents in a way similar to the low pH effect (Ellens et al., 1984, 1985). In the OA/PE system, fusion occurring upon the exposure to the low pH is greatly enhanced by addition of 2 mM of Ca^{2+} (plasma concentration of Ca^{2+}) to the system (Düzgünes et al., 1985).

Since Ca^{2+} is usually required for cellular fusion processes, these results suggest that divalent cations may be involved in cytoplasmic delivery via pH-sensitive liposomes under physiological conditions.

Based upon the above studies, fusion of the liposomal membrane with the intracellular vacuolar membrane and/or disruption of these intracellular membranes are the most probable explanation for cytoplasmic delivery. Whether membrane fusion is really necessary for the cytoplasmic delivery remains unclear.

7. Scope of the Thesis

To circumvent the lysosomotropic delivery of liposomal drugs, two different approaches have been studied at the cellular level. The first is concerned with a pH-sensitive drug and the second is dependent upon a pH-sensitive liposome composition.

The second chapter of the thesis concerns the ability of the pH-sensitive liposomes composed of unsaturated PE and cholesteryl hemisuccinate to deliver small and large molecules to the cytoplasm of cells in vitro. The efficiency of the cytoplasmic delivery is estimated using fluorescent probes and the biological active macromolecule, diphtheria toxin A chain. The mechanism leading to cytoplasmic delivery is investigated by altering the intracellular pH and extracellular divalent cation concentration.

The third chapter describes antiviral studies in vitro with membrane-impermeant phosphono antiviral agents, phosphonoformate (PF) and phosphonoacetate, encapsulated in liposomes. These drugs have three acid titratable groups which are ionized at pH 7.4 and impede drug entry into cells. The relationship between the antiviral potency and the uptake or internalization of the liposomal drug is compared to the nonencapsulated drug. The potential of cytoplasmic delivery exploiting the pH-sensitivity of the drug is discussed.

In the fourth chapter, a Rauscher murine leukemia viral model was chosen to explore the potential of passively-targeted liposomes to increase the therapeutic efficacy of phosphonoformate. In this murine model, the target cells are located in the RES organs (the liver, spleen, and bone marrow), but do not belong to the RES. The relationship between therapeutic activity and biodistribution/pharmacokinetic profile of the liposomal PF are described.

The new methods for cytoplasmic delivery studied in this thesis can increase the cytoplasmic level of the drug, but to improve the therapeutic index of the drug, the liposomes must reach the correct target cells.

CHAPTER II: EFFICIENCY OF CYTOPLASMIC DELIVERY BY pH-SENSITIVE LIPOSOMES TO CELLS IN CULTURE

1. INTRODUCTION

A variety of macromolecules that can modulate the physiology and metabolism of cells, such as antibodies (Morgan & Roth, 1988), DNA (Friedman, 1989), antisense oligonucleotides (Zon, 1988), and ribozymes (Haseloff & Gerlach, 1988) have been proposed as novel therapeutic modalities. These molecules cannot readily cross the plasma membrane, hence a delivery system to introduce them into the cytoplasm is essential for their continued development. One possibility is to use liposomes, which have been widely employed as drug carriers, functioning both as a controlled release system and to deliver encapsulated compounds into cells (Gregoriadis & Allison, 1980). However, the majority of liposomes internalized by cells enter through an endocytic pathway (Szoka et al.,1980a; Straubinger et al., 1983) and the ultimate fate of the liposome is the lysosome. Here enzymatic degradation of the lipids and their contents occur (Dijkstra et al., 1984; Szoka, 1986). Compounds that are degraded in or cannot escape the lysosomal compartment would be inactive when delivered by most liposome compositions described to date.

pH-sensitive liposomes have been developed to circumvent delivery to the lysosome. Such liposomes destabilize membranes or become fusogenic when they are exposed to an acidic environment. In the process of endocytosis the pH is reduced in the endosome, a compartment that precedes the lysosome (Tycko & Maxfield, 1982). The appropriately designed pH-sensitive liposome might then transfer its contents into the cytoplasm before the liposome can be conveyed to the lysosomes.

Straubinger and coworkers (Straubinger et al., 1985) demonstrated that a liposome composed of oleic acid (OA)/phosphatidylethanolamine (PE) can deliver membrane impermeant calcein and fluoresceinated dextran to the cytoplasm. Huang and colleagues (Connor & Huang, 1986; Collins & Huang, 1987; Ho et al., 1987) incorporated monoclonal antibodies with the fatty acid containing pH-sensitive liposomes to construct pH-sensitive immunoliposomes and were able to deliver various chemotherapeutic agents and DNA to the cytoplasm of target cells. Evidence has also been presented that cytoplasmic delivery can occur in vivo (Wang & Huang, 1987). In these prior studies the efficiency of cytoplasmic delivery via the pH-sensitive liposomes was not quantitated.

We have demonstrated that cholesterylhemisuccinate (CHEMS) behaves like cholesterol and stabilizes PE vesicles at neutral pH (Ellens et al., 1984; Lai et al., 1985b)

and that protonated CHEMS accelerates the destabilization of PE vesicles at low pH (< 6.0) by catalyzing the formation of the hexagonal H_{II} phase (Ellens et al., 1984; Lai et al., 1985b). Thus the CHEMS/PE composition is sensitive to the pH change which occurs along the endocytic pathway and liposomes composed of CHEMS/PE may become leaky or fuse with the intracellular membrane by this proton-triggering mechanism after they are endocytosed by cells.

In this report, the intracellular processing of pH-sensitive liposomes has been compared to non-pH-sensitive liposomes using a combination of fluorescent pH-sensitive dyes, radiolabelled albumin, and diphtheria toxin A chain (DTA). We show that pH-sensitive liposomes made of CHEMS/Dioleoylphosphatidylethanolamine (DOPE) deliver encapsulated fluorescent molecules and biologically active macromolecules into the cytoplasmic compartment. Cytoplasmic delivery from the pH-sensitive liposome is orders of magnitude greater than from the non-pH-sensitive composition. However, cytoplasmic delivery still accounts for less than 10% of the liposome contents that become cell associated.

2. MATERIALS AND METHODS

Materials

Dioleoylphosphatidylethanolamine, dioleoylphosphatidylcholine (DOPC), egg phosphatidylcholine (EPC), egg phosphatidylethanolamine (EPE), lysophosphatidylcholine (LysoPC), and oleic acid were obtained from Avanti Polar Lipids Inc. (Birmingham, AL.). Ammonium chloride (NH_4Cl), guanidine, dithiothreitol, trichloroacetic acid (TCA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), Triton X-100, CHEMS, fluorescein isocyanate (FITC), FITC-dextran (MW = 4200, 0.0035 mol FITC/mole glucose) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO.). Calcein, 8-hydroxy-1,3,6-pyrene-trisulfonate (HPTS), N,N'-*p*-xylenebis(pyridinium bromide) (DPX) and sulforhodamine 101 were obtained from Molecular Probes (Junction City, OR.). Poly-(D-glutamic acid-D-lysine) at a 6/4 ratio (Poly-GL, MW = 69,000) was obtained from Miles (Naperville, IL.). Nicked diphtheria toxin was obtained from Calbiochem (San Diego, CA.). ^{3}H-Inulin, ^{3}H-leucine and ^{125}I-NaI were purchased from Amersham (Arlington Heights, IL.). ^{125}I-labelled *p*-hydroxybenzamidine dihexadecylphosphatidylethanolamine was synthesized as described (Abra et al., 1982). Nuclease treated rabbit reticulocyte lysate mixture, leucine-deficient amino acid mixture and Brome mosaic virus RNA were purchased from Promega Biotec Inc. (Madison, WI.).

Cell Culture

The macrophage-like cell line RAW 264.7 and P388D1 cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% heat inactivated fetal calf serum (FCS) and RPMI 1640 with 10% FCS, respectively. For all the experiments, cells were plated as monolayers in 35 mm or 96-well culture dishes (Costar, Cambridge, MA) 16-20 hr prior to use. Cells were checked and found free from mycoplasma contamination.

Preparations of Liposomes

Liposomes (22 μM lipid) were prepared by the method of reverse-phase evaporation (Szoka & Papahadjopoulos, 1978) and extruded through 0.2 μm polycarbonate membranes (Szoka et al., 1980b). pH-sensitive liposomes were composed of CHEMS/DOPE at molar ratios of 4/6, if not otherwise indicated, while control liposomes were made of CHEMS/DOPC with the corresponding ratio. Compounds to be encapsulated were suspended in an isotonic, pH 7.4 solution with the following concentrations: 50-75 mM calcein, 50 mM FITC-dextran (FITC/dextran : 0.08/1), 30 mM HPTS/50 mM DPX, 1 mg/ml BSA/1 mM inulin, 0.6 mM fluoresceinated Poly-GL, and 10 μM DTA. Nonencapsulated calcein, FITC-dextran, and HPTS/DPX were separated by Sephadex G-75 (1 x 20 cm) gel filtration. Nonencapsulated BSA and Poly-GL (in HEPES 10 mM, 145 mM NaCl, pH 7.4 buffer) were separated from encapsulated material by Bio-Gel A 5m (1 x 20 cm) gel filtration. Nonencapsulated DTA was separated from liposome encapsulated DTA by floating the liposomes through a metrizamide gradient (Heath, 1987). Liposome diameter was determined with a laser light-scattering apparatus (NS-4; Coulter Electronics, Inc., Hialeah, FL). Phospholipid concentration was measured by the method of Bartlett (1959).

Stability of Liposomes in Serum

Calcein at 75 mM, a self-quenched fluorescence concentration, was encapsulated in liposomes composed of CHEMS/EPE (1/2), CHEMS/EPC (1/2), OA/EPC (3/10), and lysoPC/EPC (3/10). Nonencapsulated calcein was removed from the preparation by column chromatography on Sephadex G-25 and 1 μmol of lipid was placed in 1 ml of a 50% FCS/HEPES, pH 7.4, buffer at 37 ^{o}C. At intervals, a sample was removed from the incubation mixtures and the percentage of calcein remaining in the liposomes was quantitated from the dequenching of calcein fluorescence (Weinstein et al., 1984).

Preparation of FITC-Poly GL

Poly-GL was labeled with FITC as follows (Skelly et al., 1979). Peptide dissolved in 50 mM borate buffer, pH 9, was mixed with FITC in DMSO at a 1/9 (Poly-GL/FITC) molar ratio. The reaction mixture was kept in the dark at pH 9, room temperature, with constant stirring for 20 hr. Nonreacted FITC was separated on a Sephadex G-50 (1 x 28 cm) column eluted with water. The FITC-Poly-GL complex fractions were lyophilized and redissolved in 10 mM HEPES, 145 mM NaCl, pH 7.4, at a concentration of 0.6 mM before encapsulation. The final product has approximately 6 molecules of FITC conjugated to one molecule of Poly-GL.

Liposome Uptake by Cells in Culture

Cells (1.5×10^6) in 35-mm culture dishes were rinsed with FCS-free media before the addition of liposomes. Liposomes containing ^{125}I-*p*-hydroxybenzamidine dihexadecylphosphatidylethanolamine were diluted in serum-free media and incubated with cells for 1 hour at 37 ^{o}C. At the end of the incubation, cells were washed with cold PBS (6 x) and then lysed with 0.5 N NaOH. Radioactivity associated with the cell lysate was measured in a Beckman gamma scintillation spectrometer and protein concentrations were assayed by the method of Lowry (Lowry et al., 1951).

Fluorescence Microscopy of Liposomal Cytoplasmic Delivery

P388D1 cells (1×10^6) cultured in 35 mm culture dishes were rinsed with FCS-free media and then incubated with calcein, FITC-dextran or FITC-Poly-GL containing liposomes (50-1000 μmol of lipid diluted in FCS-free medium) with or without NH_4Cl (20 mM) at 37 ^{o}C for 1 hour. Cells were then washed 3 times with 2 ml of phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and refed with 1 ml of FCS free medium. A Leitz fluorescence microscope with an excitation filter in the range 450-490 nm (blue band) and a barrier filter for emission fluorescence at wavelengths greater than 515 nm was used to examine cells treated with calcein/FITC liposomes. For cells incubated with HPTS/DPX containing liposomes, one filter set consisting of a blue band excitation filter was used to observe the fluorescence from intracellular HPTS in the higher pH compartment. Fluorescence from HPTS at lower pH (< 6) was efficiently filtered using the blue band filter set. A second set of filters (violet band), excitation 350-410 nm and an emission filter for wavelengths greater than 455 nm was employed to observe the fluorescence from all HPTS inside of cells. The violet band filter permitted observation of HPTS in both the low and the high pH compartments (Matthay et al., 1989).

To investigate if the endosome/lysosome membrane can be destabilized by the pH-sensitive vesicles, P388D1 cells were incubated with the sulforhodamine 101 (12.5 μg/ml). This fluorescent dye was concentrated in the lysosomes after 3 days of incubation. Empty CHEMS/DOPE liposomes (100 μM) were added to the cell culture in dye-free medium for 4 hours, and then the nonattached vesicles were removed by washing 3 times with PBS. The cells were examined with a fluorescence microscope using the 530 to 560 nm excitation filters and an emmision filter set to pass light at wavelengths greater than 610 nm.

Fluorescence was photographed using Kodak P800/1600 film. Exposure time varied from 5 to 78 sec.

Inhibition of Protein Synthesis by Liposomal DTA

(i) Determination of encapsulated DTA concentration

Diphtheria toxin A chain was prepared by a modification of a published method (Donovan et al., 1985). Nicked diphtheria toxin was mixed with dithiothreitol (100 mM) and guanidine (500 mM) at pH 7.5 for 1 hour at 37 ^{o}C. Denatured B chain was precipitated by centrifugation at 10,000 g for 20 min. DTA remaining in the supernatant was dialyzed against 10 mM HEPES, 145 mM NaCl, pH 7.5, buffer. The DTA containing liposomes were lysed with 0.4% Triton x-100 and the amount of DTA encapsulated was determined using the reticulocyte lysate assay (Clemens et al., 1974). A standard curve was constructed by assaying known amounts of DTA with lysed empty lipid vesicles. Aliquots (3.5 μl) of lysed liposomes and DTA were mixed with 17.5 μl of reticulocyte lysate mixture, 0.5 μl of 1 mM amino acid mixture (minus leucine), 1 μl of Brome mosaic virus RNA (0.5 μg/μl) and 2.5 μl of ^{3}H-leucine (156 mCi/μmol, 1 mCi/ml) and then incubated at 33 ^{o}C for 1 hour. Amino acid incorporation into protein was assayed in a 3 μl aliquot of the reaction mixture to which 25 % TCA and 1% carrier BSA were added. The protein precipitate was collected onto Whatman GF/C filter paper and the radioactivity associated with the filter was measured in a Beckman beta scintillation spectrometer. A standard curve of DTA concentration (10^{-6} M to 10^{-9} M) versus the percentage inhibition of leucine incorporation to protein by DTA was thus constructed. This standard curve was used to determine the amount of biologically active DTA in the lysate of DTA-containing liposomes.

(ii) Inhibition of ^{3}H-leucine incorporation to cellular protein

P388D1 cells (1 x 10^5) in a 96-well dish (flat bottom) were incubated with medium, nonencapsulated DTA, empty vesicles, or DTA-encapsulated CHEMS/DOPC (2.5/7.5) or CHEMS/DOPE (2.5/7.5) liposomes for 1 hour at 37 ^{o}C. In order to obtained a more pronounced effect via pH-sensitive liposomes, the ratio of CHEMS/PE is changed to a lower ratio, which is more likely to become destabilized or fusogenic when encountering the acidic pH (Lai et al., 1985b). After two washes, cells were pulsed with ^{3}H-leucine (1 μCi/well) in fresh medium for 6 hours and then lysed with 20 μl 7M guanidine. Protein precipitated with 100 μl 25 % TCA and 50 μl 1% carrier BSA was collected onto glass fiber disks and washed with 10% TCA using a cell harvester (Skatron Inc., Sterling, VA). The radioactivity associated with the dried disks was determined in a Beckman beta scintillation spectrometer.

Fluorimetry of Cell-Associated HPTS

In order to decrease the fluorescence contributed from noninternalized HPTS-containing liposomes on the cell surface or the background fluorescence from liposomes attached to the culture dish, HPTS was coencapsulated with the collisional quencher DPX at an 1:1.7 ratio (30/50 mM) in liposomes. P388D1 cells (1.5 x 10^6) in 35-mm culture dishes were incubated with vesicles, 50 μM CHEMS/DOPE or 500 μM CHEMS/DOPC, for 1 hour and washed with medium 3 times. Then incubation was continued in fresh media without liposomes. At each indicated time point after washing, cells were removed from dishes by exposure to 3 mM EDTA/PBS and diluted to 2.5-5 x 10^5 cells/ml in PBS. Fluorescence emission was monitored at 510 nm using a SPEX Fluorolog 2 spectrofluorometer (Matthay et al., 1989). Fluorescence intensity associated with the cell suspension irradiated at excitation wavelengths of 413 and 450 nm was measured. The ratio of emitted fluorescence intensity at each excitation wavelength of 450 and 413 nm (Fl 450/413) was calculated.

The excitation spectrum of HPTS is a function of pH. The isobestic point for the excitation wavelength of this pH dependent phenomenon is 413 nm. When an excitation wavelength of 450 nm is used, the fluorescence signal increases more than 100-fold as the pH increases from 6 to 8. The ratio of the fluorescence emission for the 450 nm excitation wavelength compared to the 413 nm excitation wavelength (Fl 450/413), can be used as an indicator of the pH of the HPTS solution (Wolfbeis et al., 1983). For instance, a Fl 450/413 of 2.1 indicates that HPTS experiences a neutral pH of 7.4 and a Fl 450/413 of 0.2 represents that HPTS is in an acidic pH (pH<6) environment. The pH-dependent fluorescent pattern of nonencapsulated HPTS is not affected by the coencapsulation of

DPX with HPTS in the liposomes. Since the relationship between the pH and the Fl 450/413 value is almost linear between pH 8 (Fl 450/413 = 3.2) and pH 6.5 (Fl 450/413 = 0.59), this assay permits one to estimate the pH experienced by HPTS molecules when they become cell associated. The measured cell-associated fluorescence included contributions from HPTS in the cytoplasm, HPTS released in acidic vesicles, and to a small extent, HPTS in vesicles that were on the cell surface. The encapsulated HPTS/DPX elicited less than 4% of the maximum unquenched signal at neutral pH, thus the contribution from non-internalized liposomes containing HPTS/DPX to the total cell-associated fluorescence is small. This assumes that there is no leakage of vesicles contents at the cell surface. If 50% of the contents leak, then the residual encapsulated HPTS fluorescence would be 12% of the maximum unquenched signal and the contribution from these HPTS/DPX-containing liposomes on the cell surface is still small. The cell-associated Fl 450/413 would range between 2.1 to 0.2, since the majority of HPTS would be exposed to either an acidic environment in the intracellular compartments or the neutral environment of the cytoplasm, and the ratio would decrease as the fraction of HPTS exposed to an acidic pH increased.

Degradation of Liposomal BSA in Cell Culture

Iodinated BSA (300 μCi/mg) was prepared by the chloramine T method (Greenwood et al., 1963) and was coincorporated with ^{3}H-inulin (28 μCi/mg) into liposomes. Mixtures of double labeled CHEMS/DOPC vesicles (500 μM) plus empty CHEMS/DOPE vesicles (50 μM) and double labeled CHEMS/DOPE vesicles (50 μM) plus empty CHEMS/DOPC vesicles (500 μM) were incubated with 1.8 x 10^{6} P388D1 cells in serum free medium for 2 hr at 37 ^{o}C. Vesicles not associated with cells were removed by washing 6 times with PBS. Cells were incubated in the fresh media without FCS or liposomes for the period indicated. At each time point, cells were washed with PBS (6 times) and lysed with 0.5 N NaOH. Radioactivity (^{125}I or ^{3}H) associated with cell lysates was measured using a Beckman gamma counter and a Beckman beta scintillation spectrometer, respectively. The ratio of cell-associated ^{125}I-BSA/^{3}H-inulin was calculated and normalized to the ratio of ^{125}I-BSA/^{3}H-inulin at the beginning of the experiment.

The iodo-peptide product from BSA degradation in the lysosomal compartment is released to the extracellular medium (Dijkstra et al., 1984). When a non-metabolizable ^{3}H-labelled compound is encapsulated with the BSA, the ratio of the ^{125}I to ^{3}H is a measure of the metabolism of BSA. Inulin which is not degraded by lysosomal enzymes was selected as the non-metabolizable marker. The loss of inulin could occur due only to dissociation of vesicles from the cell, release of liposomal contents at the cell surface, and/or regurgitation

of lysosomal contents. Thus inulin coencapsulated with BSA serves as an internal standard to normalize the cell-associated liposomal contents and the BSA/inulin ratio is an indicator for the extent of degradation of liposomal BSA in the cells.

3. RESULTS

Liposome Preparation and Characterization

The charge and headgroup of phospholipids can influence the physical properties of the resulting liposomes (Gruner, 1987). In the case of PE, containing unsaturated acyl chains, a stable liposome cannot be formed at a pH less than 9.0, rather planar sheets of lipid or hexagonal H_{II} phase lipid tubes exist at room temperature. Incorporating a charged lipid with PE, such as CHEMS, results in the formation of liposomes which are stable at pH 7.0 and room temperature but destabilize as the pH is lowered (Ellens et al., 1984).

Liposomes composed of CHEMS/DOPC have a higher encapsulation volume and encapsulation efficiency than pH-sensitive (CHEMS/DOPE) liposomes (Table 1). This occurs in spite of their comparable size (diameters, 203 ± 70 nm) and surface charge density. The encapsulation volume is 1.5 to 3 fold higher for the control liposomes than for the pH-sensitive liposomes. This suggests that the pH-sensitive liposomes are oligolamellar.

Stability of Liposomes in Serum

Incorporation of cholesterol into the lipid bilayer results in a more rigid membrane (Yeagle, 1985). In the presence of serum, such vesicles are more stable than single component phospholipid vesicles. Cholesterylhemisuccinate behaves like cholesterol and stabilizes PE liposomes at neutral pH (Lai et al., 1985a). As shown in Table 2, the CHEMS/PE composition retains encapsulated calcein as well as the CHEMS/PC formulation and considerably better than formulations that contain oleic acid or lysophospholipid.

Uptake of Liposomes by Cells in Culture

Liposomes composed of CHEMS/DOPE are taken up by P388D1 cells 5-10 times more efficiently than the CHEMS/DOPC vesicles when the same amount of lipid is added to cells in culture (Figure 1a). The uptake is concentration and time dependent. The kinetics of uptake saturates about 8 hr after the start of incubation (data not shown). The

Table 1. Comparison of control and pH-sensitive liposome preparations.

Lipid composition	Encapsulated compounds	Encapsulated ratio (μl/μmol lipid)[a]	Encapsulation efficiency (%)
CHEMS/DOPC [b]	calcein	4.1	N.D.[c]
CHEMS/DOPE [b]	calcein	2.8	N.D.
CHEMS/DOPC [b]	BSA/inulin	5.1	17.7
CHEMS/DOPE [b]	BSA/inulin	3.4	12.5
CHEMS/DOPC [d]	DTA	5.2	20.9
CHEMS/DOPE [d]	DTA	1.8	7.0

a: Results are values from a single experiment of each preparation. When other preparation were measured, the CHEMS/DOPE encapsulation ratio was within 30% of the given values.
b: The molar ratio of CHEMS/DOPC or CHEMS/DOPE is 4/6.
c: N.D.: not determined.
d: The molar ratio of CHEMS/DOPC or CHEMS/DOPE is 2.5/7.5.

Table 2. Percentage of calcein remaining in liposomes in the presence of serum[a].

Time (hours)	CHEMS/EPE (1/2)	CHEMS/EPC (1/2)	OA/EPC (3/10)	LysoPC/EPC (3/10)
0	97[b]	95	95	97
1	95	85	10	29
2	94	81	10	26
3	92	78	11	26
4	91	73	12	29
24	44	29	13	19

a: Liposomes are incubated at 37 °C in the presence of serum.
b: Values are the mean of duplicate measurements from a single experiment that agreed to within 15%.

higher uptake of the PE vesicles cannot be due to the negative charge density since the PC vesicles contained the same molar ratio of CHEMS.

Ellens and coworkers showed that the PE/CHEMS vesicles tended to aggregate in the presence of high concentration of Ca^{2+}/Mg^{2+} (Ellens et al., 1985) and these divalent cations can influence the cellular uptake of certain negatively charged liposomes. The amount of CHEMS/DOPE vesicles taken up by RAW (Figure 1b) or P388D1(data not shown) cells is influenced by the Ca^{2+}/Mg^{2+} present in the medium, 1.8 mM Ca^{2+}/0.8 mM Mg^{2+} in DMEM and 0.5 mM Ca^{2+}/0.4 mM Mg^{2+} in RPMI, whereas the uptake of CHEMS/DOPC vesicles is not affected by the Ca^{2+} concentration. The reason for the different cell affinities of CHEMS/PE and CHEMS/PC liposomes is not clear. We speculate that the hydration of phospholipids, e.g., PE is less hydrated than the PC, may play a role in the phenomenon observed. Vesicles containing PE are shown to adhere to each other more strongly than do vesicles containing PC because of the difference in hydration (Kachar et al., 1986). This may explain the greater effect of divalent cations, which can promote the dehydration of phospholipid, on the uptake of the CHEMS/PE vesicles than on that of CHEMS/PC vesicles.

The cellular uptake of CHEMS/PE is higher than that of CHEMS/PC vesicles (Figure 1), and the leakage in serum is comparable, therefore the amount of contents delivered varies accordingly. Based on the data in Figure 1a, 50 μM CHEMS/DOPE and 500 μM CHEMS/DOPC were selected for the experiments reported below. These respective concentrations result in comparable amounts of cell-associated content delivery by the two liposome compositions.

Fluorescent Microscopy of Cytoplasmic Delivery of Fluorophores

Calcein is a very sensitive indicator for delivering liposome contents into the cytoplasm because it does not readily cross membranes even under mildly acidic conditions (Straubinger et al., 1983), and when a 50 mM self-quenched solution is diluted into a larger volume the fluorescence intensity increases more than 20 fold. When encapsulated in control liposomes (CHEMS/DOPC) and incubated with cells, fluorescence is present in a punctate (vacuolar) pattern in the cells (Figure 2a). If delivered by pH-sensitive liposomes, a diffuse calcein fluorescence is visible throughout the cells (Figure 2b), indicating that calcein molecules distribute in the cytoplasm. Although the cells shown in figure 2b are incubated with liposomes for one hour, the diffuse cytoplasmic fluorescence with lower intensity can be seen at as early as 15 min after addition of the pH-sensitive liposomes.

In the presence of 20 mM NH_4Cl, a concentration that dissipates the pH gradient in the acidic granules (Maxfield, 1982), a punctate fluorescence is observed in cells incubated

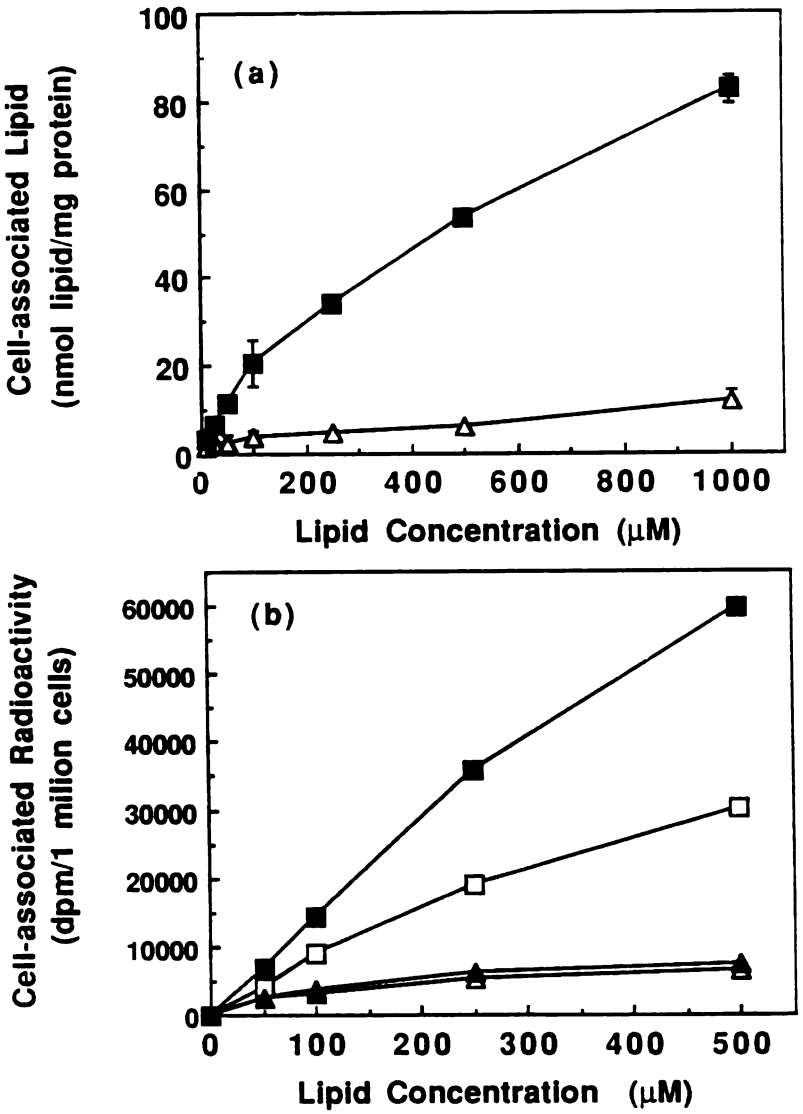


Figure 1. (a) Concentration dependent uptake of liposomes by P388D1 cells. Cells were incubated with ^{125}I-labelled liposomes of CHEMS/DOPC: 2.5/7.5 (Δ) or CHEMS/DOPE: 2.5/7.5 (■) for one hour at 37 °C and non-attached liposomes were washed off before the measurement of cell-associated radioactivity. (b) Divalent ion dependent uptake of liposomes by RAW 264.7 cells. Cells were incubated with ^{125}I-labelled CHEMS/DOPC: 4/6 (▲, Δ) or CHEMS/DOPE: 4/6 (■, □) liposomes suspended either in DMEM (1.8 mM Ca^{2+}/0.8 mM Mg^{2+}) (▲, ■) or RPMI1640 (0.5 mM Ca^{2+}/0.4 mM Mg^{2+}) (Δ , □) for one hour at 37 °C. Values are the means of duplicates in a single experiment and the bars represent the range of values from the means.

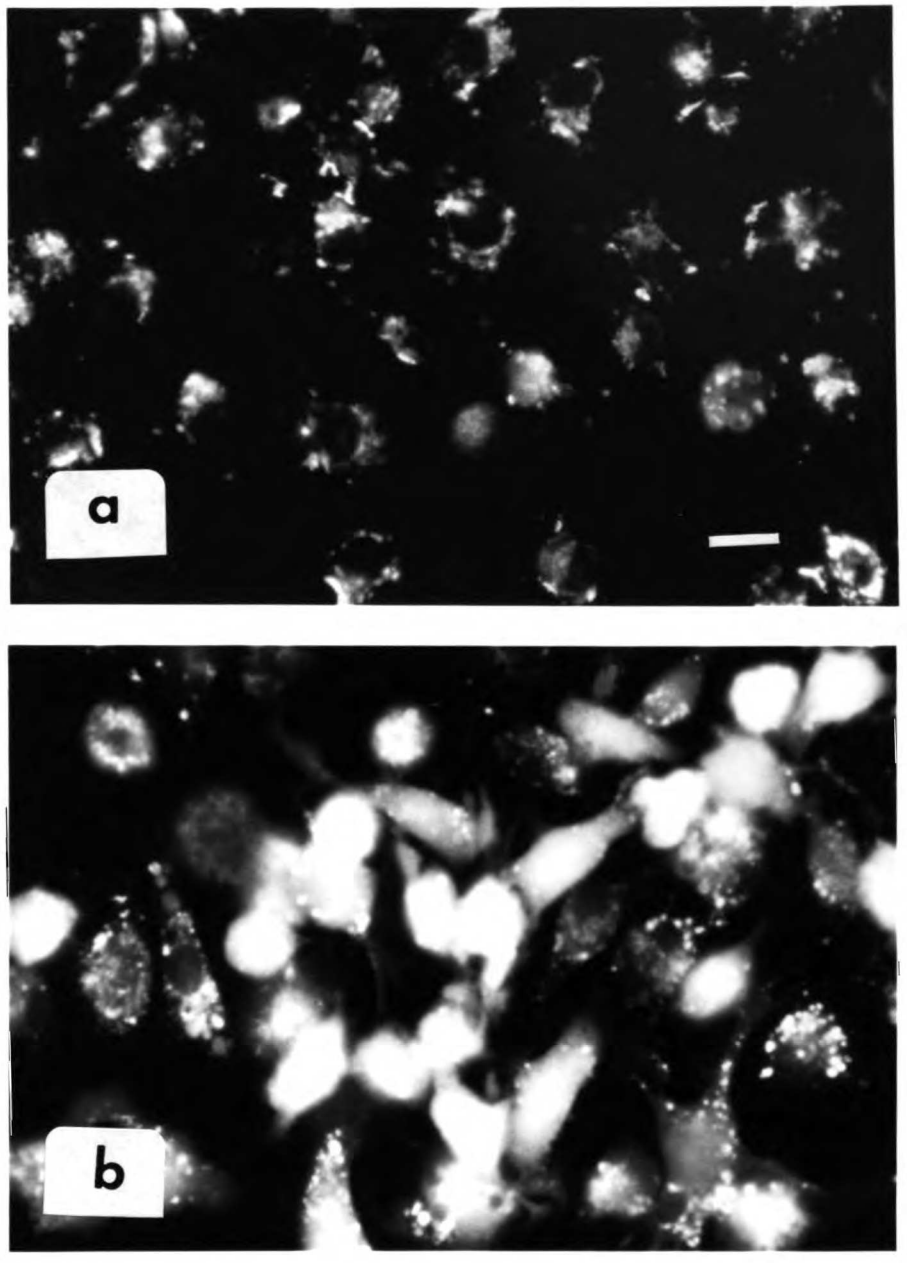


Figure 2. Fluorescence of RAW264.7 cells after incubation with calcein-containing liposomes for one hour. (a) Punctate fluorescence resulting from incubation with 500 μM CHEMS/DOPC: 4/6 liposomes. (b) Diffuse (cytoplasmic) fluorescence obtained by incubation with 100μM CHEMS/DOPE:4/6 liposomes. Bar indicates 10 μm.

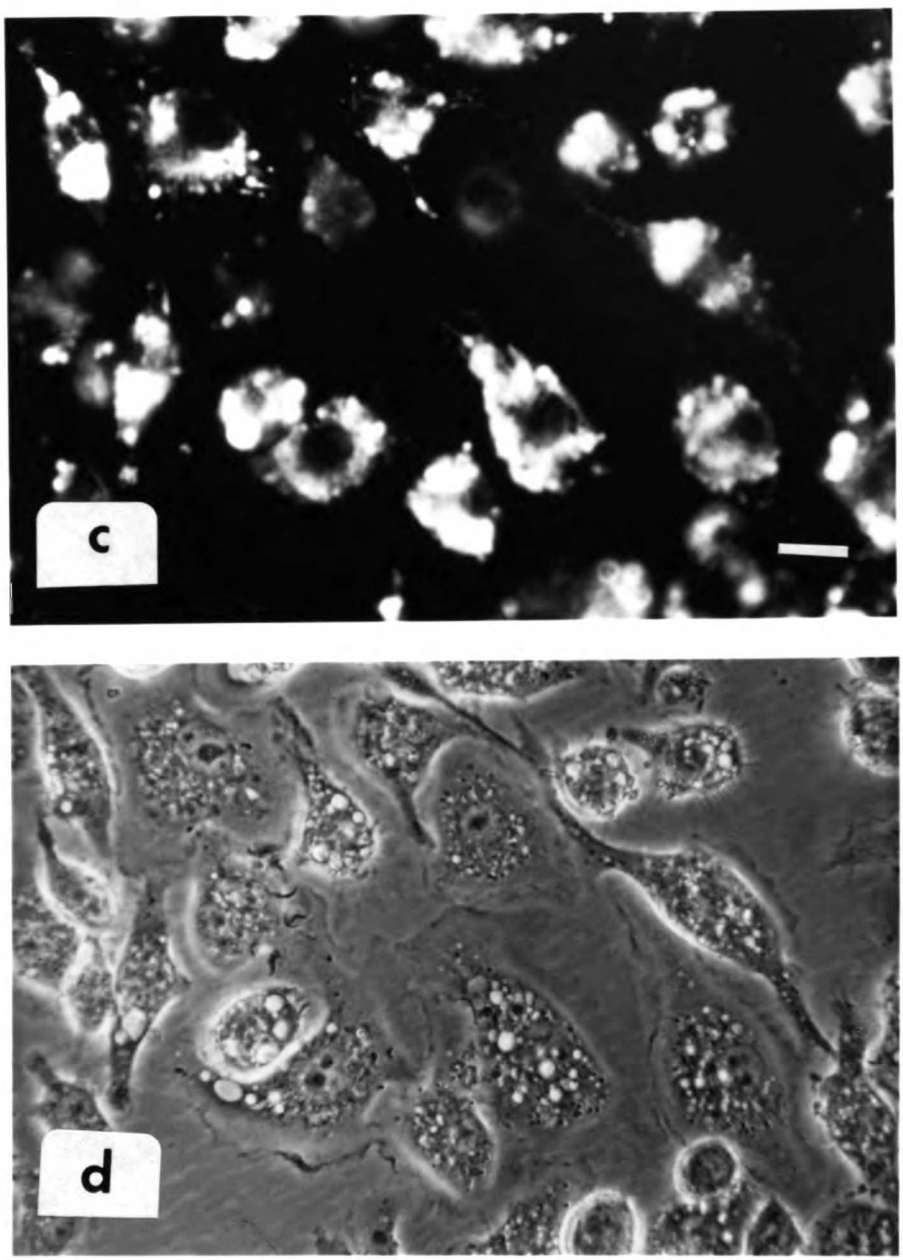


Figure 2. (continued) Fluorescence of RAW264.7 cells after incubation with calcein-containing liposomes for one hour. (c) Vacuolar fluorescence from incubation with 100 μM CHEMS/DOPE:4/6 vesicles in the presence of 20 mM NH_4Cl. (d) Phase contrast image of the field shown in (c). Bar indicates 10 μm.

with the pH-sensitive liposomes (Figure 2c). The brightly fluorescent vacuoles indicate that calcein is released and confined in these compartments. This supports the idea that transfer of calcein to the cytoplasm is a pH dependent phenomenon.

Divalent cations, Ca^{2+}/Mg^{2+}, are also required for cytoplasmic delivery via pH-sensitive liposomes. When divalent cations were chelated by the addition of 1 mM EDTA in DMEM, cytoplasmic calcein fluorescence from pH-sensitive liposomes was not observed (data not shown). Only the punctate fluorescence which was similar to that of cells treated with NH_4Cl was obtained, even the fluorescence intensity observed under these two conditions, NH_4Cl and EDTA treatment, was similar. These findings indicate that both protons and divalent cations are involved in the destabilization/fusion of CHEMS/DOPE vesicles resulting in cytoplasmic delivery. All the phenomena describing the cytoplasmic delivery via CHEMS/DOPE vesicles and the factors altering the outcomes are similar in RAW 264.7 and P388D1 cells. No appreciable differences in their response to the pH-sensitive liposomes were observed.

When either P388D1 or RAW 264.7 cells were incubated with sulforhodamine 101 for 3 days, fluorescence was found to be concentrated in the perinuclear vacuoles (lysosomes) as previous described (Wang & Goren, 1987). Incubation of these cells with fresh medium containing empty CHEMS/DOPE or CHEMS/DOPC vesicles does not change the fluorescent distribution in the cell, in other words, fluorescence is observed only in the perinuclear vacuole and not in the cytoplasm (data not shown). This result indicates that pH-sensitive liposomes are not likely to destabilize the lysosomal membranes and cause release of sulforhodamine 101 from these intracellular compartments into the cytoplasm. The possibility of fusion between pH-sensitive liposomes and the endosomal/lysosomal membrane or destabilization of endosomal membranes by the pH-sensitive liposomes cannot be excluded. Since the intracellular vacuoles are known to become disrupted due to the hypertonicity of a 20% glycerol solution (Okada & Rechsteiner, 1982), sulforhodamine in the perinuclear vacuoles could be released into the cytoplasm by exposing the cells to 20% glycerol for 5 min. This treatment of glycerol results in a bright cytoplasmic fluorescence in the control cells and the cells incubated with the pH-sensitive or non-pH-sensitive liposomes. The glycerol treatment also serves as a positive control demonstrating the rupture of the intracellular vesicles and redistribution of the dye into the cytoplasm. Whether the effect of hypertonic glycerol treatment is limited only to the disruption of lysosomal or involves other intracellular membranes is not clear, so that other mechanisms that induce redistribution of the dye from the lysosomes to the cytoplasm cannot be ruled out.

When large fluorescent molecules, such as FITC-dextran (MW=4200) and FITC-Poly GL (MW=69,000) are delivered in pH-sensitive liposomes, cytoplasmic fluorescence is observed in the cells, which can be differentiated from punctate fluorescence in the vacuoles. Only punctate fluorescence is observed when these two macromolecules are delivered in the non-pH-sensitive CHEMS/DOPC composition. However, due to the background fluorescence of vesicles containing the non-quenched fluorophore adhering to the cell surface and the substratum, photographs of cells (P388D1 and RAW 264.7) treated with the pH-sensitive liposomes do not conclusively document the phenomenon.

The efficiency of cytoplasmic delivery from one other pH-sensitive composition, oleic acid/DOPE, was also examined on the P388D1 and RAW 264.7 cells using calcein as a fluorescent marker. A very dim diffuse fluorescence, much less than that seen with the CHEMS/DOPE vesicles (Figure 2b), is observed (data not shown).

In cells (P388D1) incubated with pH-sensitive liposomes containing HPTS/DPX, cytoplasmic fluorescence is observed when the blue band filter (high-pH form of HPTS) is used (Figure 3b). When the violet band filter (both pH forms of HPTS) is used, a punctate and diffuse fluorescence pattern is observed in cells incubated with the pH-sensitive liposomes (Figure 3a). A similar result is obtained when RAW 264.7 cells are used (data not shown).

These results indicate that membrane-impermeant fluorophores can reach the cytoplasm when they are delivered by pH-sensitive liposomes but end up predominantly in the perinuclear vacuoles while delivered by non-pH-sensitive liposomes.

Protein Synthesis Inhibition by Liposomal DTA

Diphtheria toxin is a highly potent inhibitor of protein synthesis. Estimates as low as one molecule per cell, under appropriate conditions, can totally inhibit protein synthesis (Yamaizumi et al., 1978). The A chain of diphtheria toxin is unable to cross either the plasma membrane or the endosomal membrane without the assistance of the B chain of the toxin. Hence DTA is not inhibitory to cells. The inhibition of leucine incorporation by DTA delivered by liposomes is shown in Figure 4. Neither nonencapsulated DTA (up to 10^{-6} M) plus empty vesicles, equivalent to the concentration of DTA-containing liposomes, nor DTA-containing control liposomes (up to 5×10^{-8} M DTA/10^{-3} M lipid) inhibit leucine incorporation into cellular protein. Inhibition is observed only when cells are incubated with DTA-containing pH-sensitive liposomes. A dose-dependent inhibition from DTA encapsulated in pH-sensitive liposomes is observed (Figure 4a). The concentration of CHEMS/DOPE vesicles that caused a 50% inhibition (IC_{50}) is 40 μM which contain about 7.0×10^{-10} M (or 1.4×10^{-13} mol) of DTA. Ammonium chloride blocks the inhibition of

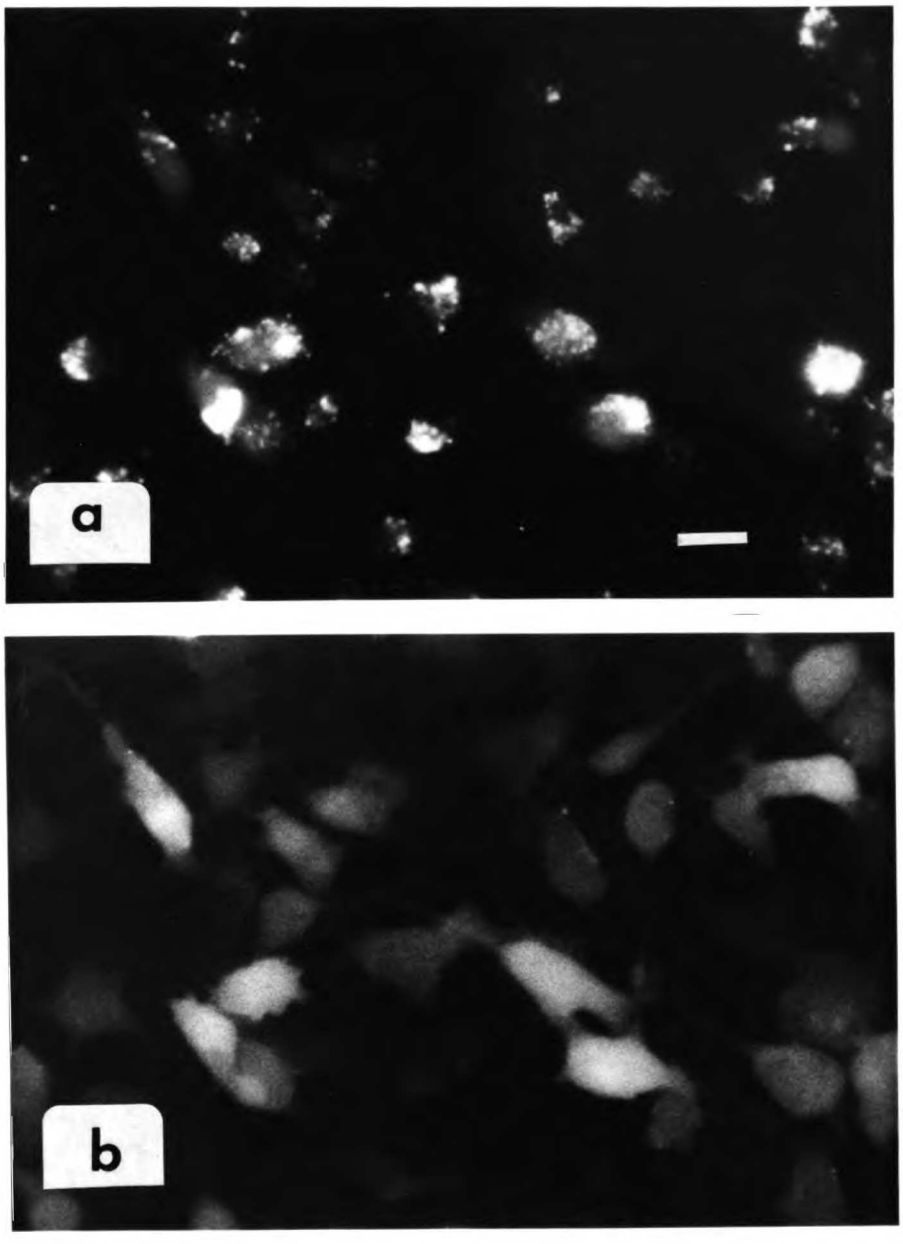


Figure 3. Cellular location of liposomal HPTS in P388D1 cells. (a) Vacuolar fluorescence and diffuse fluorescence observed under violet band filter set after incubation with 50 μM CHEMS/DOPE: 4/6 vesicles containing HPTS/DPX. (b) Cytoplasmic fluorescence viewed under blue band filter set after incubation with 50 μM corresponding pH-sensitive liposomes. Bar indicates 10 μm.

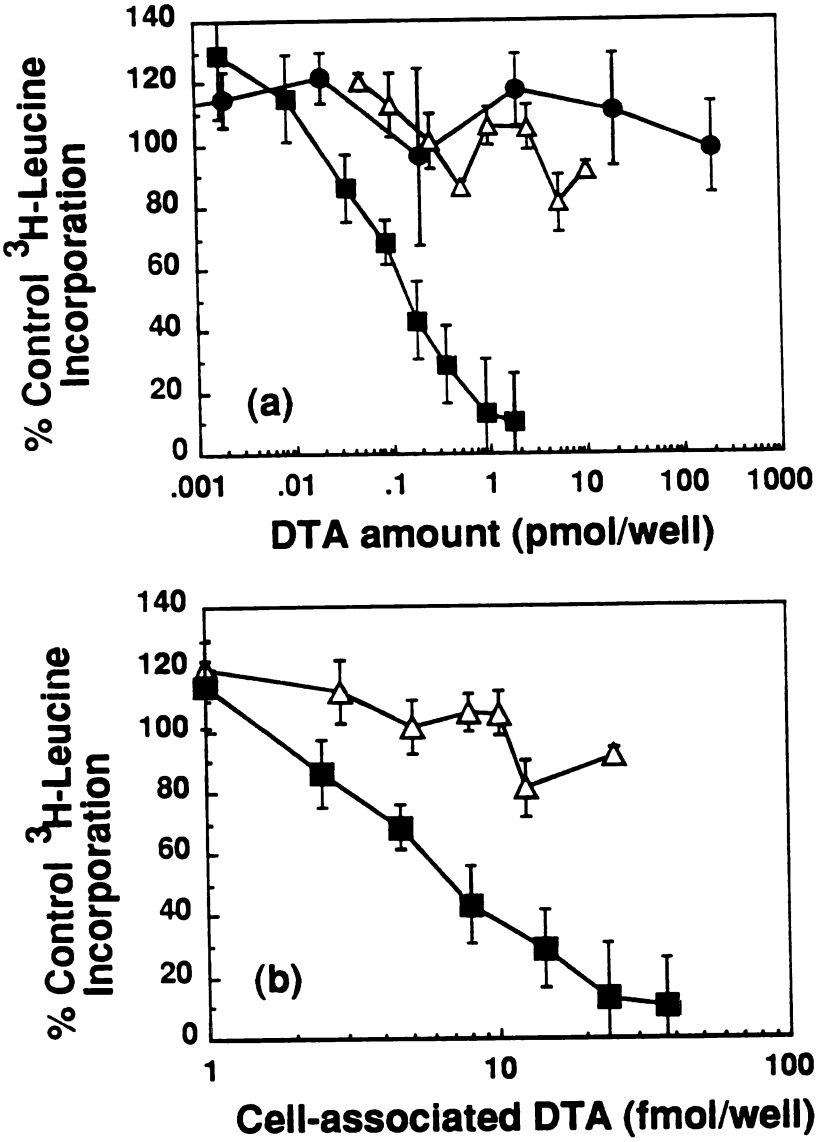


Figure 4. Inhibition of ^{3}H-leucine incorporation by liposomal DTA in P388D1 cells. (a) Result expressed as amount of DTA in DTA-containing CHEMS/DOPC: 2.5/7.5 vesicles (Δ), in DTA-containing CHEMS/DOPE: 2.5/7.5 vesicles (■), or in a nonencapsulated form (●) incubated with 1 x 10^5 cells for one hour. (b) Result expressed as cell-associated DTA after one hour incubation with DTA-containing CHEMS/DOPC (Δ) or DTA-containing CHEMS/DOPE vesicles (■). The cell-associated amount of DTA was calculated based on the result shown in Figure 1a and the encapsulation ratio of DTA in the liposomes (mol DTA/mol lipid). Values are the means of quadruplicate measurements in a single experiment and bars represent the standard deviations from the means.

protein synthesis elicited by DTA-containing pH-sensitive liposomes (data not shown). This indicates that a low pH pathway is involved in the introduction of DTA from the pH-sensitive liposomes into the cytoplasm. This inhibition of protein synthesis experiment was not examined in RAW 264.7 cells, alhough the outcome is expected to be similar to that obtained form P388D1 cells due to the similar behaviors observed in other experiments.

Cell-associated DTA can be calculated based upon the amount of DTA encapsulated in liposomes (17.5×10^{-15} mol DTA/nmol CHEMS/DOPE lipid or 52.1×10^{-15} mole/nmol CHEMS/DOPC lipid) and the measured amount of vesicle uptake per cell (Figure 1a). This assumes that all the cells take up liposomes, encapsulated molecules do not leak from the liposomes and one million cells corresponds to 0.3 mg of cellular protein. When the dose response curves are plotted on the basis of the amount of cell associated DTA (Figure 4b), the pH-sensitive composition is at least 100 times more efficient at delivering DTA to the cytoplasm than the non-pH-sensitive composition.

Acidification of HPTS

The fluorescence ratio from cell-associated HPTS/DPX as a function of time after incubation is shown in Figure 5. Since the Fl 450/413 of cell-associated HPTS correlates with the pH of the environment HPTS is exposed to, this assay is used to estimate the pH of the cell-associated HPTS. In this experiment, the liposomes containing HPTS/DPX have a Fl 450/413 of 2.1 at pH 7.4, which is not significantly different from the value obtained using the nonencapsulated, unquenched HPTS solution at pH 7.4. At the zero-time point, shown in Figure 5, the P388D1 cells have already been incubated with the HPTS/DPX-containing liposomes for 1 hour at 37 ^{o}C. Vesicles not firmly attach to the cells are removed using three successive washes at 4 ^{o}C. At the zero-time point, the assay indicates that the average HPTS molecule experiences a pH 6.6 environment. This is because a fraction of the liposomes have been internalized and they have delivered their contents into a low pH compartment. As the incubation continues, the Fl 450/413 ratio progressively declines until the Fl 450/413 reaches 0.25 (corresponding to pH 6.1) after 4.5 hr (Figure 5). The Fl 450/413 ratio remains constant at 0.25 for 24 hr, the last time point measured. There is no significant difference in the rate of acidification of intracellular HPTS whether the fluorophore is delivered in the pH-sensitive or non-pH-sensitive lipid vesicles. The similar result is obtained when RAW 264.7 cells were studied (data not shown). Only the Fl 450/413 ratio is slightly higher at 4.5 hr after washing. The variability of this assay is about 15%. Although fluorescence microscopy indicates cytoplasmic delivery from the pH-sensitive liposomes, the result with the

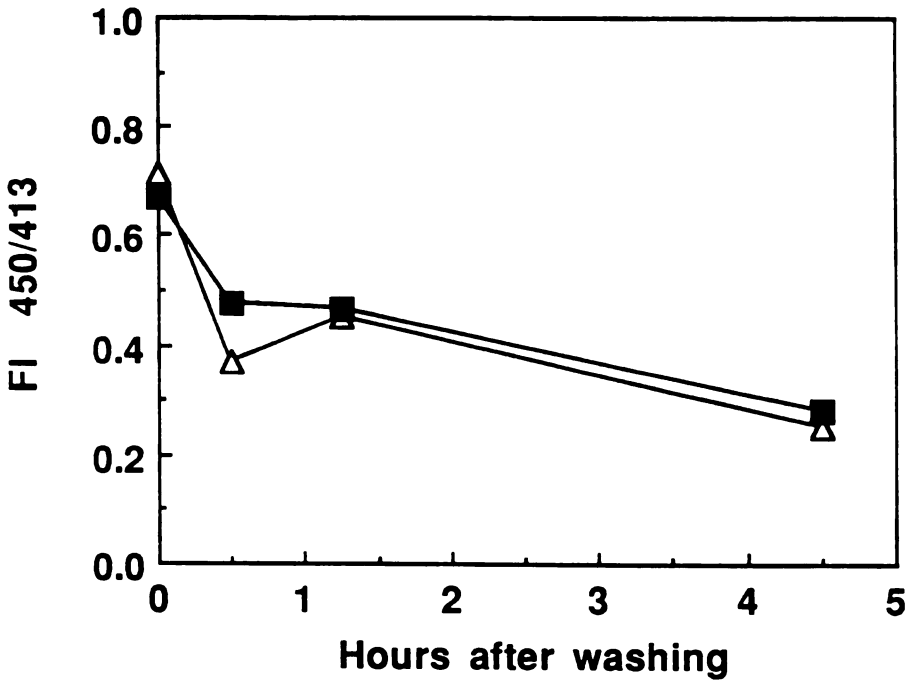


Figure 5. Time course of acidification of liposomal HPTS in P388D1 cells. Cells were treated with HPTS/DPX-containing CHEMS/DOPC: 4/6, 500μM. (Δ), or CHEMS/DOPE: 4/6 50 μM (■) vesicles for one hour. Non-attached vesicles were removed and fresh medium was added to cells at time 0. At the time indicated, cell-associated fluorescence observed at 510 nm when excited at wavelengths of 450 nm and 413 nm were measured as described in the methods. Values represent one measurement from a single experiment. The variation between experiments is within 15% of the value reported.

spectrofluorimetric assay suggests that the fraction of HPTS delivered into cytoplasm via the pH-sensitive liposomes is less than 10% of the cell-associated contents.

Degradation of BSA

The degradation rate of BSA was investigated to estimate the fraction of BSA that escaped the lysosomotropic pathway. This assay is based on the fact that BSA degradation in the cytoplasm (half-life = 20-34 hr) (Neff et al., 1981; Zavortink et al., 1979) is much slower than BSA degradation in the lysosomes (half-life < 1 hour) (Dijkstra et al., 1984). The degradation products of BSA are released into the extracellular medium while the inulin is resistant to lysosomal enzymes and remains within the cells. Thus the cell-associated BSA/inulin ratio can be used as an indicator for the extent of degradation of liposomal BSA in the cells. This experiment was conducted so that a similar level of lipid became cell-associated with both the pH-sensitive liposomes and the non-pH-sensitive liposomes. The initial BSA/inulin ratio was 1.0. After a two hour exposure to liposomes, the cells were washed and the incubation continued in the absence of liposomes. Immediately after the wash the ratio was 0.73 for control liposomes and 0.65 for pH-sensitive liposomes because the BSA was being degraded inside the cells during the incubation. This ratio progressively declines, regardless of the liposome composition, to 0.34 by 3 hr (Figure 6). The BSA/inulin ratio in duplicate determinations varied 10% from the mean value. Thus cytoplasmic delivery of less than 10% of the contents would be undetectable by this method. The similarity in the BSA degradation rate between the two compositions indicates that the cytoplasmic delivery from the pH-sensitive composition is less than 10% of the cell-associated contents. A similar conclusion is reached when BSA-containing CHEMS/DOPE and CHEMS/DOPC liposomes were incubated with RAW cells (data not shown).

4. DISCUSSION

pH-sensitive liposomes were originally developed to release their contents in the pH environment (pH 6.6-7.2) found in the vicinity of certain tumors (Yativin et al., 1980). Since the pH change from the plasma to the tumor is rather modest, investigators turned their attention to the endosome. Liposomes are internalized into cells via the endosome and the pH in this pre-lysosomal compartment is reduced from pH 7.4 to between 5.3 and 6.5 within minutes after the endosome is formed. The pH-sensitive liposomes are designed to mimic enveloped viruses which have membrane fusion proteins that undergo a

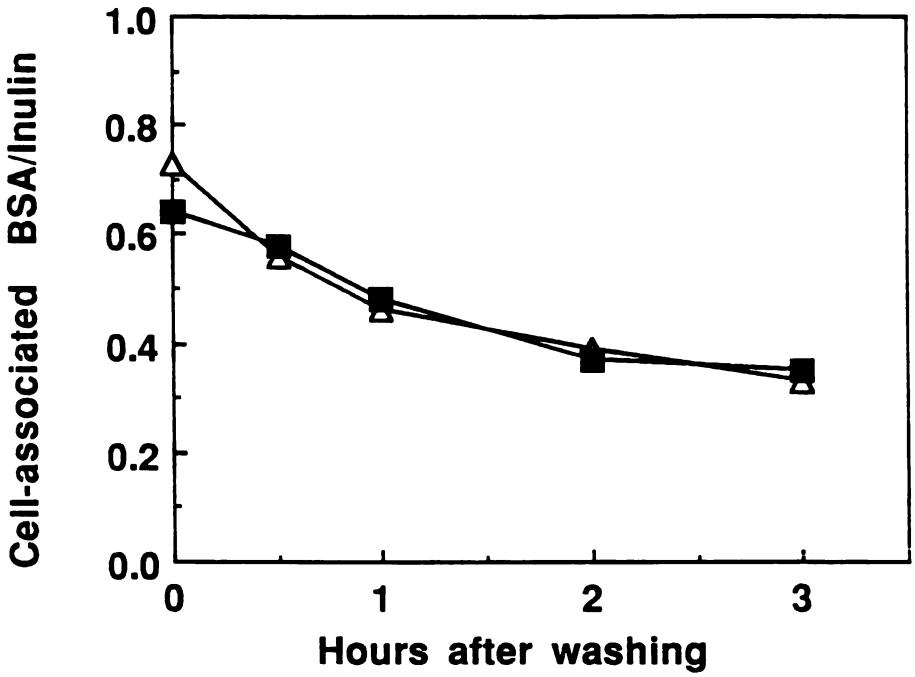


Figure 6. Time course of degradation of liposomal BSA in P388D1 cells. Empty CHEMS/DOPC:4/6 liposomes (500 μM) plus ^{125}I-BSA/^{3}H-inulin encapsulated CHEMS/DOPE:4/6 vesicles (50 μM) (■) and empty CHEMS/DOPE:4/6 vesicles (50 μM) plus ^{125}I-BSA/^{3}H-inulin containing CHEMS/DOPC: 4/6 vesicles (500 μM) (Δ) were incubated with cells for 2 hours. At time 0, non-attached liposomes were washed off and fresh media was added for continued incubation. Cell-associated radioactivity was measured at the indicated times after washing. Values are the means of duplicates in a single experiment. The ranges of values are within 10% of the means.

conformational change when the pH is reduced to about 6.0. The change in protein conformation induces fusion between the viral and the endosomal membrane and the viral genetic material is delivered into the cytoplasm. Lipid mixtures composed of PE and a pH-titratable fatty acid or lipid derivative have been developed to achieve this goal (Nayar & Schroit, 1985; Düzgünes et al., 1985). Biophysical studies have demonstrated that such vesicles either fuse or leak their contents at low pH. Ellens and colleagues have demonstrated that CHEMS/DOPE vesicles, triggered by H^+, become destabilized at pH < 5.5 and fuse at pH < 5.0 (Ellens et al., 1984; 1985).

In this work, we demonstrate that CHEMS/DOPE vesicles deliver various fluorophores (calcein, FITC-dextran, FITC-Poly GL) and biologically active DTA into the cytoplasm. The fact that both low and high molecular weight molecules are transferred into the cytoplasm implies that disruption of the intracellular membranes or fusion between the liposomal membrane and the intracellular compartment membranes is the most likely mechanism involved in the cytoplasmic delivery. The destabilization/fusion events are most likely to occur in the endosomes, since these vacuoles are the first compartment where liposomes encounters a low pH. Cytoplasmic fluorescence is not observed in the presence of agents that dissipate the pH gradient in intracellular vacuoles; a pH-sensitive event is necessary for the cytoplasmic delivery from the CHEMS/DOPE vesicles. It is also interesting that calcium/magnesium is required for cytoplasmic delivery since biophysical studies have indicated a requirement of millimolar levels of these divalent cations for fusion to occur in model systems (Ellens et al., 1985).

The inhibition of protein synthesis by DTA-containing CHEMS/DOPE vesicles but not by non-encapsulated DTA, empty liposomes, or DTA-containing CHEMS/DOPC liposomes further confirms that DTA is introduced into the cytoplasm by pH-sensitive liposomes. There is a greater than a 5 order of magnitude difference between the cytotoxicity of CHEMS/DOPE-DTA and that of non-encapsulated DTA. Moreover, the CHEMS/DOPE composition is at least 100-fold more efficient in delivering cytotoxic DTA than the CHEMS/DOPC composition. A 50% inhibition was obtained by incubating 7 x 10^{-10} M DTA, encapsulated in 40 μM pH-sensitive liposomes, with cells for 1 hour (Figure 4).

The calculated amount of DTA which becomes cell associated after one hour incubation with CHEMS/DOPE vesicles is 30,000 molecules per cell, if we assume that no leakage occurs during the incubation. The actual cell-associated DTA is probably lower than the amount calculated due to the leakage of contents induced by cell contact (Szoka et al., 1979). To estimate the fraction of the 30,000 cell-associated DTA molecules that reach the cytoplasm in an active form, we can use data from studies on the efficiency of

translocation of intact diphtheria toxin into the cytoplasm of susceptible cells (Moynihan & Pappenheimer, 1981). In this work the reciprocal half time for inhibition of protein synthesis in a cell-free system is a linear function of DTX concentration, with a slope of 1.2 nM^{-1} min^{-1}. If we assume the cytoplasmic volume to be 2 pl and the half-time of inhibition of protein synthesis by 40 μM DTA-containing DOPE/CHEMS liposomes to be 360 minutes (assay was carried out with a pulse of ^{3}H-leucine for 6 hr after removal of non-attached liposomes), the amount of DTA required to elicit this degree of inhibition is 3 molecules per cell. Thus the cytoplasmic delivery efficiency of the CHEMS/DOPE system is at least 0.01% of the cell-associated contents.

The correlation between diphtheria toxin effects on protein synthesis reported in the cell-free system (Moynihan & Pappenheimer, 1981) and DTA effects in the P388D1 cells can only be inferred, thus we emphasize that this estimate is a lower bound on the efficiency of cytoplasmic delivery by the CHEMS/PE system. It is interesting, however, that Moynihan and Pappenheimer conclude that only 0.4% of the diphtheria toxin that becomes cell associated reaches the cytoplasm. Colombatti and coworkers have shown that the cytotoxicity of diphtheria toxin is about 1000 times greater than antibody-linked DTA in target cells (Colombatti et al., 1986). This indicates that the CHEMS/DOPE vesicles are about 2 orders of magnitude more efficient than antibody-linked DTA and within an order of magnitude as efficient as intact toxin at transporting DTA molecules into the cytoplasm.

The CHEMS/DOPE composition seems to be the most efficient liposomal composition reported to date to deliver DTA-inhibitory activity. McIntosh and Heath (1982) showed that DTA-containing phosphatidylserine (PS) vesicles did not inhibit protein synthesis in lymphoma cells even after 21 hr of lag time. Ikuta and colleagues (1987) demonstrated that DTA-containing liposomes (EPC/cholesterol/PS) could kill HIV-infected leukemia cells but not non-infected cells with continuous incubation for 3 days. The killing resulted from the facilitated transport of DTA through the altered plasma membrane of the infected cells rather than from cytoplasmic delivery by the liposomes. Using a similar lipid composition (DOPC/cholesterol/PS or DOPC/cholesterol/DOPE), Jansons and Panzner (1983) reported that approximately 50% inhibition of protein synthesis in human lymphoblastoid cells was obtained by incubating DTA-containing (DTA=1.2 x 10^{-8} M) vesicles with cells for 2.5 hr. They were not able to get greater than 70% inhibition with longer incubation or higher concentration. Attaching antibodies to the surface of liposomes, Collins and Huang (1987) showed that only the DTA-containing pH-sensitive immunoliposomes could inhibit protein synthesis (IC_{50} = 1.4 x10^{-9} M) in target cells after 3 hr of incubation while non-targeted pH-sensitive liposomes or non-pH-sensitive immunoliposomes were ineffective. The cell associated DTA using the pH-

sensitive immunoliposomes was not quantitated, so the efficiency of delivery is unknown. In the absence of targeting, we can obtain a 50% inhibition of protein synthesis at 7.0×10^{-10} M of DTA after a one hour incubation of CHEMS/DOPE-DTA with P388D1. However, the macrophage-like nature of P388D1 cells may also contribute to the higher apparent delivery efficiency of the CHEMS/DOPE vesicles when compared to results in the other studies.

When the efficiency of cytoplasmic delivery by CHEMS/DOPE was compared with another pH-sensitive composition, OA/DOPE, the CHEMS/DOPE vesicles delivered more calcein to the cytoplasm. Moreover, CHEMS/DOPE seems to be able to deliver macromolecules better than the OA/PE composition as well. Baldwin and coworkers (Balwin et al., 1986) employed OA/PE vesicles to deliver pokeweed antiviral protein, a DTA-like protein synthesis inhibitor which cannot readily cross the cell membrane. They showed that the OA/PE-encapsulated compound was 2500-fold more potent than nonencapsulated molecules in inhibiting CV-1 cell growth after 8 hr of incubation, while CHEMS/DOPE-DTA is at least 10^5 times more potent than nonencapsulated DTA in our system. In addition, the greater stability of CHEMS/DOPE in the presence of serum makes CHEMS/DOPE a more suitable formulation for in vivo use.

Both HPTS acidification and BSA degradation experiments clearly show that pH-sensitive liposomes are taken up by cells by an endocytotic pathway. This pathway routes the majority of liposomes and their contents into an acidic environment and eventually into the lysosomes where iodo-albumin is degraded. This is the same as the fate of non-pH-sensitive liposomes. The sensitivity of these two assays to discern the fate of the encapsulated molecules is limited by experimental variation which is always between 3 and 15%. Since there is no significant difference found in the rates of HPTS acidification and BSA degradation between pH-sensitive and control liposomes, the efficiency of the cytoplasmic delivery is less than the sensitivity of the assays.

In summary, we conclude that the CHEMS/DOPE composition can deliver macromolecules to the cytoplasm of cells. This process requires a low-pH environment and the presence of divalent cations to effect cytoplasmic delivery. Whether a true fusion of the liposomal membrane with the endosomal and/or lysosomal membranes occurs cannot yet be determined. However cells whose lysosomes have been labeled with sulforhodamine 101 do not show cytoplasmic fluorescence when incubated with the pH-sensitive liposomes. This indicates that the pH-sensitive liposomes do not cause extensive leakage of the endosomal/lysosomal contents. It suggests that leakage from these intracellular compartments is probably not a significant delivery pathway as opposed to endosome/lysosome liposome fusion for the CHEMS/DOPE composition. The efficiency

of cytoplasmic delivery in the endocytotically active cells studied here is greater than 0.01% and less than 10% of the cell associated liposomal contents. This is superior to other liposome compositions reported to date but still leaves much room for improvement.

Incorporating ligands onto the surface of pH-sensitive vesicles as has been done with antibodies (Connor & Huang, 1986; Collins & Huang, 1987) may increase the efficiency of cytoplasmic delivery. This is because cellular uptake of liposomes is more efficient by receptor-mediated endocytosis than by nonspecific adsorptive endocytosis (Matthay et al., 1984) and the receptor-ligand binding may bring the liposome membrane and the endosome membrane into close proximity. The latter can accelerate the fusion between the two membranes (Szoka et al., 1981). An alternative approach is to incorporate viral fusion proteins (Stegmann et al., 1987) or synthetic peptides (Subbarao et al., 1987) into the liposome bilayer, where they may assume a fusogenic conformation in the acidic endosome and induce fusion between the liposomal and the endosomal membranes (Parente et al., 1988). At present, we are exploring both approaches to increase the efficiency of cytoplasmic delivery from pH-sensitive liposomes.

5. SUMMARY

To circumvent the lysosomotropic delivery of liposomal contents, pH-sensitive liposomes were constructed such that liposmes would fuse with or destabilize the endosomal/lysosomal membrane when encountered the acidic pH in the vacuoles. The intracellular processing of pH-sensitive liposomes composed of cholesterylhemisuccinate (CHEMS) and dioleoylphosphatidylethanolamine (DOPE) by eukaryotic cell lines has been compared to non-pH-sensitive liposomes made of CHEMS and dioleoylphosphatidyl-choline (DOPC). The pH-sensitive liposomes can deliver encapsulated fluorescent molecules [(calcein, fluoresceinated dextran, fluoresceinated polypeptide, and diphtheria toxin A chain (DTA)] into the cytoplasm. Cytoplasmic delivery can be blocked in the presence of ammonium chloride or EDTA, indicating that the process requires a low pH environment and the presence of divalent cations. Inhibition of cellular protein synthesis by DTA delivery from the pH-sensitive liposome is orders of magnitude greater than from the non-pH-sensitive liposome composition. The delivery of DTA into the cytoplasm by pH-sensitive liposomes is at least 0.01% of cell-associated liposomal DTA. There is no significant difference in the degradation rate of bovine serum albumin (BSA) or the rate of acidification of pH-sensitive dye, 8-hydroxy-1,3,6-pyrene-trisulfonate (HPTS), when delivered to cells in pH-sensitive and non-pH-sensitive liposomes. Thus the efficiency of

cytoplasmic delivery is less than 10% of the cell-associated liposome contents, which is the smallest difference that can be detected by these two assays. Based upon the various assays used to measure liposome content disposition in the cell, we conclude that the efficiency of cytoplasmic delivery by the CHEMS/DOPE liposomes is greater than 0.01% and less than 10% of the cell associated liposomal contents.

(This work was published in Pharmaceutical Research, 7: 824-834, 1990)

CHAPTER III: INCREASED EFFICACY OF INHIBITION OF HERPES SIMPLEX VIRUS TYPE 2 REPLICATION BY LIPOSOME ENCAPSULATED PHOSPHONOFORMATE AND PHOSPHONOACETATE

1. INTRODUCTION

Phosphonoformate (PF) and phosphonoacetate (PAA) have been shown to be effective inhibitors of viral replication for a variety of DNA-containing (PF and PAA) (Helgstand et al., 1978; Öberg, 1983; Overby et al., 1977; Overby et al., 1974) and RNA-containing (PF) viruses (Öberg, 1983; Sandstrom et al., 1985; Sundquist & Öberg, 1979). These compounds are potent inhibitors of cell-free DNA polymerases (Öberg, 1983; Overby et al., 1977) and reverse transcriptase (Sandstrom et al., 1985; Sundquist & Öberg, 1979). However, greater than 100-fold more drug is necessary to obtain antiviral effects in tissue culture and animals (Öberg, 1983; Overby et al., 1977).

The reason for the decreased activity in intact cells is due to the low permeability of the phosphono compounds. The pK_as of the three titrable groups of PF are 7.3, 3.4, and 0.5 (Warren & Williams, 1971), while the analogous values for PAA are 8.2, 5.0, and 2.6 (Mao et al., 1985). The poor penetration can be attributed to the multianionic nature of the phosphono compounds at physiological pH. Attempts to enhance their transport properties by chemical modifications have failed to produce more active analogs, even though a large number of congeners have been synthesized and tested (Eriksson et al., 1980; Herrin et al., 1977; Mao et al., 1985).

An alternative approach to increase the transport of PF and PAA into cells that are endocytotically active is to encapsulate the drugs in liposomes, a versatile biodegradable drug carrier (Szoka et al., 1987; Szoka & Papahadjopoulos, 1980). Endocytosis of the liposome-encapsulated agent places the liposome and its contents into the low pH environment of the lysosome (pH 4.0 to 5.5) (Dijkstra et al., 1984; Szoka, 1986) where the charge on the drug will be decreased owing to titration (Figure 1). Neutralization of charge should enhance the penetration of the drug from the lysosome into the cytoplasm. This approach substantially increases the efficacy of aspartylmethotrexate (Heath et al., 1983) and fluoroorotic acid (Heath et al., 1985a). Agents showing enhanced efficacy when encapsulated in liposomes and applied to cell cultures under steady-state conditions have been designated liposome-dependent drugs (Heath et al., 1985a; Heath et al., 1983).

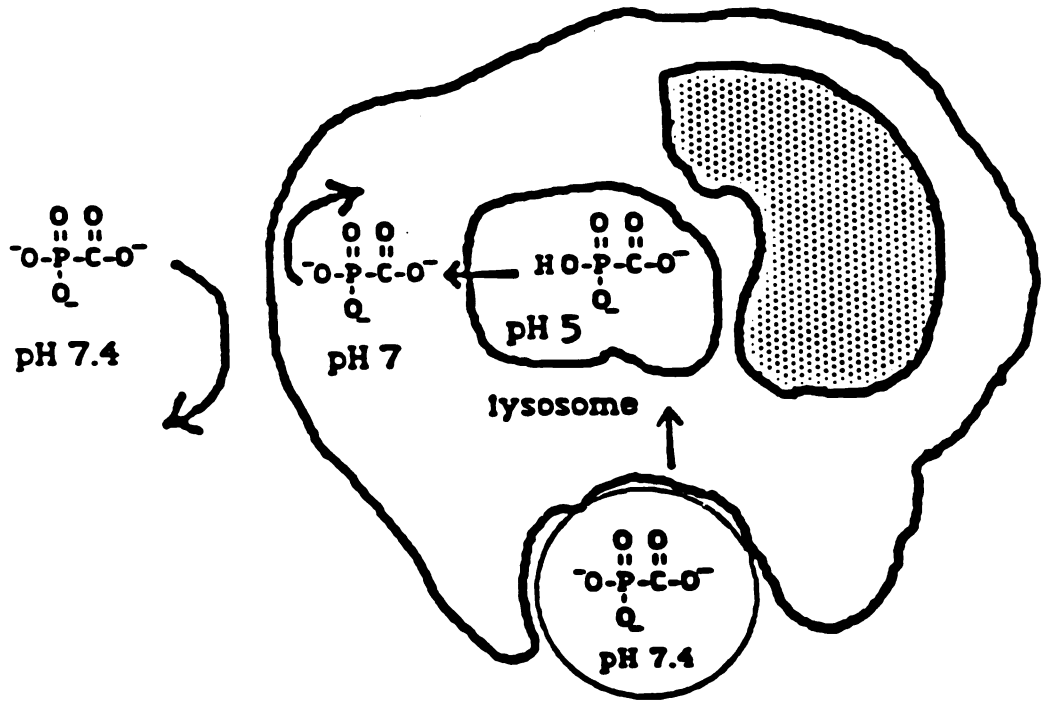


Figure 1. Concept of a liposome-dependent drug illustrated with PF. PF has three titrable groups. At pH 7.4, it will exist predominantly as the trianionic compound; thus, it will have poor membrane permeability. The liposome-encapsulated drug can be internalized into the lysosome of an endocytotically or phagocytically active cell. In this acidic compartment, the drug will be partially protonated upon release from the liposome. In this form it will have a greater membrane permeability. In the cytoplasm it will be deprotonated again, hence effectively trapped in this compartment.

In this study, we demonstrated that PF and PAA behave as liposome-dependent drugs in their inhibitory action against herpes simplex virus type 2 (HSV-2) replication in tissue culture.

2. MATERIALS AND METHODS

Materials

Phosphonoformate trisodium salt (PF), PAA, and cholesterol were obtained from Sigma chemical co., St. Louis, Mo. Egg phosphatidylcholine and egg phosphatidylglycerol were purchased from Avanti Polar Lipids, Birmingham, Ala. The p-hydroxybenzamidine dihexadecylphosphatidylethanolamine was synthesized and iodinated as described previously (Abra et al., 1982). ^{3}H-thymidine, ^{14}C-PF (25 mCi/mmole) and ^{125}I-sodium iodide were products of Amersham Corp., Arlington Heights, Ill..

Drug and Liposome Preparation

Drug solutions were prepared from 8 to 80 mM. The pH was adjusted to 7.4 with either HCl or NaOH, and the osmolality was adjusted to 300 mosM with NaCl before encapsulation. Reverse-phase evaporation liposomes (REV) composed of egg phosphatidylcholine/egg phosphatidylglycerol/cholesterol in a molar ratio of 9:1:8 were prepared and extruded through a 0.2 μm polycarbonate membrane as described previously (32). Nonencapsulated drugs were removed by dialysis against a 2x100-fold excess of 5 mM HEPES (*N*2-hydroxyethylpiperazine-*N*'-2-ethane-sulfonic acid)-140 mM NaCl-0.1 mM diethylene-triamine-pentaacetic acid (pH 7.4) buffer. Non-drug-containing (empty) liposomes were prepared by encapsulating the HEPES buffer. The concentrations of encapsulated drugs and phospholipids were determined by assaying the phosphate content after acid digestion by the method of Bartlett (1959). The vesicle sample was extracted in a two-phase system by the method of Bligh and Dyer (1959). The methanolic aqueous phase contained the drug, and the chloroform phase contained the phospholipid. Liposome diameter was determined with a laser light-scattering apparatus (NS-4; Coulter Electronics, Inc., Hialeah, Fla.).

Virus and Cell Culture

HSV-2 strain G and Vero cells were obtained from Dr. Epstein, Palo Alto, Calif. Vero cells were routinely grown in Eagle minimum essential supplemented with 5% heat-inactivated fetal calf serum obtained from the cell culture facility at the University of

California, San Francisco. The virus was propagated in Vero cells, and titers of various viral preparations were determined by plaque assay in Vero cells (Mogensen, 1976).

CPE assay

A modified version of the cytopathic effect (CPE) assay developed by Epstein et al. (Epstein et al., 1981) was performed as follows. Vero cells (10^4) were cultured in a 96-well plate for 2 days at 37°C. After removal of culture fluid cells were infected by adsorbing HSV-2 (800 PFU per well) to cells at 37°C for 1 hour before drug treatment. Various concentrations of drugs, liposomal drugs, and drugs plus empty liposomes (empty-REV) were added to each well. Each dose was tested in quadruplicate. Controls included in each plate comprised a cell control and a virus control. After 48 hr, cells were rinsed with phosphate-buffered saline and then fixed and stained with Armstrong solution (0.5% [wt/vol] crystal violet, 50% [vol/vol] ethanol, 5% [vol/vol] Formalin, 0.85% [wt/vl] NaCl (Epstein et al., 1981). Excess dye was washed off, and the dye incorporated by the viable cells was eluted with dimethyl sulfoxide. The optical densities (OD) were read at 550 nm with a multichannel spectrophotometer (Titertek-Multiskan). The percentage of dye uptake was calculated as $(OD_{treatment} - OD_{virus\ control})/(OD_{cell\ control} - OD_{virus\ control}) \times 100$. The ED_{50} is the concentration of the drug at which the test well demonstrates 50% of the control well dye uptake.

Virus Yield Assay

Vero cells (10^6) cultured in a 6-well (35-mm-diameter) plate were inoculated with HSV-2 with a multiplicity of infection of 0.05. Drug treatment started 1 hour after virus adsorption. Two days later, cell suspensions were subjected to three cycles of freezing and thawing to release virus. The number of viruses in the supernatant of the cell cultures was determined by plaque assay in Vero cells with a 1.5% Sea Plaque agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) overlay. After virus plaques had developed (2 days), cells were stained with a 0.01% neutral red solution (Eppstein et al., 1983).

Cytotoxicity Assay

Vero cells without virus infection were exposed to drug treatment for 44 hr under the same culture conditions as described above for the CPE assay. Then, 1 μCi of ^{3}H-thymidine was added to each well for 6 hr. At the end of the pulse, the cells were lysed and collected onto glass fiber filters with a multiple-channel cell harvester (Skatron Inc., Sterling, Va.). Radioactivity (counts per minute) associated with each filter disk was

counted in a Beckman liquid scintillation spectrometer. The percentage of ^{3}H-thymidine incorporation was expressed as (counts per minute$_{treatment}$/counts per minute$_{control}$) x 100. The IC_{50} is the concentration of the drug required to reduce thymidine incorporation to 50% of the control value.

A second measure of drug cytotoxicity was cell growth in the presence of drug or encapsulated drug. Vero cells (10^5) were diluted in growth medium to various concentrations and then added to cell cultures in duplicate at time zero. Cells were removed from dishes treated with 0.05% trypan blue, and counted with a hemacytometer at the indicated times.

Uptake of PF and Liposomes by Vero Cells

A trace amount of ^{125}I-labeled *p*-hydroxybenzamidine dihexadecylphosphatidyl-ethanolamine or a trace amount of ^{14}C-PF (0.31 μCi/μmol PF) was used as a lipid or internal marker respectively for liposome uptake studies (Abra et al., 1982). Vero cells (1.2 x 10^6) in 35-mm culture dishes were incubated with various amounts of ^{125}I-labeled liposomes, ^{14}C-PF containing liposomes (3.4 mM PF/31 mM lipid), or nonencapsulated ^{14}C-PF (2.94 μCi/μmol PF). After incubation for the period indicated, nonattached liposomes or drug were removed by rinsing the cultures six times with phosphate buffered saline, and the cells were then lysed with 0.5N NaOH. Radioactivity associated with the cell lysate was measured in a Beckman scintillation spectrometer. The concentration of cellular protein in the lysate was determined by the method of Lowry et al. (1951).

Efflux of PF and Liposomal PF from Vero Cells

Release of PF from Vero cells was determined by measuring ^{14}C-radioactivity in the medium after cells (1.2 x 10^6) had been pulsed with nonencapsulated PF 500μM or liposomal PF 50 μM for 24 h and then chased in 1 ml of drug-free medium for the times indicated. Cell-associated radioactivity at these time points were also determined as described above. To determine the fraction of nonencapsulated PF in the medium, the medium collected at each time point was dialyzed against a 10-fold volume of the HEPES buffer at 4 ^{o}C for 24 hr. Nonencapsulated PF is able to diffuse from the dialysis bag and, an equlibrium has been attained between the inside and the outside of the dialysis bag in 24 hr. Encapsulated PF is retained in the dialysis bag. The total amount of nonencapsulated PF in the medium thus obtained by multiplying the amount of PF in the dialysate by a factor of 1.1. The amount of drug remaining encapsulated in the medium was computed by subtracting the amount of nonencapsulated drug in the medium from the total amount of PF in the medium at each time point.

3. RESULTS

Liposome Preparations

PF and PAA are highly water soluble compounds that can be readily encapsulated in liposomes at high concentrations. Liposome encapsulation of 80 mM drug solution resulted in 0.20 μmol of PF per μmol of lipid and 0.31 μmol of PAA per μmol of lipid. Separate preparations of encapsulating 8, 20, 35, and 71 mM of PAA produced liposomes with a lower PAA-to-lipid ratio, 0.024, 0.048, 0.10, and 0.20, respectively. Owing to the negative charges on the phosphono compounds at pH 7.4 , there is little leakage (less than 1% per week) of either PF or PAA from the liposomes upon storage at 4 °C. The diameter of the liposomes as measured by dynamic light scattering ranged between 0.16 and 0.22 μm for various preparations.

Antiviral Effects

To measure the antiviral efficacy of the liposome-encapsulated compounds, we used a quantitative dye-binding CPE assay (Epstein et al., 1981). In this assay, nonencapsulated PAA had an EC_{50} of about 60 μM, and the addition of empty liposomes did not change this value (Figure 2a; Table 1). Liposome encapsulation of PA (0.31 μmole PA/μmole lipid) resulted in a 150-fold increase in efficacy (EC_{50} = 0.4 μM) (Figure 2a; Table 1). Nonencapsulated PF in the presence or absence of empty liposomes had an EC_{50} of about 210 μM. Encapsulation of PF (0.20 μmol PF /μmole lipid) resulted in about a 30-fold increase in efficacy, EC_{50} = 7 μM (Figure, 2b; Table 1). Plaque reduction assays in the presence of liposome-encapsulated drug could not be done because the agarose overlay restricted the diffusion of the liposome. However, virus yield assays confirmed the increased effectiveness of the liposome-encapsulated PF. In replicate virus yield assays, a 6- to 100-fold increase in efficacy was seen with the liposome-encapsulated PF compared with the nonencapsulated PF and a 100- to 500-fold increase in efficacy compared with free PAA was obtained with liposome-encapsulated PA.

Cytotoxicity

The inhibition of incorporation of thymidine into cellular DNA and effect on cell growth were used as a measure of the cytotoxicity of the drugs. Thymidine incorporation assays were performed under the same tissue conditions as for the CPE assay.

PAA had an IC_{50} of 507 μM which was considerably reduced to 2 μM when the drug was encapsulated in liposomes (0.31 μmol PA/μmol lipid) (Figure 3a; Table 1). In contrast, PF exhibited an IC_{50} of 880 μM which was not appreciably reduced by liposome

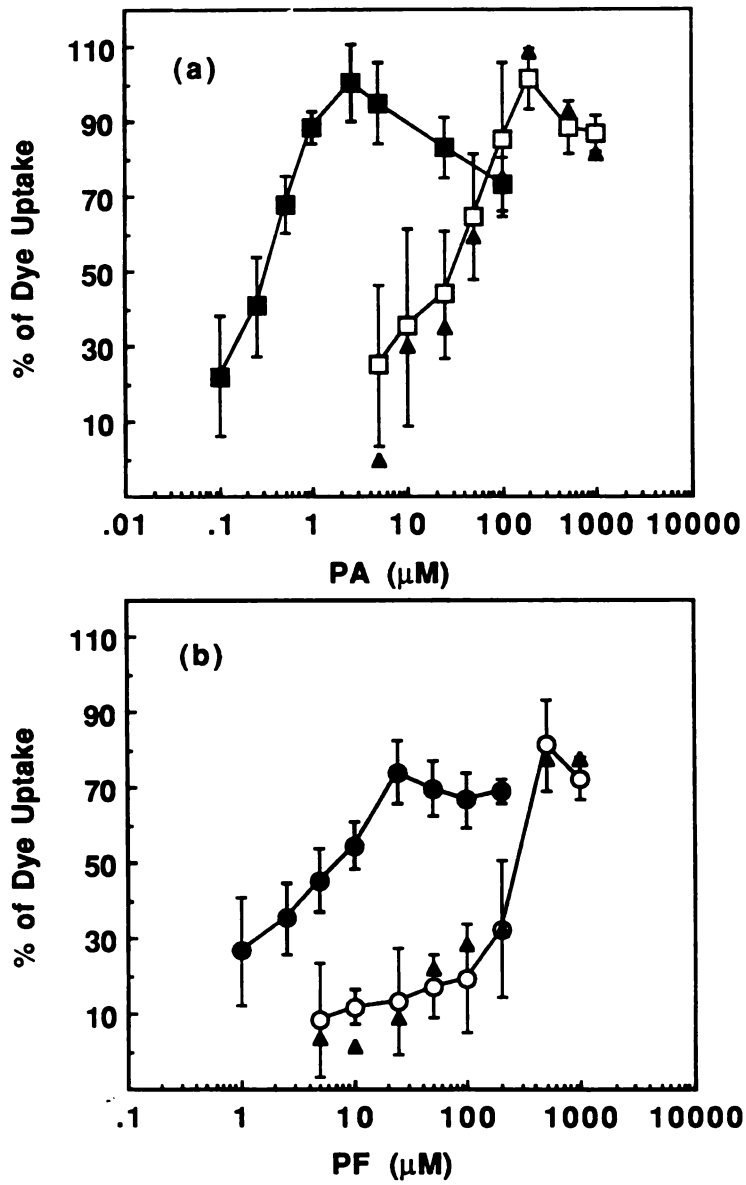


Figure 2. Antiviral effects of encapsulated and nonencapsulated phosphono compounds. (a) Comparison of the antiviral effect of PAA and liposomal PAA assessed by the CPE assay. Vero cells were infected with HSV-2 for 1 hour before drug treatment. At 48 hr after drug treatment, viable cells were fixed and stained and the percentage of dye uptake was calculated as described in Materials and Methods. Symbols: (□) PAA; (▲), PAA plus NDC-Lipo; (■), Lipo-PAA. (b) Comparison of the antiviral effect of PF and liposomal PF assessed by the CPE assay. Symbols: (○), PF; (▲), PF plus NDC-Lipo; (●), Lipo-PF. Values are the mean ± SD (bars), n = 4.

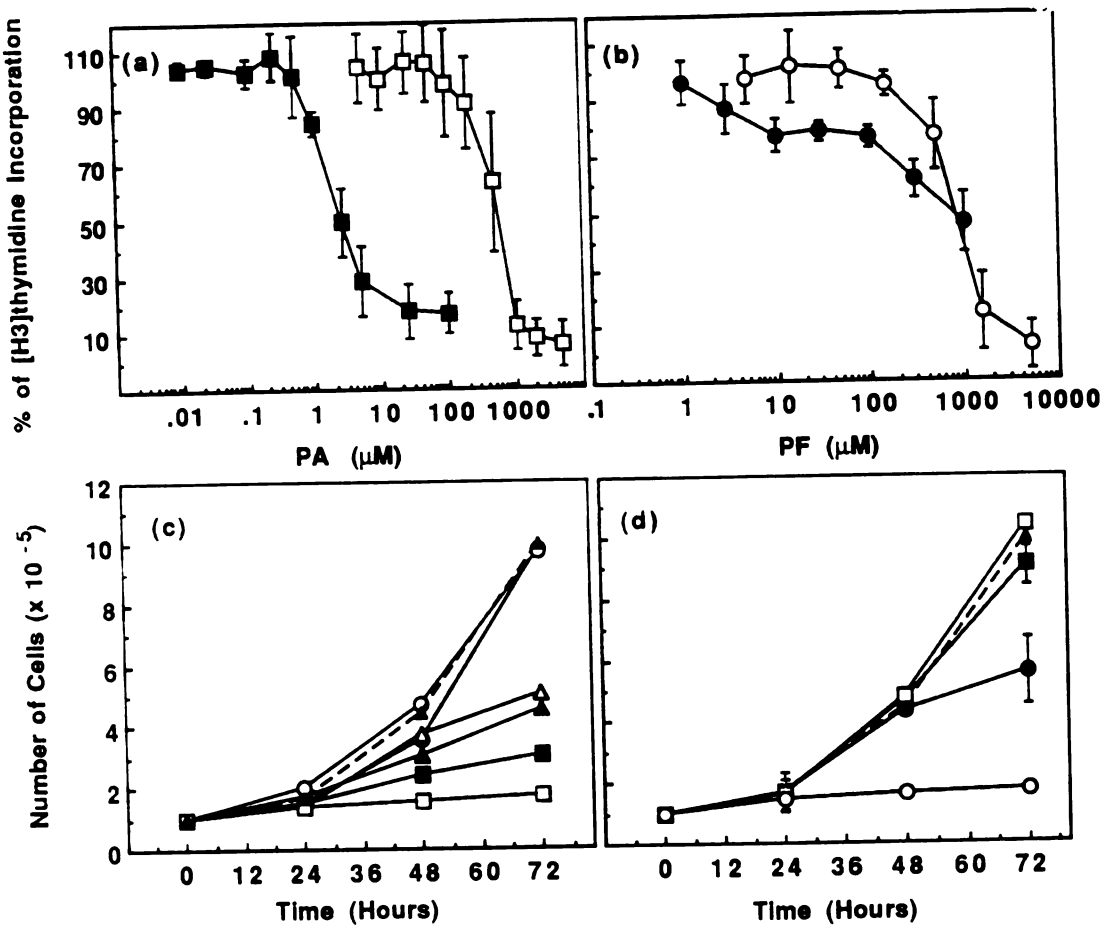


Figure 3. Inhibition of thymidine incorporation and cell growth by encapsulated and nonencapsulated phosphono compounds. (a) Inhibition of thymidine incorporation by PAA and liposomal PAA in Vero cells. Vero cells were incubated with the drug for 44 h, and then thymidine incorporation was measured as described in Materials and Methods. Symbols: (□), PAA; (■), Lipo-PAA. Values are the mean ± SD (bars), n = 4. (b) Inhibition of thymidine incorporation by PF and liposomal PF in Vero cells. Incubation conditions were the same as described for panel A. Symbols: (○), PF; (●), Lipo-PF . Values are the mean ± SD (bars), n = 4. (c) Inhibition of Vero cell growth by PAA and Lipo-PAA. Symbols: (--▲--), control; (○), PAA, 100 μM; (Δ), PAA, 500 μM; (□), PAA, 1000 μM; (●), Lipo-PA, 0.4 μM; (▲), Lipo-PA, 10 μM; (■), Lipo-PA, 50 μM. Each point is the mean of duplicate cell counts that agreed to within 10%. (d) Inhibition of Vero cell growth by PF and Lipo-PF. Symbols: (--▲--), control; (□), PF, 500 μM; (○), PF, 1000 μM; (■), Lipo-PF, 300 μM; (●), Lipo-PF, 1000 μM. Each point is the mean of duplicate cell counts that agreed to within 10%.

Table 1. Comparison of efficacy and toxicity of liposome-encapsulated PF and PAA with those of the nonencapsulated compounds.

Agent	EC_{50} ($\mu M \pm SD$)[a]	IC_{50} ($\mu M \pm SD$)[a]	Selectivity (IC_{50}/EC_{50})	Enhancemem t[b]
PAA	60 ± 25 (2)[c]	507 ± 162 (3)	8.4	
Lipo-PAA[d]	0.4 ± 0.1 (2)[c]	2.1 ± 0.1 (2)[c]	5.3	0.63
PF	210 ± 84 (4)	880 ± 57 (5)	4.1	
Lipo-PF[d]	7 ± 2.9 (4)	>800 (5)[e]	114	27

a: The value in parentheses is the number of independent determinations.
b: Enhancement = the ratio of the selectivity of the liposomal drug to the selectivity of the nonencapsulated drug.
c: These values are the range, rather than the SD.
d: Lipo-PAA, Liposome-encapsulated PAA; Lipo-PF, liposome-encapsulated PF.
e: In one experiment, the Lipo-PF was as cytotoxic as the nonencapsulated PF. In four additional experiments, the Lipo-PF was less cytotoxic than the nonencapsulated PF and the IC_{50} was not reached.

encapsulation (0.20 μmol PF/μmol lipid) (Figure 3b; Table 1). Measurement of cell growth in the presence of the drugs, either encapsulated or nonencapsulated, confirmed the results of the cytotoxicity measured by thymidine incorporation (Figure 3c and 3d). Liposomal PA was more effective than free drug at reducing cell growth; an approximately 50% reduction was observed at 10 μM (Figure 3c). Nonencapsulated PAA at 500 μM reduced cell growth to about 50% of control cell growth (Figure 3c), in close correspondence to its effect on thymidine incorporation. Liposomal PF at 1000 μM reduced cell growth to less than 50% of control (Figure 3d).

Although PA in liposomes was approximately 150-fold more active as an antiviral agent, it was also 250-fold more cytotoxic. Therefore, its selectivity ratio (IC_{50}/EC_{50} = 5.3) was less than that for the free drug (Table 1). Encapsulation of PF in liposomes resulted in a 30-fold increase in antiviral activity but no increase in cytotoxicity. Thus, the selectivity ratio of the PF was increased from 20 to approximately 100 by liposome encapsulation. This unexpected increase in the selectivity ratio for liposomal PF was replicated in five separate experiments.

Antiviral Activity and Cytotoxicity of Liposomal PAA with Various Drug/Lipid Ratios

Encapsulation of 8, 20, 35, and 71 mM of PAA resulted in liposome preparations with a PAA/lipid ratio of 0.024, 0.048, 0.10, and 0.20, respectively. Antiviral activity was determined by the CPE assay and the cytotoxicity was measured by the thymidine incorporation method described above. The results from these liposomal PAA and non-drug containing liposomes are shown in Figure 4 and summarized in Table 2. In this experiment, EC_{50} obtained with the two highest encapsulation preparations was about 0.3 μM and IC_{50} is about 1.5 μM. However, the antiviral activity of the lowest encapsulation (0.024) preparation did not achieve a 100% protection while the cytotoxicity of this preparation was the same as the non-drug containing liposomes. This suggests that the toxicity observed is induced by the lipid, not by the drug, when the preparation with the lowest drug/lipid ratio was used. Liposomes with an encapsulation ratio of 0.048 exhibited a low level of cytotoxicity and a maximum antiviral activity of 60% protection (Figure 4). Thus, reducing the encapsulation ratio (mol of PAA/mol of lipid) from 0.2 to 0.048 decreases the cytotoxic effect such that the IC_{50} is closer to that of the non-drug containing liposomes; however, the antiviral effects are concomitantly reduced (a 100% protection was not achieved). At the lower PAA/lipid ratio (0.048), the $IC_{50} > 100$ μM and the $EC_{50} \cong 0.3$ μM, the selectivity increased to be greater than 333, while selectivity was about 5 at the higher drug/lipid ratio. Thus, varying the amount of drug encapsulated

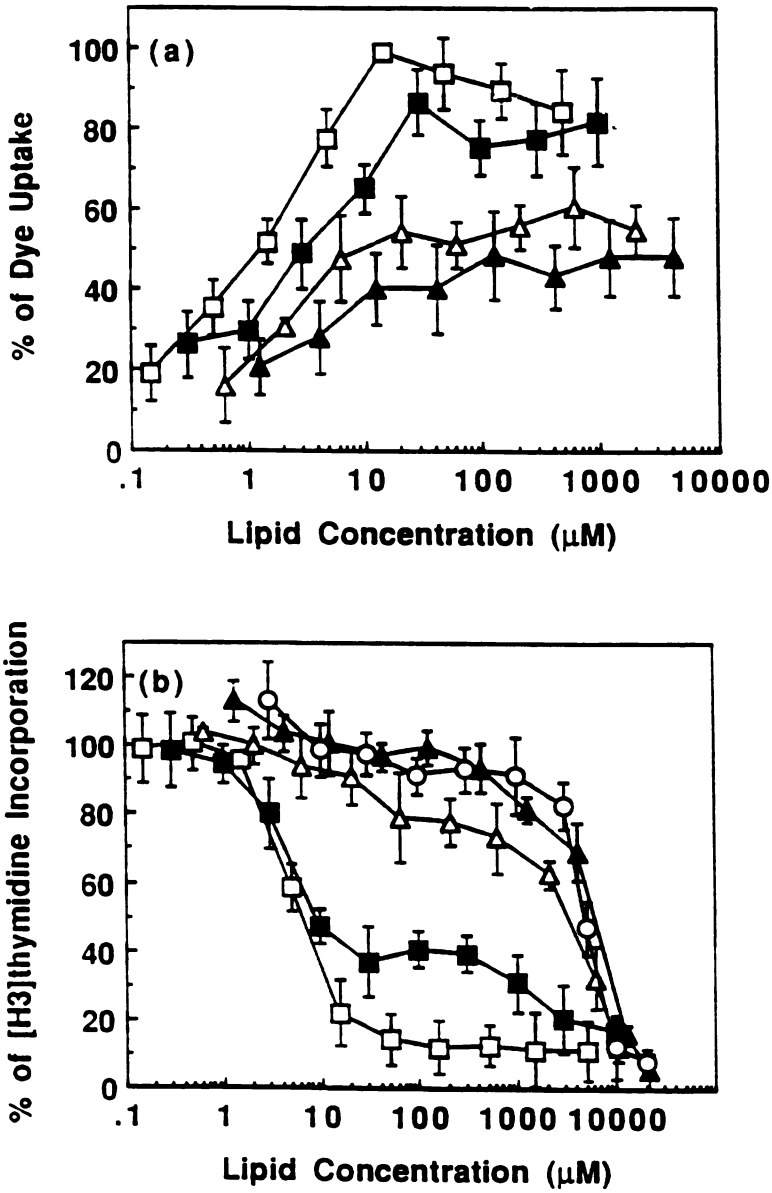


Figure 4. Antiviral activity and cytotoxicity of liposomal PAA of various encapsulation ratios. (a) Comparison of the antiviral effect of liposomal PAA assessed by the CPE assay. Vero cells were infected with HSV-2 for 1 hour before drug treatment. At 48 hr after drug treatment, viable cells were fixed and stained and the percentage of dye uptake was calculated as described in Materials and Methods. (b) Inhibition of thymidine incorporation by non-PAA containing liposomes and Lipo PAA in Vero cells. Vero cells were incubated with the drug for 44 hr, and then thymidine incorporation was measured as described in Materials and Methods. Symbols: (□), Lipo-PAA with 0.20 mol PAA/mol lipid; (■), Lipo-PAA with 0.10 mol PAA/mol lipid; (Δ), Lipo-PAA with 0.048 mol PAA/mol lipid; (▲), Lipo-PAA with 0.024 mol PAA/mol lipid; and (○), non-PAA containing liposomes. Values are the mean ± SD (bars), n = 4.

Table 2. Antiviral activity and cytotoxicity of liposomal PAA with various drug/lipid ratios

PA concentration for encapsulation	Drug/Lipid ratio	IC_{50} (μM)	EC_{50} (μM)	Selectivity (IC_{50}/EC_{50})
8 mM	0.024	> 100 [a]	3.0 [b]	_
20 mM	0.048	> 100 [a]	0.3 [c]	> 333
35 mM	0.102	1.5 [d]	0.3	5.0
71 mM	0.198	1.5	0.3	5.0

a: No cytotoxicity were observed up to 100 μM of the drug added. The higher concentrations of the drug showed some cytotoxicity due to the high concentrations of lipid required to provide the drug concentration indicated.
b: CPE assay showed a plateau level of 50% effectivity with higher concentrations (> 3 μM) of drug delivered from this liposomal PA preparation.
c: CPE assay showed that the effectivity levels off at 60% with higher concentrations (> 10 μM) of drugs delivered by this liposomal preparation.
d: The plateau level of cytotoxicity resulted from this liposomal PAA preparation with drug concentrations greater than 3 μM is ≅ 60% .

in the liposome modulates the selectivity of the drug and increases the therapeutic index of liposomal drugs.

Uptake of PF and Liposome by Vero cells

A possible explanation for the increase in the selectivity ratio for liposomal PF and liposomal PA with a low drug/lipid ratio but not for liposomal PA at the highest encapsulation ratio tested is that liposome uptake by the Vero cells is saturable. The maximum amount of liposome-encapsulated drug that becomes cell associated is a function both of the saturation level of cell-associated liposomes and of the concentration of the drug in the liposome. In the case of low encapsulation preparation, an intracellular drug concentration that inhibits viral replication but not cellular DNA synthesis is attained due to the saturation of liposome uptake by the cells. Thus selectivity ratio of the drug is increased. Whereas using the high encapsulation ratio preparation, the selectivity ratio of the drug is not changed because an intracellular drug level that inhibits both viral replication and cellular DNA synthesis is attained.

Measurement of the cellular association of a nonexchangeable radiolabeled lipid marker demonstrated that liposome uptake plateaued after 10 h (Figure 5a). Moreover, the uptake saturated at a liposome dose of about 300 μM (Figure 5b). Varying the amount of drug per liposome will affect the amount of drug that becomes cell associated. The higher encapsulation ratio for PA then causes a higher cell-associated amount of PA than of PF when delivered by liposomes. It is evident that a majority of drug uptake from the liposome forms occurs at low levels of added liposome-encapsulated drug. The consequence of this for drug action is discussed below.

Since ^{14}C-PF is not metabolized in cells (Öberg, 1983), the cell-associated ^{14}C-radioactivity is the amount of cell-associated drug. ^{14}C-PF containing liposomes (molar ratio of PF/lipid = 0.11) showed a similar saturable uptake pattern (Figure 5c & 5d) as that of ^{125}I-labeled liposomes. However the cell associated drug saturates at 7 h and declines after 24 h of incubation (Figure 5c) while the cell-associated lipid is maintained at the level reached at saturation. Similarly, the concentration-dependent uptake of ^{14}C-PF containing liposomes begins to level off at 25 μM liposomal PF (231 μM lipid) and stays at the same level even with higher liposome concentrations in culture (Figure 5d). This result corresponds to the uptake of ^{125}I-labeled empty liposomes, though there is discrepancy between cell-associated lipid and PF at various liposome concentrations. These discrepancies are caused by two major factors. (i) The leakage of PF from liposome-cell complex. (ii) The release of PF from cells after liposomes degradation in the lysosomes is more rapid than the release of the degradation product of ^{125}I-labeled lipids.

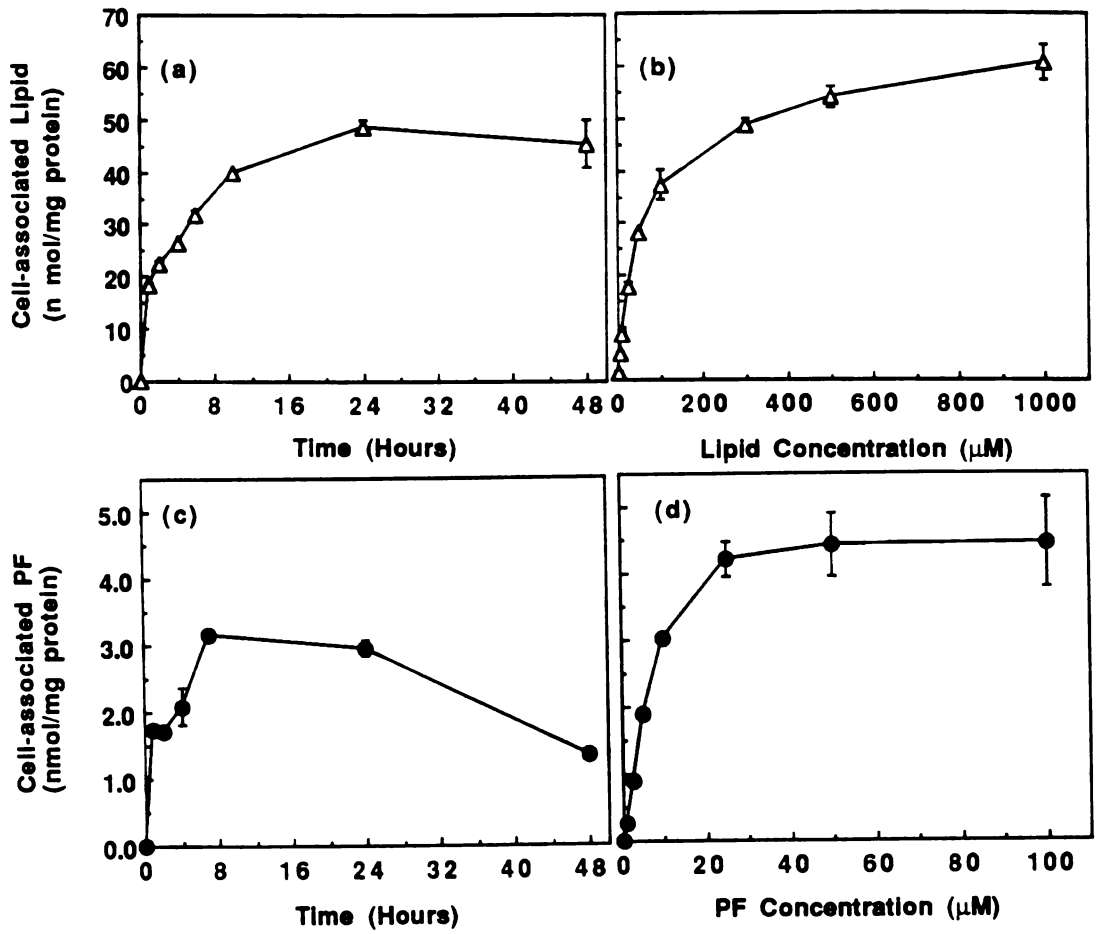


Figure 5. Uptake of liposomes and liposomal PF by Vero cells. (a) ^{125}I-labeled liposomes (300 μM) were added to the cell culture for various periods. At the end of each incubation, non-cell-associated liposomes were washed off and cell-associated radioactivity was counted. (b) Various concentrations of ^{125}I-labeled liposomes were incubated with Vero cells for 24 hr. (c) ^{14}C-PF containing liposomes (50 μM PF/462 μM lipid) were incubated with Vero cells for the times indicated. (d) Various concentrations of ^{14}C-PF containing liposomes (1-100 μM PF/9.2-920 μM lipid) were added to cell culture for 24 hr. Cell-associated radioactivity was measured as described in Materials and Methods. Values are the mean ± SD (bars), n = 3.

Uptake of nonencapsulated PF is found to be a linear function of external drug concentration (Figure 6a). The efficiency of this uptake process is low (less than 0.2% of external drug after 24 hr incubation) when compared with uptake of liposomal drug (about 4.6% of 50 μM liposomal PF under the same condition). The kinetics of uptake of nonencapsulated PF shows a two components kinetics, an initial linear phase up to 2-4 hr and a slower phase after that (Figure 6b). Since PF carries three negative charges at the physiological pH, the permeability of the drug through the cell membrane is very low (Öberg, 1983, 1989). The initial cell association of nonencapsulated drug is most likely due to a pinocytosis mechanism, as had been demonstrated by various investigators for the uptake of sucrose, polyvinylpyrrolidone, and Lucifer Yellow (Sasaki et al., 1987; Roberts et al., 1977; McKinley & Wiley, 1988), and the second phase is a result of the slow permeation of PF through the plasma membrane.

Efflux of PF and Liposomal PF from Vero cells

The efflux of PF from cells was measured in Vero cells which had been incubated with either nonencapsulated PF, 500 μM, or liposomal drug, 50 μM, for 24 hr. Cells were washed to remove nonassociated drug and placed in the drug-free medium at time 0. For the nonencapsulated drug treatment, about 60-70% of the initial cell-associated amount remains with cells at the end of a 24 h chase. For cells exposed to the liposomal drug, only about 18-22% of the initial cell-associated amount remains with the cells at the end of a 24 hr chase (Figure 7).

The efflux of PF from Vero cells incubated with nonencapsulated drug shows a similar kinetic pattern to that of other pinocytotic markers. The rapid release of PF at the first hour (Figure 8a, Table 3) is similar to the diacytosis phenomenon, a process that occurs as endosomes rapidly return their contents to the extracellular medium (Besterman et al., 1981). However, the rate observed here is much slower (half-life = 22 min; apparent first order rate constant = 1.9 hr^{-1}) than previously reported (half-life= 5-10 min; rate constant = 5.2-8.3 hr^{-1}). The second phase of PF release from Vero cells (rate constant = 0.011 hr^{-1}) is also slower than the release of other pinocytotic markers (rate constant = 0.069-0.23 hr^{-1}) from a deeper compartment, which has been suggested to be the lysosomes (Besterman et al, 1981; McKinley & Wiley, 1988). The first time point taken in this experiment is at one hour after the beginning of chase, therefore early diacytosis, which occurs within 5-10 min after the commencement of the chase, is not detected in this study. We speculate that the fast phase of efflux in our study is the combination of the efflux from the endosomes and the lysosomes. The third mechanism of release is the efflux of the cytoplasmic PF via exocytosis or via permeation through the

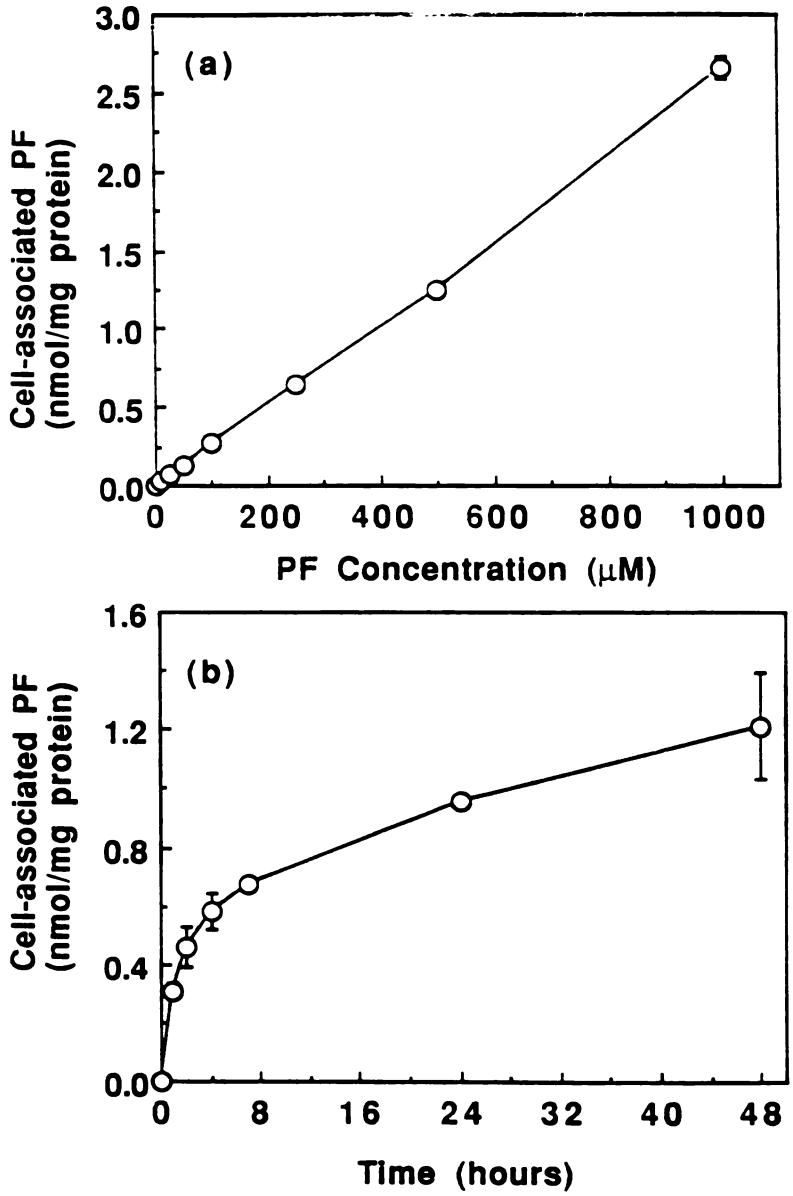


Figure 6. Uptake of nonencapsulated ^{14}C-PF by Vero cells. (a) Various concentrations of nonencapsulated ^{14}C-PF were added to cell culture for 24 hr. (b) Nonencapsulated ^{14}C-PF, 500 μM, were incubated with Vero cells for the times indicated. Nonattached drugs were washed off and cell-associated radioactivity was measured. Values are the mean ± range (bars) of duplicate measurements.

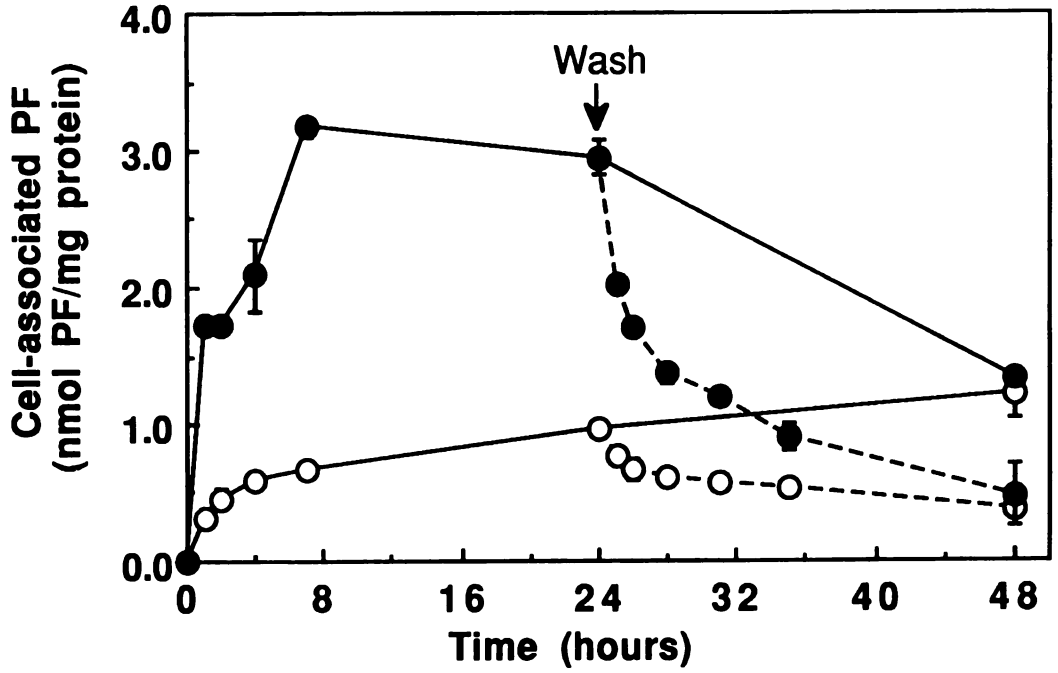


Figure 7. Uptake and efflux of ^{14}C-PF from Vero cells. Nonencapsulated ^{14}C-PF, 500 μM and ^{14}C-PF containing liposomes, 50 μM were incubated with cells. Some cells were washed after 24 hr incubation (indicated by the arrow) and reincubated in the drug-free medium for extended times. At the times indicated, nonattached drugs were washed off and cell-associated radioactivity was measured. Symbols: (○), nonencapsulated PF; (●), Lipo-PF. The dashed line represents the cell-associated PF after washing at 24 hr. Values are the mean ± range (bars), n = 2.

Table 3. Kinetic parameters of PF release from Vero cells.

Treatment	Rapid phase		Slow phase	
	Rate constant[a] (hr^{-1})	Fraction[b] (%)	Rate constant[a] (hr^{-1})	Fraction[b] (%)
PF[c]	1.90	32.3	0.011	67.6
Lipo PF[d]	1.27	41.2	0.043	58.8

a: Apparent first order rate constant at each phase, obtained by curve-peeling method described in the text.
b: Fraction of the total cell-associated drug released at each phase.
c: ^{14}C-PF released from Vero cells treated with nonencapsulated PF, 500 μM, for 24 hr.
d:.^{14}C-PFreleased from Vero cells treated with liposomal PF, 50 μM, for 24 hr.

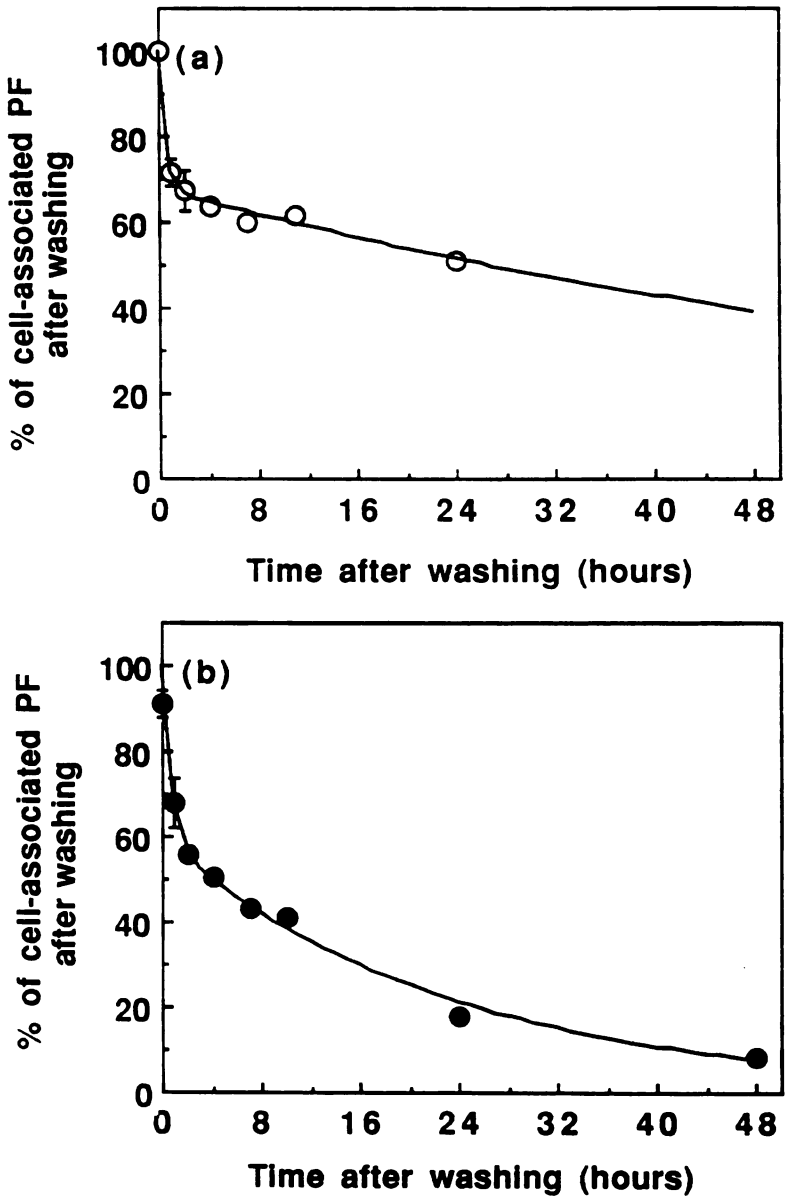


Figure 8. Release of ^{14}C-PF from Vero cells pulsed with encapsulated- and nonencapsulated- drug. Cells were pulsed with (a) nonencapsulated PF, 500 μM; and (b) Lipos PF, 50 μM, for 24 hr and then chased in the drug-free medium for the periods indicated. ^{14}C-PF remained with cells at the end of reincubation was determined and expressed as percentage of the cell-associated PF at time 0. Solid line represents the data calculated from the equation with two exponential terms (Table 3). Experimental data are the mean ± range (bars) of duplicate measurements.

plasma membrane. Thus the later phase of PF release, which account for the efflux of 67% of the total cell-associated drug, may result from a combination of release from the lysosomes and efflux from the cytoplasm.

Release of PF from cells pulsed with liposomal PF also exhibits a two phase kinetics (Figure 8b, Table 3). The half-life of the first fast phase is about 28 min which is similar to the result obtained for the nonencapsulated drug treatment. The rapid phase of drug release from cells exposed to liposome encapsulated drug may be due to a combination of diacytosis as well as dissociation of liposomes from the cell surface, since only a small amount of nonencapsulated PF appears at the early phase of the release (Figure 9). It is conceivable that regurgitation of degraded lipid and PF from the lysosomes contributes to the efflux of the drug, since the degraded proteins can be regurgitated from the lysosomes (Burktenica et al., 1988).

To further understand the kinetics of lysosomal regurgitation, we investigated the appearance of nonencapsulated PF in the medium during the chase period (Figure 9). About 90% of the drug released from the cells in the first hour is liposome-encapsulated form, only a small amount of nonencapsulated PF appears at the early phase of the release. This indicates that in the early stages of the release, the encapsulated form is returned to the medium from the cell surface/the endosomes. The proportion of intact liposomal PF declines as the incubation proceeds. In the latter phase of PF release, nonencapsulated drug is the predominant form. This suggests that a second phase occurs later and consists mostly of the nonencapsulated drug from the lysosomes or the cytoplasm after the drugs are freed from degraded liposomes.

Thus a complex process is involved in the efflux of drug from liposome treated cells: the dissociation of liposome from the cell surface, diacytosis, regurgitation from the lysosomes, degradation of liposomes in the lysosomes, leakage of liposomal contents in the medium or in the endosomes, permeation of drug from the endosomes/lysosomes to the cytoplasm, interaction of drug with cytoplasmic components, exocytosis, and permeation of drug through the plasma membrane. These mutiple components make it impossible to dissect each individual step using a two-components kinetic profile. Efflux studies also cannot be used to estimate the fraction of the cell-associated PF which reaches the cytoplasmic compartment when delivered by liposomes.

A comparison of the terminal phase of efflux between cells exposed to nonencapsulated PF and those exposed to liposomal PF (Table 3) indicates that the liposomal PF is leaving more rapiidly. This may indicate that the majority of cell-associated liposomal PF is rapidly released from intracellular vacuoles. Perhaps the liposome treatment stimulates membrane recycling and then facilitates the influx and efflux

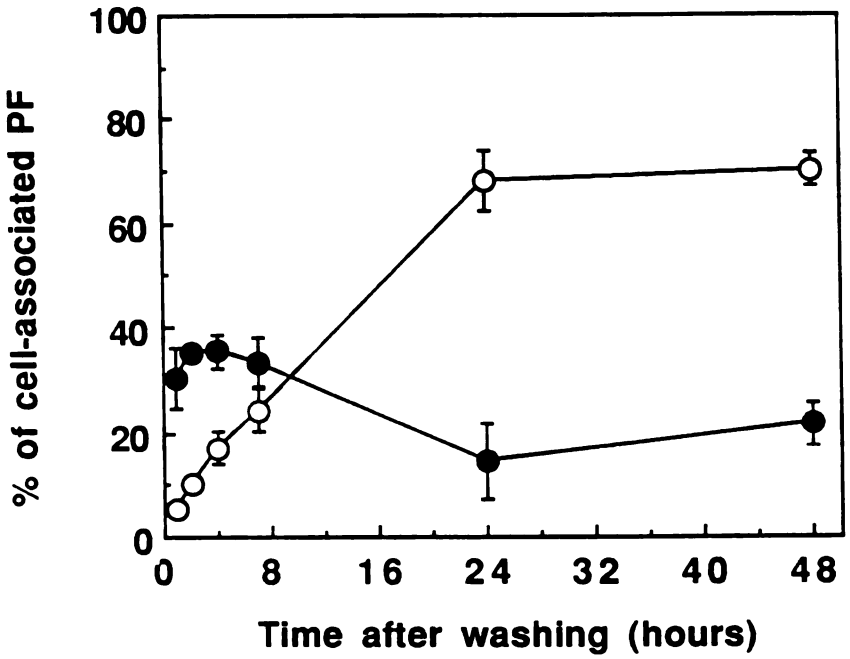


Figure 9. Release of encapsulated- and nonencapsulated- ^{14}C-PF from Vero cells pulsed with liposomal drug. Cells were incubated with ^{14}C-PF containing liposome 50 μM for 24 hr and reincubated in fresh medium at time 0 as described in Figure 8. The medium was collected at each time point during the chase and then dialized against a buffer for 24 hr. Radioactivity associated with cells and the dialysate were measured. Data are expressed as the percentage of radioactivity associated with cells at time 0. Symbols: (○), dialyzable form in the dialysate; and (●), encapsulated form obtained by subtracting the amount of dialyzable form in the medium from the total amount of PF in the medium at each time point. Values are the mean ± range (bars) (n=2).

of the liposome-entrapped drug. In spite of this, cells exposed to liposomal PF, 50 μM, have the same absolute level of cell-associated PF as cells exposed to 500 μM PF even at 48 hr (Figure 7).

4. DISCUSSION

The order of magnitude increase in efficacy against HSV-2 achieved by the encapsulation of PF or PAA in liposomes illustrates the concept of a liposome-dependent drug (Figure 1) (Heath et al, 1983, 1985a). For the nonencapsulated compounds, the EC_{50s} for both PAA (60 ± 25 μM) and PF (210 ± 85 μM) were consistent with previously published values (PAA = 60 to 100 μM and PF = 60 to 200 μM) against various strains of HSV (Eriksson et al., 1980; Helgstand et al., 1978; Mao et al., 1985; Öberg, 1983; Overby et al., 1977; Overby et al., 1974). Thus, the increase in efficacy of the liposome-encapsulated compounds (PAA = 0.4 μM, PF = 7 μM) could be important for in vivo antiviral activity of PAA and PF.

For PF, the increased efficacy was achieved without any substantial increase in cytotoxicity as measured by inhibition of thymidine incorporation. Thus, the selectivity ratio of liposomal PF was significantly better than that of the nonencapsulated compound. In the contrast, at 0.3 μmol of drug per μmol of lipid, PAA exhibited a 150-fold increase in efficacy but also had a 250-fold increase in cytotoxicity so that the selectivity of the drug was slightly decreased compared with the free drug. The selectivity ratio could be increased by decreasing the drug-to-lipid ratio in the liposome. For both drugs, the cytotoxicity of the nonencapsulated compounds, as measured by either thymidine incorporation or growth rates, was similar to previously reported values (Öberg, 1983; Overby et al., 1977). In purified DNA polymerase alpha preparations, PAA (K_i = 35 μM) has a lower inhibition constant than PF (K_i = 50 μM), so that the rank order effect of the two nonencapsulated agents on thymidine incorporation observed in this study was consistent with the inhibition constants (Öberg, 1983).

Thus, the possibilities of manipulating the selectivity ratio of these compounds by varying the drug/lipid ratio provides an alternative to drug modification for improving drug therapy. We postulate that the difference between the two phosphono compounds at the high drug/lip ratios relates to three factors: (i) the difference in the encapsulation ratio, that is, the micromoles of drug per micromoles of lipid, achieved in the preparation of the liposomes; (ii) the relative sensitivity of the Vero cell to the cytotoxic effects of the two drugs; and (iii) the fact that liposome uptake by the Vero cells saturates (Figure 5 and 7).

In Figure 10 this concept is illustrated graphically. It is assumed that the antiviral effect and the cytotoxic effect occur at the same intracellular drug concentration, regardless of whether the drug is delivered in liposomes or as the nonencapsulated compound. The drug level at intracellular sites can be estimated from the nonencapsulated PF uptake data (Figure 6A) and the fraction of cell-associated PF that released from a deeper intracellular compartment during the chase period (67%, Table 3). The assumption of a linear relationship between cell-associated drug and internal drug levels allows the construction of the dashed line in Figure 10 which is plotted based on the values equal to 67% of the cell-associated PF level obtained from uptake study shown in Figure 6A. The intracellular drug level at EC_{50} of the nonencapsulated PF (210 μM) is designated as point A. Given that the same intracellular drug concentration would exist at EC_{50} for liposome-encapsulated (7 μM) or nonencapsulated compound, point B can be obtained from knowing the inhibitory drug level at EC_{50} for the nonencapsulated drug (point A). At point B the intracellular PF levels equals to approximately 13% of the cell-associated drug level obtained with 7 μM of liposomal PF in culture (Figure 5d). Thus 13% is assumed to be the fraction of the cell-associated liposomal PF which reaches the intracellular active site. Multiplying this factor to the measured cell-associated amount (Figure 5d), we can get a plot which relates liposomal PF concentration in culture to the intracellular PF levels. Then a curve that rises drastically and starts to level off at a relative low concentration of external liposomal PF is obtained (Figure 10). At the IC_{50} (880 μM) of nonencapsulated PF, the intracellular concentration (point C) exceeds that attainable by the liposomal form (point D). Thus, the liposome can increase the efficacy without increasing cytotoxicity of the encapsulated PF. Assuming that release of PAA from liposomes and cells are similar to that of PF, we can obtain the intracellular PAA level by using the same factor (13%) to multiply the amount of cell-associated PAA. The cell-associated PAA was obtained indirectly by multiplying the fraction of liposomal PF which become cell-associated to the external liposomal PAA concentrations, assuming that the extent of cellular uptake of both liposomal drugs were the same. If the same dotted line of intracellular PF (Figure 10) is used to represent the intracellular concentration attained by the nonencapsulated PAA, then the extracellular concentration of PAA that caused cytotoxicity (point E) results in an intracellular concentration of PA that can be achieved by a low level of external liposomal PAA (point F). Thus, both the efficacy and toxicity would be increased for PAA when it is encapsulated at the highest drug-to-lipid ratio.

Based on this model, a decrease in the PAA concentration encapsulated in liposomes should still result in an enhancement of the antiviral effect but should limit the maximal concentration of PAA in the cell to below the cytotoxic range. When this was

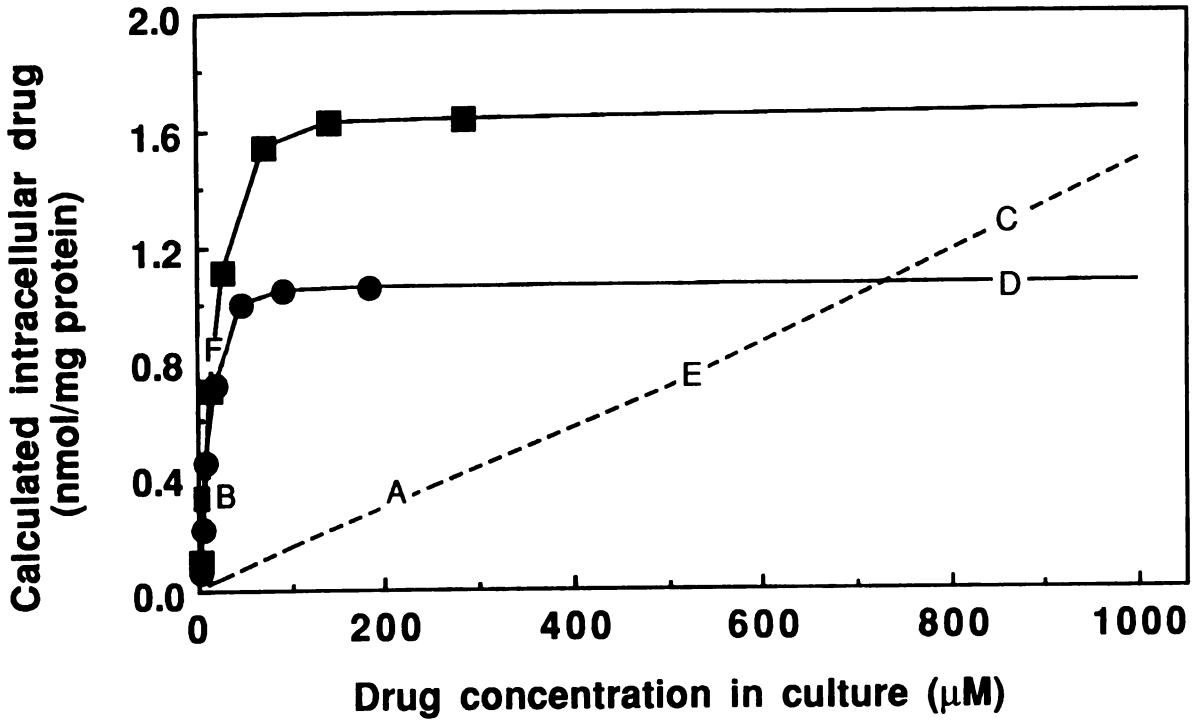


Figure 10. Comparison of intracellular level of PF and PAA in Vero cells after delivery in liposomes or in a nonencapsulated form. The amount of intracellular drugs was estimated by multiplying the measured cell-associated drug at 24 hr by 0.67 and 0.13 for non-encapsulate PF and liposomal PF, respectively. Symbols: (●), Lipo-PF; (■) , Lipo-PAA. The dashed line originating at the origin and proceeding through the points marked A, E, and C is the curve relating extracellular nonencapsulated drug concentrations to intracellular drug concentrations. See Discussion for a detailed explanation. Point A is the intracellular drug concentration at the EC_{50} for the non-encapsulated PF at 210 μM. Point B is at the same intracellular concentration as point A but is attained at an extracellular encapsulated drug concentration of 7 μM. Point C is the intracellular PF concentration at the IC_{50} of nonencapsulated PF. Point D represents the intracellular drug concentration obtained with the Lipo-PF at 880 μM. Point E is the intracellular drug concentration at the IC_{50} for nonencapsulated PAA. Point F is the intracellular PA concentration for the Lipo-PAA at IC_{50}. Notice that the Lipo-PF is not able to attain the necessary intracellular drug concentration to reach the IC_{50}, whereas the Lipo-PAA attains an inhibitory intracellular concentration at a much lower level of added compound than the nonencapsulated PAA.

done, the efficacy was not changed, while the cytotoxicity decreased from that obtained at the highest drug-to-lipid ratio. The model has been tested using four different PAA-containing liposome preparations with a PAA/lipid ratio ranging from 0.024 to 0.20. Their antiviral activity and cytotoxicity are shown in Figure 4 and Table 2. Both antiviral activity and cytotoxicity are dependent on the lipid concentration in culture and both activities leveled off at a lipid concentration around 100 μM due to the saturation of the liposome uptake. Liposomes with the highest drug/lipid ratio have the lowest EC_{50} and IC_{50}, while liposomes with the low drug/lipid ratio cannot elicit a 100% antiviral activity and are not toxic to cells except at extremely high lipid concentrations. At intermediate drug/lipid ratios, antiviral efficiency is increased substantially but cytotoxicity is hardly increased. Thus the selectivity is increased with lower PAA encapsulation.

Studies of efflux of ^{14}C-PF from cells exposed to nonencapsulated or encapsulated PF shed some light on the intracellular distribution of the drug once they are internalized. It seems that only a minor portion of the liposomal drug has entered the cytoplasmic compartment. However, a more efficient cytoplasmic delivery of PF is obtained using liposomes than using nonencapsulated drug. Since liposomes are transported and concentrated in the lysosomes, the concentration gradient of the drug, which is released from degraded liposomes, between the lysosomes and the cytoplasm is much greater than that between the extracellular space and the cytoplasm when non-encapsulated drug is used. In addition, the permeation coefficient of the partially protonated PF at the acidic pH is greater than that of highly charged PF at neutral pH. These two factors overcome the disadvantage of the smaller diffusion area of the lysosomal membrane than that of the plasma membrane and contribute to the facilitated transport of liposomal PF into the cytoplasm.

The acidic vacuoles are suggested to be involved in the entry of Herpes virus into the cells (Koyama & Uchida, 1984). Addition of NH_4Cl to the culture medium to raise the pH in the intracellular vascuoles decreases the viral infectivity. Therefore, the influence of an increased pH in the lysosomes on the antiviral activity of liposomal PF cannot be clearly demonstrated.

As discussed by Heath et al. (1983, 1985a), for a drug to be considered a liposome-dependent drug several conditions must be met. First, the free drug must be relatively impermeable owing to a potentially reversible chemical feature. Second, the target cell must be able to internalize the liposome through an active endocytotic pathway that terminates in a low pH compartment. Third, the liposome must retain the drug until it is internalized by the cell. Liposomes that are too fragile will release the drug prior to internalization, and no enhancement of activity will be observed. All these conditions for a

liposome-dependent drug appeared to have been met in the system tested here for PF and PAA. In regard to the first point, it is important to realize that a freely permeable drug will redistribute from the cell, even if the liposome is initially able to concentrate it in the cytoplasm. Such compounds must be contrasted with PF and PAA, which have low permeabilities in their ionized forms. Once protonated in the lysosome, these two compounds can more readily diffuse into the cytoplasm. The pH of the cytoplasm is about 7; thus, the phosphono compounds would revert to the ionized form and become trapped in this compartment.

The optional use of liposome-dependent drugs in vivo will depend on targeting of the liposome. This can be done either by an active scheme in which a targeting ligand is employed or by exploiting the known capacity of macrophages to take up liposomes. If the latter approach is taken, viral diseases that infect the macrophage would be ideal targets for such therapy (Koff & Fidler, 1985). Clearly, PF and PAA appear to be good candidates for liposome encapsulation for therapy of virally infected macrophages.

The antiviral potency of the phosphono compounds is hindered by their inability to cross membranes. Nonetheless, PF has been used to treat cytomegalovirus infections in recipients of bone marrow and renal transplants (Ringden et al., 1986). Moreover, the potent anti-reverse transcriptase activity of PF (Sandstrom et al., 1985; Sundquist et al., 1979) has already been exploited in a limited clinical trial to treat human immunodeficiency virus infections (Farthing et al., 1987; Gaub et al., 1987). In these studies, up to 15 g of PF per day has been infused on a continuous basis for a period of 21 days. The large dose and continuous intravenous infusion is necessary because PF is cleared rapidly from the blood and is poorly absorbed when taken orally. A 100-fold enhancement of efficacy would permit liposome-encapsulated PF to be given as an intravenous bolus rather than as a continuous infusion. In addition, human immunodeficiency virus is known to infect macrophages (Crowe et al., 1987; Gartner et al., 1986; Levy et al., 1985; Lifson et al., 1986; Ruscetti et al., 1986), and the macrophage has been suggested by some to be a significant factor in the persistence of the virus and its transport into the brain in vivo (Crowe et al., 1987; Sodroski et al., 1986). The liposome effect demonstrated here is not virus specific. We would expect that other virus infections sensitive to the phosphono drugs in endocytotically active cells would behave in a fashion similar to that of the HSV system. Thus, it could be of considerable therapeutic importance to extend these results to other viral systems, including a model human immunodeficiency virus macrophage infection (Crowe et al., 1987).

5. Summary

Phosphonoformate and phosphonoacetate encapsulated in liposomes have substantially greater activity against herpes simplex virus type 2 in Vero cell tissue culture than the nonencapsulated compounds at the same dose. Encapsulation of phosphonoformate in liposomes resulted in 30-fold increase of the antiviral effect with no increase in cytotoxicity measured by inhibition of thymidine incorporation into normal Vero cells. Thus, the selectivity of the liposomal drug increased 27-fold compared with the nonencapsulated compound. Liposome encapsulation of phosphonoacetate at a ratio of 0.3 mmol/mmol of lipid resulted in a 150-fold increase of antiviral activity with a concomitant 250-fold increase in cytotoxicity. However, the selectivity of phosphonoacetate could be increased by reducing the drug-to-lipid ratio. Liposome uptake by Vero cells, measured by the cell association of a nonexchangeable radiolabeled lipid, plateaued after 24 hr of incubation and saturated at 60 nmol of lipid per mg of cellular protein at a lipid concentration of 300 μM. The saturation of liposome uptake on the Vero cells may account for the 27-fold increase in selectivity observed with the liposomal phosphonoformate. The greater activity of the encapsulated phosphono compound is most likely due to their increased transport into the cytoplasm; this occurs subsequent to the uptake and processing of the liposome in the lysosomes of the cell. Liposome encapsulation of these agents may result in superior efficacy against viral infections residing in endocytotically and phagocytically active cells such as macrophages.

(The majority of this work was published in Antimicrobial Agents and Chemotherapy, 32: 858-864, 1988)

CHPATER IV: ANTIVIRAL ACTIVITY AND PHARMACOKINETICS OF LIPOSOME-ENCAPSULATED PHOSPHONOFORMATE IN RAUSCHER MURINE LEUKEMIA VIRUS INFECTED MICE.

1. INTRODUCTION

With the advent of the AIDS epidemic the control of viral infections has become a more urgent problem in drug therapy. In addition to the development of new antiviral agents, attempts are underway to achieve site specific delivery of existing agents. Targeting of drugs can increase local concentrations of the drug at the target site and/or minimize exposure of sensitive tissue to the drug, both events can lead to improvements in the therapeutic index. The interaction of the physicochemical properties of the drug with the anatomical and physiological attributes of the target and toxic sites play critical roles in a successful outcome for targeted drug delivery. For instance, Stella and Himmelstein have emphasized that retention of active drug at the target site is one of the prerequisite for successful targeted delivery (Stella & Himmelstein, 1980). Hunt and colleagues have predicted that drugs with high total-body clearance values would benefit more from a targeted carrier than those with low clearance values (Hunt et al., 1986). They also indicate that the therapeutic index of drugs which are directly eliminated at the target site or at the toxic site would be drastically improved via targeted delivery.

PF appears to be a good candidate for targeted delivery, since it meets a number of these criteria. The in vitro antiviral activity of phosphonoformate is enhanced by liposomal delivery in a Herpes simplex virus model (Szoka & Chu, 1988). The enhanced antiviral effect is a result of the increased delivery of the liposome encapsulated drug to an intracellular site. PF has three negative charges at physiological pH (Öberg, 1989), so the ionized drug, once in the cytosol, will not readily redistribute to extracellular sites and the intracellular concentration will be sustained. Conversely, the therapeutic potential of PF in vivo is limited due to its poor penetration into cells and its rapid clearance by the kidney which results in a short plasma half-life (Öberg, 1989). Therefore, large amounts of PF must be administered (e.g., 5-18 g/day) through a continuous intravenous (i.v.) infusion to maintain the plasma concentration needed to inhibit cytomeglovirus in immunosuppressed patients (Ringden et al., 1986). Renal failure is the most prevalent side effect caused by these large amounts of PF.

The possibility of enhanced potency of liposomal PF via a passively targeted mechanism can be tested on a Rauscher murine leukemia virus (RMLV) model. RMLV, a

RNA retrovirus, infects and transforms erythroblasts in BALB/c mice. The transformed erythroleukemia cells proliferate rapidly and cause splenomegaly, hepatomegaly and viraemia in the infected mice (Rauscher, 1962). Ruprecht has successfully used this model to screen potential anti-retroviral agents for prophylactic chemotherapy and for therapy of chronic viraemia after virus exposure (Ruprecht, 1989). A majority of liposomes are cleared from the circulation by cells of the reticuloendothelial system (RES). This results in a passive targeting of the liposomes to the liver and spleen in vivo. Employing this model, liposomes and the encapsulated drug may localize in the enlarged spleen and liver as RMLV infection progresses, and result in a high local concentration of the drug. Whether delivery of the drug to the virally infected target cells would ensue is an open question. A second unknown is whether saturation of the RES with a large dose of liposomes would compromise the host defense mechanism which previously has been shown to be important in the control of progression of RMLV disease (Knyszynski & Danon, 1977; Buchman et al., 1973).

We investigated how pharmacokinetic parameters of PF in RMLV infected animals are altered when PF is delivered in liposomes. We also studied the effect of liposome-encapsulated PF on the progressive changes in the spleen and liver of RMLV infected mice via passive targeting of the liposomes to these organs. Although liposome delivery results in a significant increased drug level in the target tissue, a concomitant increase in drug potency in the RMLV model was not observed.

2. MATERIALS and METHODS

Materials

Phosphonoformate trisodium salt, cholesterol and tocopherol were obtained from Sigma Chemical Co., St. Louis, Mo.. Egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG) were purchased from Avanti Polar Lipids, Pelham, Ala. p-Hydroxybenzamidine dihexadecylphosphatidylethanolamine was synthesized and iodinated as described previously (Abra et al., 1982). ^{14}C-PF (25 mCi/mmole) and ^{125}I-sodium iodide were products of Amersham Corp., Arlington Heights, Ill.. Purity of the ^{14}C-PF was assessed by quantitative thin layer chromatography on cellulose plates, developed in CCl_3COOH:10% NH_4OH:H_2O:CH_3OH = 5g:15ml:30ml:50ml (Gawell, 1983). A single spot was found to be greater than 98% of radioactivity loaded, when ^{14}C-PF comigrated with an authentic sample of PF. The RMLV viral stock was a generous gift of professor M. Strand, Johns Hopkins University (Scheinberg & Strand, 1982). BALB/c mice were

purchased from Jackson Laboratories (Bar Harbor, Maine) and monitered in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Liposome and Drug Preparation

Reverse-phase evaporation liposomes (REV), composed of EPC/EPG/cholesterol/ tocopherol in a molar ratio of 9/1/8/0.1 (30-40 mM total lipid), were prepared and extruded through a 0.2 μm polycarbonate membrane as described previously (Szoka et al., 1980b). Non-drug-containing liposomes (empty Lipo) were prepared by encapsulating 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid)-140 mM NaCl-0.1 mM diethylenetriaminepentaacetic acid (pH 7.4). The concentrations of encapsulated drug and phospholipid after the extraction in a two-phase system were determined as previously described (Szoka & Chu, 1988). Liposome diameters were determined with a laser light-scattering apparatus (NS-4; Coulter Electronics, Inc., Hialeah, Fla.).

Propagation of RMLV

Diluted (1:20) stock RMLV solution was injected into 6 week old female BALB/c mice via the tail vein. Three weeks after inoculation, the enlarged spleens were removed and teased into smaller cell aggregates and single cells. The single cell suspension was subjected to three rounds of freezing and thawing in a dry-ice/ethanol bath and was passed through a 20 gauge needle 3 times. The supernatant obtained after centrifugation of the cell lysate at 3000 g for 30 min was sterilized by filtration through a 0.45 μm Milipore membrane and maintained at -70 ^{o}C as the virus stock.

Establishment of Animal Model

Female BALB/c mice (7-10 weeks old) were infected with 0.25 ml of 1:20 dilution of stock RMLV solution by an i.v. injection into the tail vein. Spleens were fixed in Bioun's solution and macroscopic foci (0.5-3 mm diameter) were counted as spleen foci forming (SFF) units at the 9th day post-infection (Pluznik & Sachs, 1964). Spleen and liver weights were measured at various time points as the disease progressed during the three weeks following viral inoculation (Chirigos, 1964).

Biodistribution of Liposomes

^{125}I-p-hydroxybenzamidine dihexadecylphosphatidylethanolamine (< 0.01 mole %) was incorporated into liposomes (^{125}I-REV) as a lipid marker. At different times after the virus inoculation, infected mice received an aliquot (8-10 μmol of lipid) of ^{125}I-REV or

^{14}C-PF containing liposomes by i.v. injection into the tail vein and were sacrificed 3 hours later. Three mice were used at each time point. The blood was collected from a retro-orbital venous site, while the heart, lung, spleen, liver, kidney, stomach and intestine were removed and weighed. The radioactivity associated with each organ was measured in a Beckman gamma spectrophotometer and was corrected for blood volume in each organ (Szoka, 1986). The blood volume in the enlarged spleen of moderately anemic animal was found to be proportional to the weight of infected spleen (Morse et al., 1978). Thus the blood volume in the enlarged spleens of RMLV infected mice is corrected by the following formula : Spleen blood volume$_{RMLV}$ = Spleen blood volume$_{normal}$ x (Spleen weight$_{RMLV}$ / Spleen weight$_{normal}$).

Pharmacokinetics of Nonencapsulated and Liposome-encapsulated PF

Trace amounts of ^{14}C-PF were incorporated into 80 mM PF solution (0.093-0.36 μCi/μmol PF) before encapsulation into liposomes. An aliquot (0.31-0.46 μCi/dose) of ^{14}C-PF-encapsulated liposomes was injected via i.v. (0.25ml) or intraperitoneal (i.p.). (0.5ml) route into RMLV infected (three weeks after infection) or noninfected normal mice (12 weeks old). Due to the variation in the encapsulation efficiency of different liposome preparations, doses of PF given in these studies varied since the lipid dose was kept constant (0.4-0.5 mmole/kg). In the case of nonencapsulated ^{14}C-PF, 24 mg PF/kg in 0.25 ml was injected intravenously into normal mice and 491 mg PF/kg in 0.5 ml was given intraperitoneally into RMLV infected mice (1-1.3 μCi/dose). Three mice/group were sacrificed at 0, 0.25, 0.5, 1, 3, 6, and 24 hr post injection, and the blood, heart, lung, liver, spleen, and kidney were removed and weighed. To each gram of the tissues, 4-8 ml of PBS was added and a tissue homogenate was made using a Polytron homogenizer. The tissue homogenate was solubilized and decolorized at 60 ^{o}C using a tissue solubilizer (BTS-450, Bechman, Fullerton, CA.) and 30 % H_2O_2. Then the solubilized tissue was neutralized with glacial acetic acid before the associated radioactivity was measured using a Beckman scintillation spectrophotometer. The drug level associated with each organ was calculated based on the specific activity of the compound. The data were fitted into a 2 or 3 compartment model using the residual method (Abraham et al., 1983; Rahman et al., 1986; Gibaldi & Perrier, 1982) to determine the terminal rate of the drug elimination in these organs. The area under the tissue drug level-time curve (0-24 hr) was calculated using the trapezoidal method and the approximate clearance of the drug was estimated by dividing the administered dose with $AUC_{blood,\ 0\text{-}24\ hr}$ (Gibaldi & Perrier, 1982). Unpaired Student's t-test is used for statistical analysis.

Chemotherapy

Seven to nine week old mice were infected with 0.25 ml of diluted (1:20) RMLV suspension (120 ± 58 SFFU/ml) on day 0. The control group received PBS instead of virus suspension. Drug treatments started at 24 hr after the viral inoculation. For the control and virus control groups, PBS was administered by i.p. injection at the same schedule as the drug treatment groups. Four to eight mice were used in each group. On day 20, all mice were sacrificed and their spleens and livers weighed. Chemotherapy experiment 1 was designed to test the effect of nonencapsulated PF and liposome-encapsulated PF (Lipo-PF). Nonencapsulated PF (500 mg/kg) was given by i.p. injection on days 1, 2, 3, 5, 7, 9, 11, 13, 15, and 17. Lipo-PF (20 mg PF/0.56 mmole lipid/kg) was given by i.v. injections on days 1, 2, & 3, followed by i.p. injection on days 5, 7, 9, 11, 13, 15, and 17. A high dose of Lipo-PF was tested in chemotherapy experiment 2. In this trial, either nonencapsulated PF (500 mg/kg) or Lipo-PF (100 mg PF/2 mmole lipid/kg) was administered by i.p. injection on days 1, 2, 3, 4, 5, 6, 8, 10, 13, 15, and 17. The dose of Lipo-PF was reduced to 50 mg/kg after day 6. A corresponding dosage of non-drug containing liposomes was administered to examine the effect of lipid alone. Chemotherapy experiment 3 was designed to demonstrate the prophylactic effect of nonencapsulated PF and Lipo-PF. Mice received one dose of either drug at 3 hr before virus inoculation and the second dose 24 hr after the virus inoculation. Nonencapsulated drug (500 mg/kg) was given by i.p. injection, and liposomal PF (25 mg PF/0.6 mmole lipid/kg) or non-drug containing liposomes (0.6 mmole lipid/kg) were given via an i.v. route. Animals receiving non-drug containing liposomes plus nonencapsulated PF at the appropriate doses were also included as a control for a liposome-drug interaction.

Uptake of Liposomes by Nonadherent Spleen Cells

Enlarged spleens from mice infected with RMLV for 6 weeks were removed and teased into smaller cell aggregates. A single cell suspension, collected after filtration through steel mesh, was washed with Hank's balanced salt solution 2 times and then plated onto plastic culture flasks for one hour at 37 ^{o}C in RPMI 1640 medium containing 10% fetal calf serum. Non-adherent cells were harvested after 2 consecutive platings. Ten million nonadherent spleen cells were incubated with ^{125}I-REV in culture tubes set in a constant rocking motion (approx. 30 cycles/min). At the end of each incubation, cells were washed with PBS for 3 times and the radioactivity associated with cell pellets was measured. The concentration of cellular protein in the sodium hydroxide lysate of cell pellets was determined by the method of Lowry using bovine serum albumin as the standard.

3. RESULTS

Liposome Characterizations

Phosphonoformate trisodium salt is highly water soluble at neutral pH, and at 80 mM concentration it is easily encapsulated in liposomes. The encapsulation efficiency in liposomes with diameters of 0.16-0.22 μm varied from 5% to 11%, which approaches the expected efficiency for a water soluble compound in unilamellar vesicles of this size and at the lipid concentration used for encapsulation. The resulting liposome preparations have a PF/lipid molar ratio of 0.09-0.16 and the ratio was maintained at a constant level during storage. Less than 2% of encapsulated PF had leaked from the vesicles when stored at 4 ^{o}C for 3 weeks. The majority (96%) of encapsulated PF remains associated with liposomes when incubated with 85% fetal calf serum at 37 ^{o}C for 24 hr. Thus these liposomes are very stable in the presence of serum in vitro.

RMLV Murine Model

Serial dilutions of stock RMLV suspension were assayed for viral activity using the spleen foci forming assay (Pluznik & Sachs, 1964) and spleen weight assay (Chirigos, 1964). Spleen weight and spleen foci units were directly related to the amount of virus inoculated (data not shown). The 1:40 dilution results in 15 ± 7.3 SFF units at 9 days post infection. An i.v. injection of 0.25 ml of 1:20 dilution of stock virus suspension (corresponding to 30 ± 14 SFF units) was used in this model. In mice receiving the viral inoculation, spleens gradually enlarged (Figure 1). Marked increases in the spleen weight were observed at two weeks after viral inoculation, while the liver was slightly enlarged. The mean survival time of 18 mice infected with this dose of virus is 76 ± 23 days post-infection.

Liposome Disposition in RMLV Infected Mice.

Passively targeted delivery is based on the assumption that liposomes are taken up by the RES in vivo. Whether or not the biodistribution pattern of liposomes is altered by RMLV infection is the imminent question to be addressed. The disposition of ^{125}I-labelled liposomes in RMLV infected mice is similar to that observed in normal mice (at day 0), i.e., the majority of liposomes were found in the liver, spleen, and blood at 3 hr after dosing (Figure 2a). As the disease progressed (at day 3, 9, 15, & 21), about 80% of liposomes were still found in these three compartments. Approximately, 35-55% of ^{125}I-REV was found in the blood, 15-30% in the liver, and 10-20% in the spleen. A similar

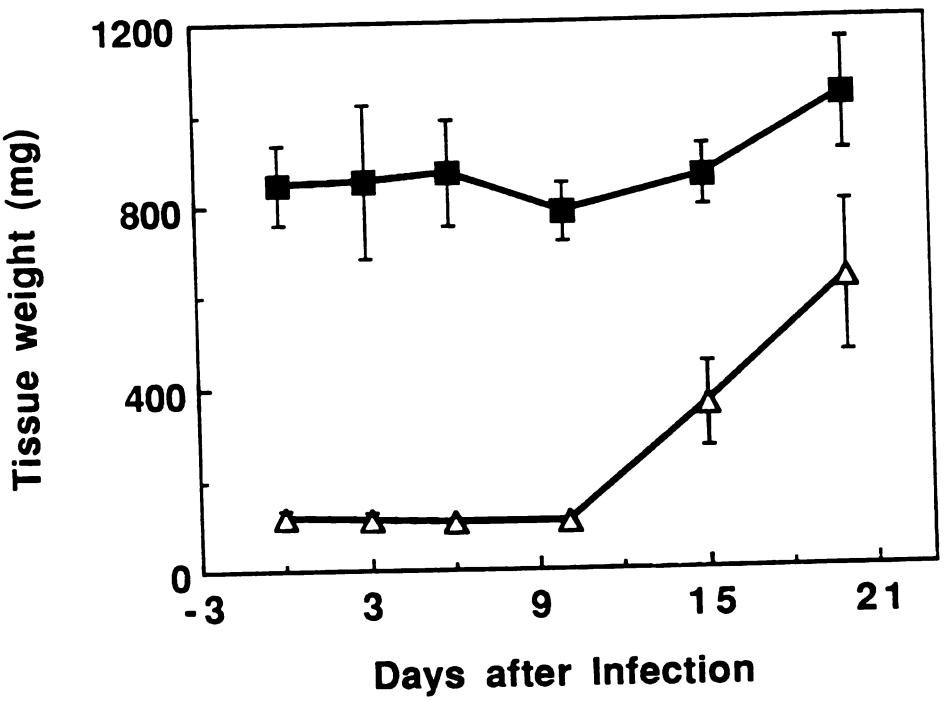


Figure 1. Spleen weights and liver weights of infected mice at different stages of RMLV infection. BALB/c mice were injected with 1:20 dilution of RMLV solution at day 0. The liver (■) and spleen (Δ) of infected mice were weighed at the time indicated. The values are the mean of three animals and the bars represent the standard deviation (SD).

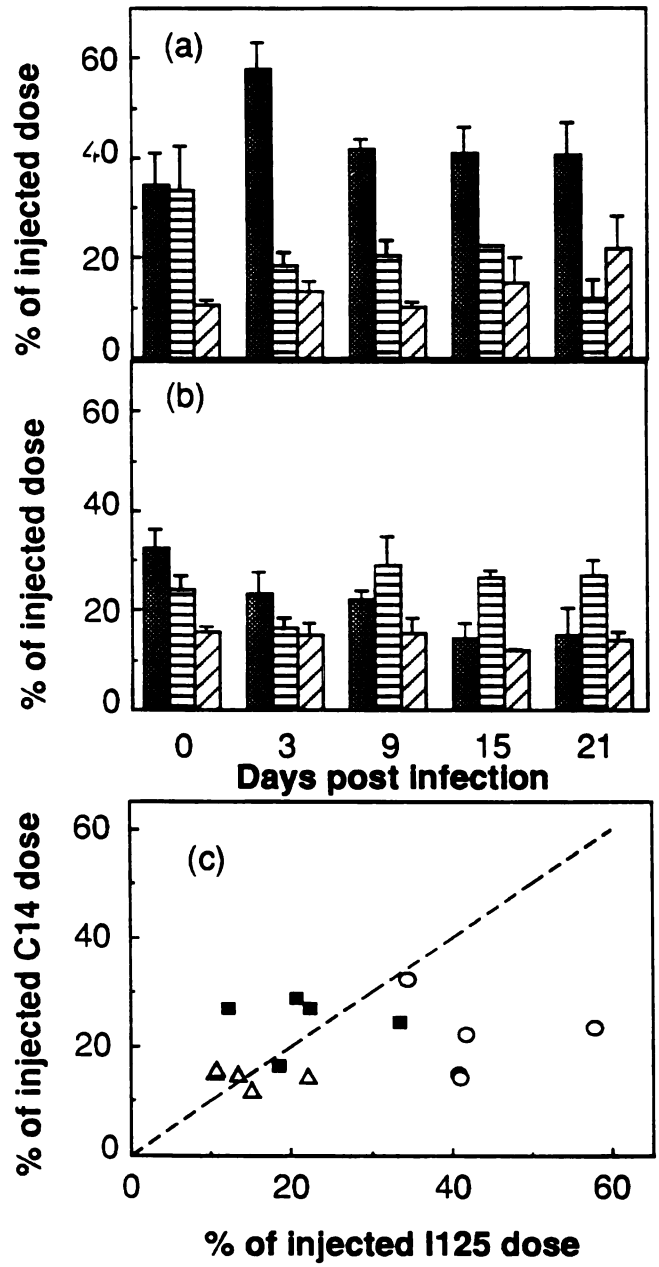


Figure 2. Distribution of liposomes in the RMLV infected mice at different stages of infection. An aliquot (8-10 μmole) of (a) ^{125}I-labelled liposomes or (b) ^{14}C-PF containing liposomes were injected intravenously to RMLV infected mice at various time after the virus inoculation. Radioactivity associated with the blood (■), liver (⊟), and spleen (▨) was measured at 3 hours after dosing. Values represent the mean and the SD of three mice. (c) Correlation of distribution of ^{14}C- and ^{125}I-radioactivity in the blood (○), liver (■), and spleen (Δ). The dotted line represents the relationship with a correlation coefficient of 1.

pattern of distribution of ^{14}C-PF is obtained when ^{14}C-PF-containing liposomes were used (Figure 2b), though the total ^{14}C radioactivity associated with these three organs were about 60% of the injected dose, that is, 15-35% of ^{14}C-PF in the blood, 15-30% in the liver, and 15% in the spleen. The major discrepancy between the biodistribution of ^{125}I-labeled lipid and ^{14}C-labeled liposomal contents lies in the blood (figure 2c). The most prominent discrepancy between ^{125}I and ^{14}C distribution in the blood occurs in the RMLV infected mice where significantly greater levels of ^{125}I remain in the blood compared to ^{14}C PF. These results suggest that a portion of EPC/EPG/cholesterol liposomes containing ^{14}C-PF are rapidly taken up by the liver and spleen while the remainder of the liposomes circulate in the blood and gradually release their encapsulated contents.

Pharmacokinetics of PF and Liposomal PF

It is known that both the tissue distribution and the pharmacokinetic properties of encapsulated drugs are altered by liposomal delivery. Higher drug levels were maintained in the blood, liver, and spleen for an extended time after an i.v. bolus administration of liposomal PF (18 mg PF/0.53 mmol lipid/kg) than after an i.v. bolus of nonencapsulated PF (24 mg PF/kg) (Figure 3). The area under the blood, liver, or spleen drug concentration-time curve is about 60-80 times larger in the liposomal treatment than in the nonencapsulated case. Most remarkably, PF levels in the liver and spleen decline very slowly when liposomal drug is given and a significant level of PF (20-32 nmol/g tissue) can be detected in these two organs at 72 hr after dosing (Figure 4). The slowly declining drug level in the liver and spleen is probably due to a slow efflux of the drug from macrophages or hepatocytes which have internalized liposomes (Storm et al., 1988), or less possibly due to a slow leakage of PF from liposomes adsorbed to the tissue surface. The rate of the release is so slow that it becomes the rate-limiting step in the elimination of the drug from the body as represented by the parallel declining lines among the drug level-time curves in the blood (central compartment) and in the liver and spleen at the terminal phase of the study (48-72 hours after dosing). These results confirm that the residence time of PF in the body, especially in the RES, of normal mice can be extended by liposomal delivery. In general, these findings, e.g. extended blood half-life, elevated drug levels in the liver and spleen, larger AUC in the blood, liver and spleen, and lower total body clearance, are in agreement with pharmacokinetic profiles obtained using various liposomal drug preparation (Abraham et al., 1983; Rahman et al., 1986).

The pharmacokinetics of the nonencapsulated PF was also studied in RMLV infected mice. To match the regimen of nonencapsulated drug used in the following chemotherapy studies, a 20-fold higher dose of nonencapsulated PF was given by i.p.

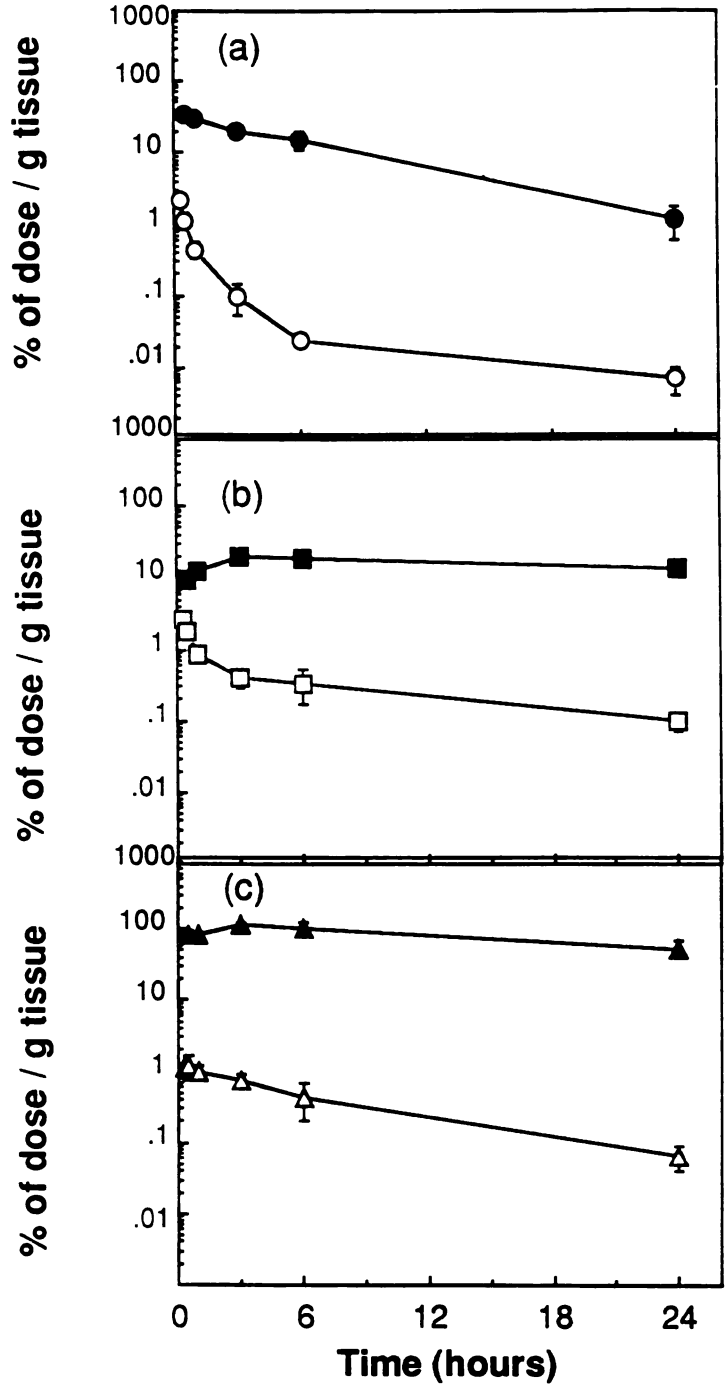


Figure 3. Tissue levels of ^{14}C-PF after intravenous administration of nonencapsulated ^{14}C-PF or liposomal PF into normal mice. A single dose of 24 mg/kg of nonencapsulated ^{14}C-PF (open symbols) or 18 mg PF/0.53 mmol lipid/kg of ^{14}C-PF containing liposomes (solid symbols) was administered intravenously into normal mice. At each time point, three animals were used to determine the radioactivity associated with the (a) blood, (b) liver, and (c) spleen. Values are the mean ± the SD.

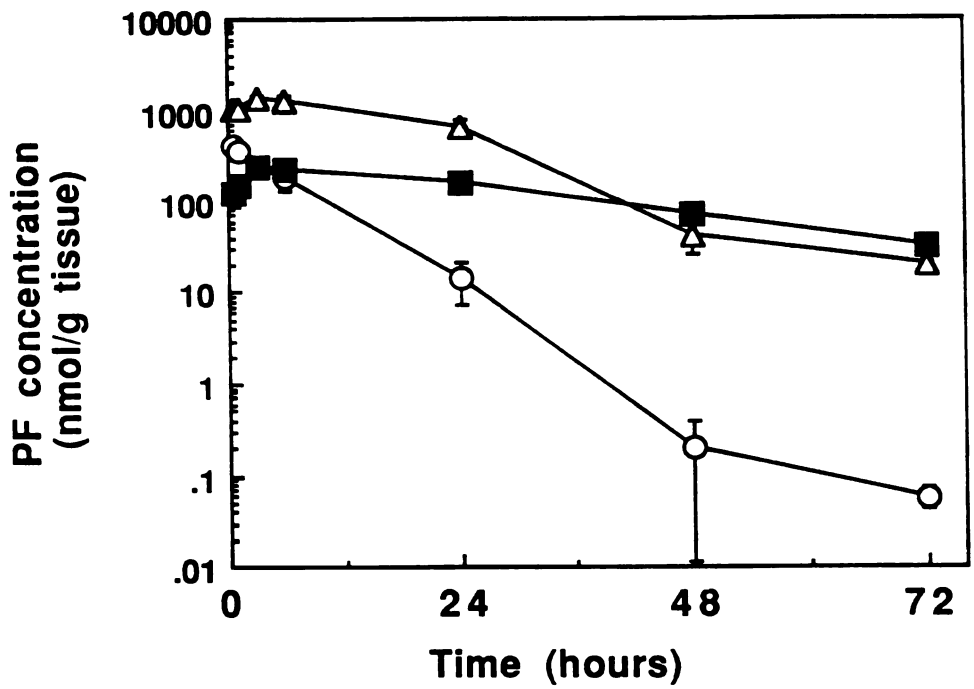


Figure 4. Tissue levels of ^{14}C-PF in normal mice receiving an i.v. bolus of liposomal PF. A single dose of 18 mg PF/0.53 mmol lipid/kg of ^{14}C-PF containing liposomes was administered intravenously into normal mice. At each time point, three animals were used to determine the radioactivity associated with the blood (○), liver (■), and spleen (Δ). Values are the mean ± the SD.

injection to RMLV infected mice. The drug level in the blood, liver and spleen following this administration is compared with that of normal mice receiving an i.v. bolus of PF as shown in Figure 5. In spite of the differences in (i) the condition of the animal (normal versus RMLV infected), (ii) dosage (24 mg/kg versus 491 mg/kg), and (iii) the route of administration (i.v. versus i.p.), pharmacokinetic profiles in the blood, liver, spleen and other organs after these two treatments do not significantly differ from each other. In both cases, nonencapsulated PF is rapidly excreted from the body, and only 0.1-0.2 % of the injected dose was found in the blood at 3 hr after dosing while less than 0.1% of the dose was detected in the liver or spleen at 24 hr post dosing. Thus PF appears to be handled by the body in a similar fashion whether given as an i.v. or i.p. dose, since it reaches the circulation very rapidly after the i.p. administration.

The pharmacokinetic parameters of liposomal PF are altered by the disease state of animals (3 weeks after infection) (Figure 6). The drug level in the spleen is remarkably different between RMLV infected and noninfected animals, after receiving an i.v. bolus of liposomal drug, 13 mg PF/0.4 mmol lipid/kg, and 18 mg PF/0.53 mmole lipid/kg, respectively (Figure 6c). The fraction of the injected dose associated with the unit weight of the spleen is about 10 times higher in normal mice than in infected mice. This implies that the virally infected spleen is taking up less liposomes and their encapsulated drugs than the normal spleen. A slightly greater rate in the decline of drug levels in the blood, liver, and spleen of RMLV infected animals is also observed (Figure 6). This suggests that liposomal PF is eliminated faster in the ailing mice than in normal mice. The faster release of PF from these organs in the diseased mice may result from the following mechanisms. (i) The macrophages in the virally infected liver and spleen are more active in degrading the ingested liposomes and releasing the drug than the macrophages in the normal liver and spleen or (ii) non-internalized liposomes account for a greater proportion of total liposomal PF in the virally infected organs than in the normal liver and spleen, and drug leakage from this population of liposomes is faster than from liposomes internalized by macrophages.

Intraperitoneal administration was employed in chemotherapy study due to the difficulty in repeated i.v. injection into the tail vein and limited dose used in i.v. administration. The effects of the i.p. route of administration and dosage on the pharmacokinetics of liposomal PF were also investigated. The kinetic profile of PF in RMLV infected mice (3 weeks after infection) receiving a large dose of the drug (74 mg PF/2 mmole lipid/kg Lipo-PF) given intraperitoneally is shown in figure 7, together with the kinetic profiles following an i.v. bolus dose of the drug (13 mg PF/0.4 mmole lipid/kg). The parallel declining curves in the blood, liver, and spleen following the i.v. or i.p. injection suggest that the terminal elimination rate of the drug is not affected by the

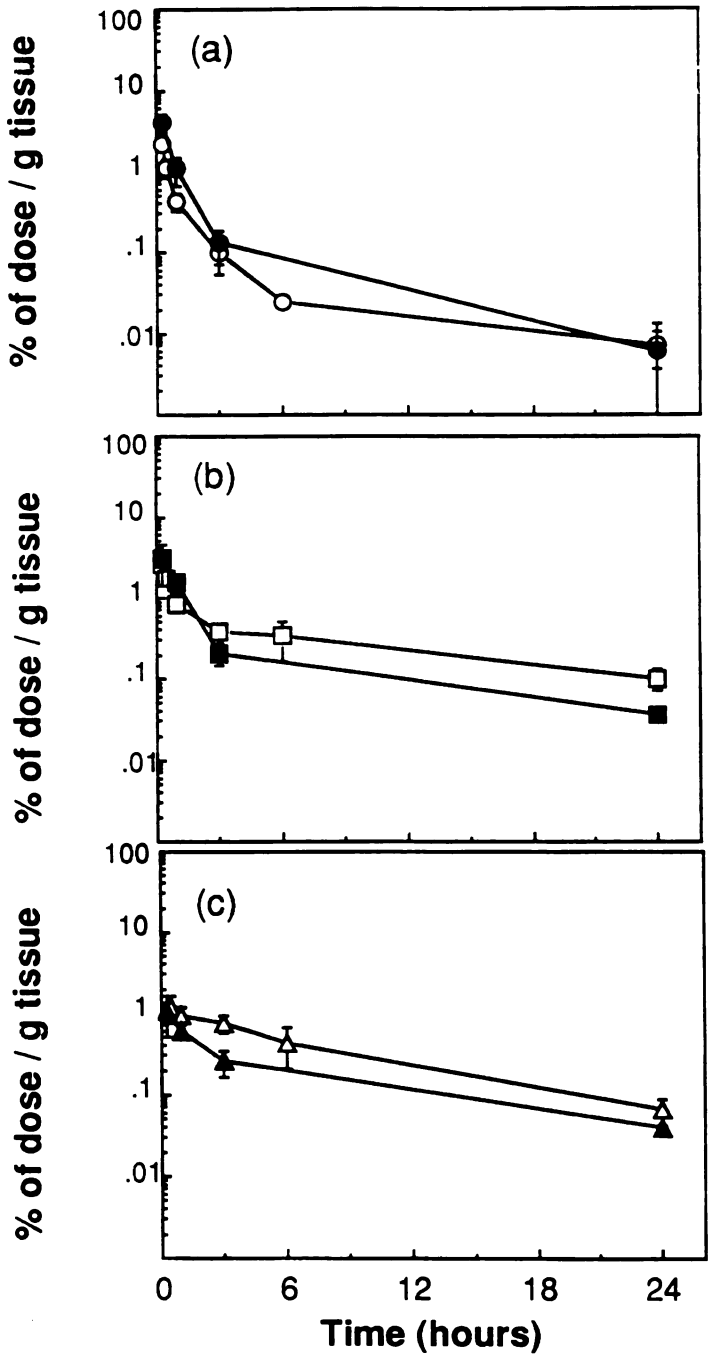


Figure 5. Tissue Level of ^{14}C-PF following one i.v. injection of nonencapsulated PF into normal mice or after one i.p. injection of nonencapsulated PF into RMLV infected mice. A single dose of 24 mg/kg of ^{14}C-PF was given intravenously into normal mice (open symbols) and one dose of 491 mg/kg of ^{14}C-PF was administered by i.p. injection into RMLV infected mice (solid symbols). At each time point, three animals were used to determine the radioactivity associated with (a) blood, (b) liver, and (c) spleen. Values are the mean ± the SD.

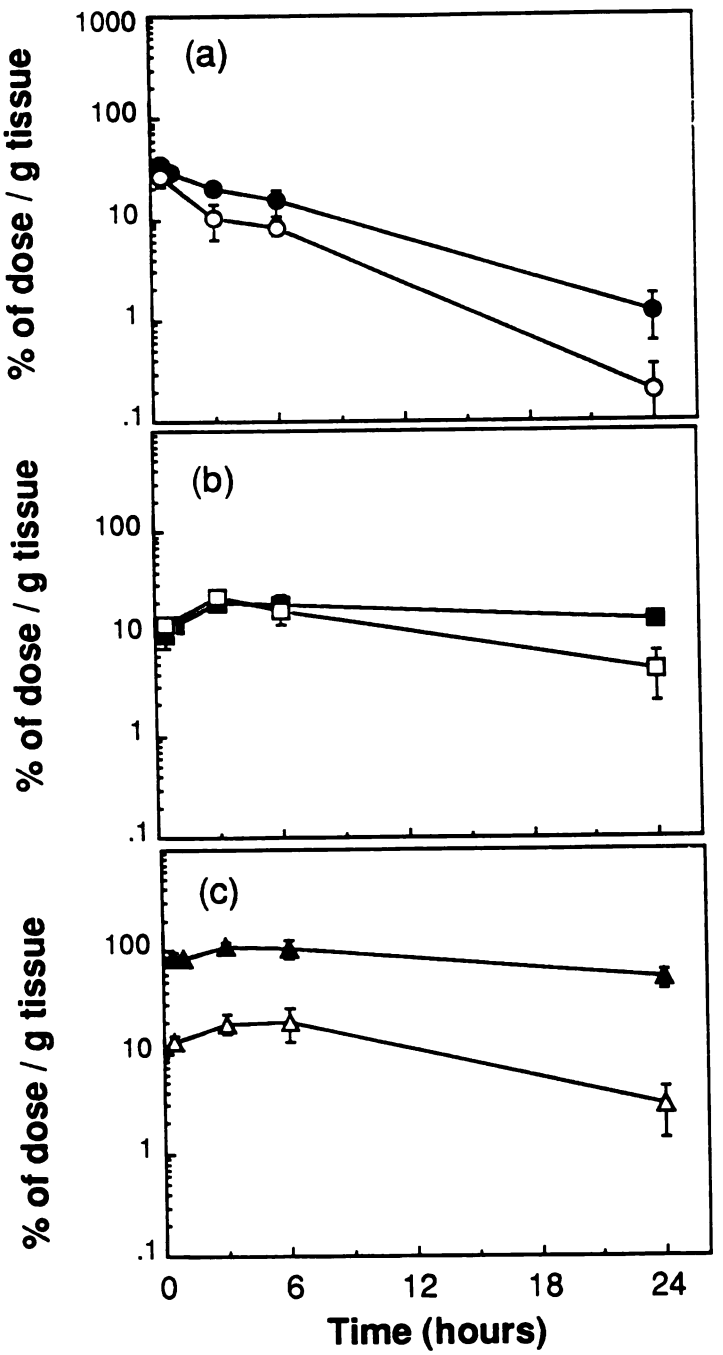


Figure 6. Tissue levels of ^{14}C-PF in normal or RMLV infected mice following an i.v. bolus of ^{14}C-PF containing liposomes. A single dose of 18 mg PF/0.53 mmole lipid/kg of ^{14}C-PF containing liposomes was given to normal mice (solid symbols) and one dose of 13 mg PF/0.4 mmole lipid/kg of ^{14}C-PF containing liposomes was administered to RMLV infected mice (open symbols). At each time point, three animals were used to determine the radioactivity associated with (a) blood, (b) liver, and (c) spleen. Values are the mean ± the SD.

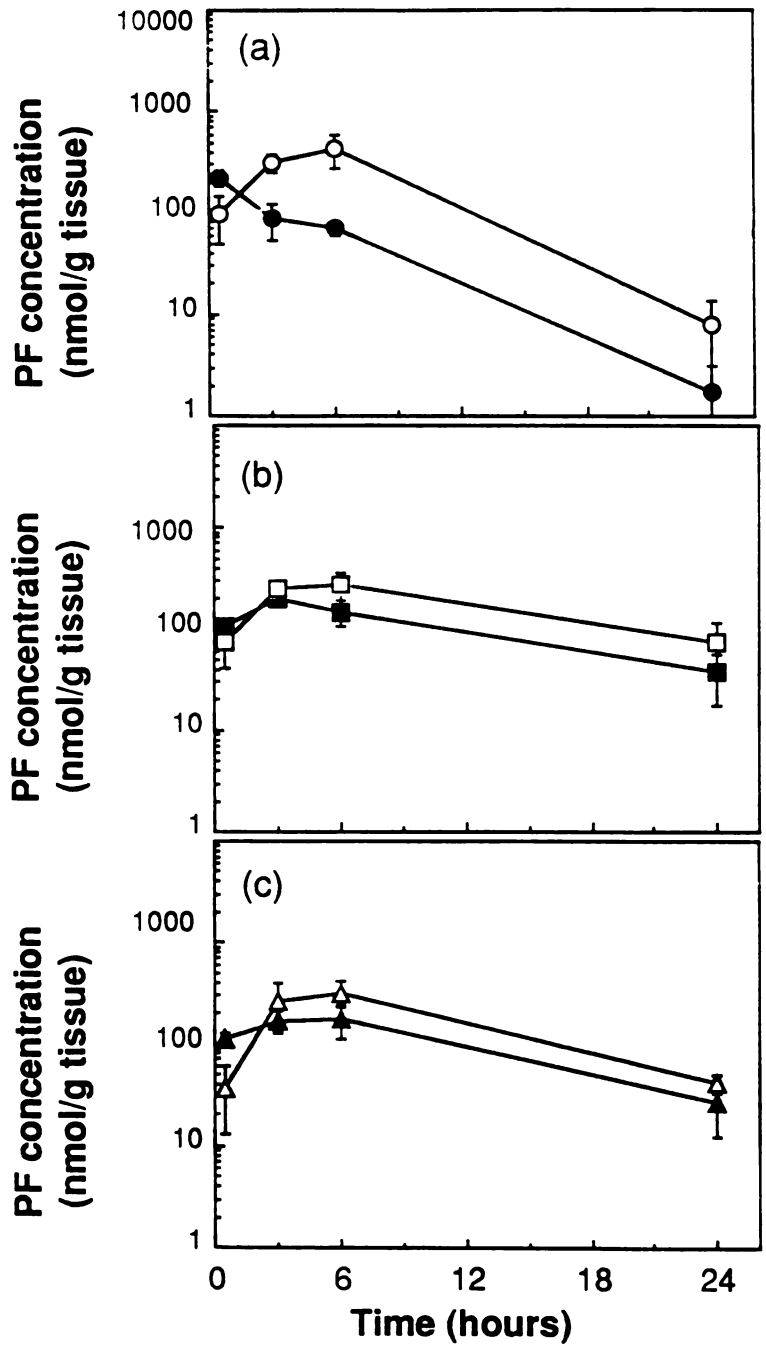


Figure 7. Tissue levels of ^{14}C-PF after i.v. and i.p. administration of liposomal PF into RMLV infected mice. A single dose of 13 mg/0.4 mmole lipid/kg of ^{14}C-PF containing liposomes was given intravenously (solid symbols) or 74 mg/2 mmole lipid/kg of ^{14}C-PF containing liposomes was administered intraperitoneally (open symbols) into RMLV infected mice. At each time point, three animals were used to determine the radioactivity associated with the (a) blood, (b) liver, and (c) spleen. Values are the mean ± the SD.

route of administration, nor is the kinetic profile of liposomal PF in the liver and spleen (Figure 7b & 7c). The clearance of liposomal PF from the body after these two treatments is not significantly different from each other (Table 1). Only the peak concentration time reached in the circulation is delayed via the i.p. route (Figure 7a).

The clearance of nonencapsulated PF is fast relative to processes governing disposition of liposomes (Table 1), the majority of PF remains in the body must be encapsulated in liposomes or trapped in the cells following internalization of intact liposomes. Thus we may assume that pharmacokinetics of liposomal PF is representing the pharmacokinetics of encapsulated drug exclusively. The clearance of the liposomal PF is not significantly different when administered via the i.v. or i.p. route, the bioavailability of the liposomal drug appears to be reduced by i.p. administration. The area under the blood curve via the i.p. injection is about 3.8 times larger than the corresponding AUC via the i.v. injection, although the i.p. dose was 5.7 times larger than the i.v. dose (Figure 7a, Table 1). This result implies that only 67% of the i.p. injected liposomal PF reaches the circulation as the encapsulated form. It is conceivable that liposomes, when they are given intraperitoneally, are absorbed by lymph nodes before entering the circulation (Hirano & Hunt, 1985; Parker et al., 1982) and nonencapsulated drug released from these nodes are eliminated rapidly.

The extraction ratio of liposomal drug in the RES can be estimated by the ratio of $AUC_{liver+spleen}$/total $AUC_{blood+liver+spleen}$. Following the i.p. injection of 74 mg PF/2 mmole lipid/kg, the ratio is 0.61, while the corresponding extraction ratio for the i.v. injection of 13 mg PF/0.4 mmole lipid/kg is 0.78. Thus, a smaller fraction of liposomal PF was taken up by the liver and spleen, when the larger dose was given. This apparent less efficient uptake of liposomes, as evidenced by the lower extraction ratio, can be accounted for by the saturation of the RES with a high dose of liposomes (2 mmol lipid/kg) (Bosworth & Hunt, 1982). Due to the lower bioavailability of the i.p. administration and the lower extraction efficiency of a saturating dose of liposomes, a 5.7 fold larger dose of liposomal PF given intraperitoneally yields a only 1.7 fold larger AUC in the liver and spleen than the i.v. liposome dose.

Comparison of pharmacokinetic profiles following the i.p. injection of nonencapsulated PF (491 mg PF/kg) to the i.v. injection of liposome-encapsulated PF (13 mg PF/kg) in RMLV infected mice shows that the AUC in the liver and spleen of nonencapsulated PF was 1.3-1.7 times lower than corresponding AUC for liposomal PF (Table 1). Moreover, a 2-3 fold higher drug level in the liver and spleen was observed with the liposomal treatment than that obtained after the nonencapsulated drug treatment at a

Table 1. Pharmacokinetic parameters of nonencapsulated and liposome-encapsulated ^{14}C-PF in RMLV infected mice at 3 weeks after infection. Mice were sacrificed at 0.25, 1, 3, & 24 hr after nonencapsulated ^{14}C-PF or at 0.5, 3, 6, & 24 hr after liposomal ^{14}C-PF administration to determine the drug levels associated with the tissues. Values shown are estimated based on the mean values ± standard deviation of three animals at each time point.

Form	Route	Dose (mg PF/kg)	Organ	Terminal $t_{1/2}$ [a] (hr)	$AUC_{0\text{-}24\ H}$ [b] (hr·nmol/g)	Clearance [c] (g/hr)
PF	i.p.	491	Blood	4.8 ± 1.1	1880 ± 630	18.8 ± 6.9
Lipo-PF	i.v.	13	Blood	3.4 ± 0.8	1460 ± 310	0.61 ± 0.13
Lipo-PF	i.p.	74	Blood	3.1 ± 0.6	5610 ± 1960	0.96 ± 0.36
PF	i.p.	491	Liver	8.6 ± 0.8	2230 ± 730	N.A.[d]
Lipo-PF	i.v.	13	Liver	9.2 ± 2.0	2590 ± 720	N.A.
Lipo-PF	i.p.	74	Liver	9.7 ± 0.6	4350 ± 1350	N.A.
PF	i.p.	491	Spleen	7.8 ± 2.1	1610 ± 490	N.A.
Lipo-PF	i.v.	13	Spleen	6.6 ± 0.9	2690 ± 910	N.A.
Lipo-PF	i.p.	74	Spleen	6.1 ± 0.6	4480 ± 1410	N.A.

a : Calculated by fitting data into a two-compartment model using least-squares regression analysis.
b : $AUC_{0\text{-}24\ hr}$ is calculated by the trapezoidal method.
c : Clearance = dose administered/$AUC_{Blood,\ 0\text{-}24\ hr}$.
d : Not applicable.

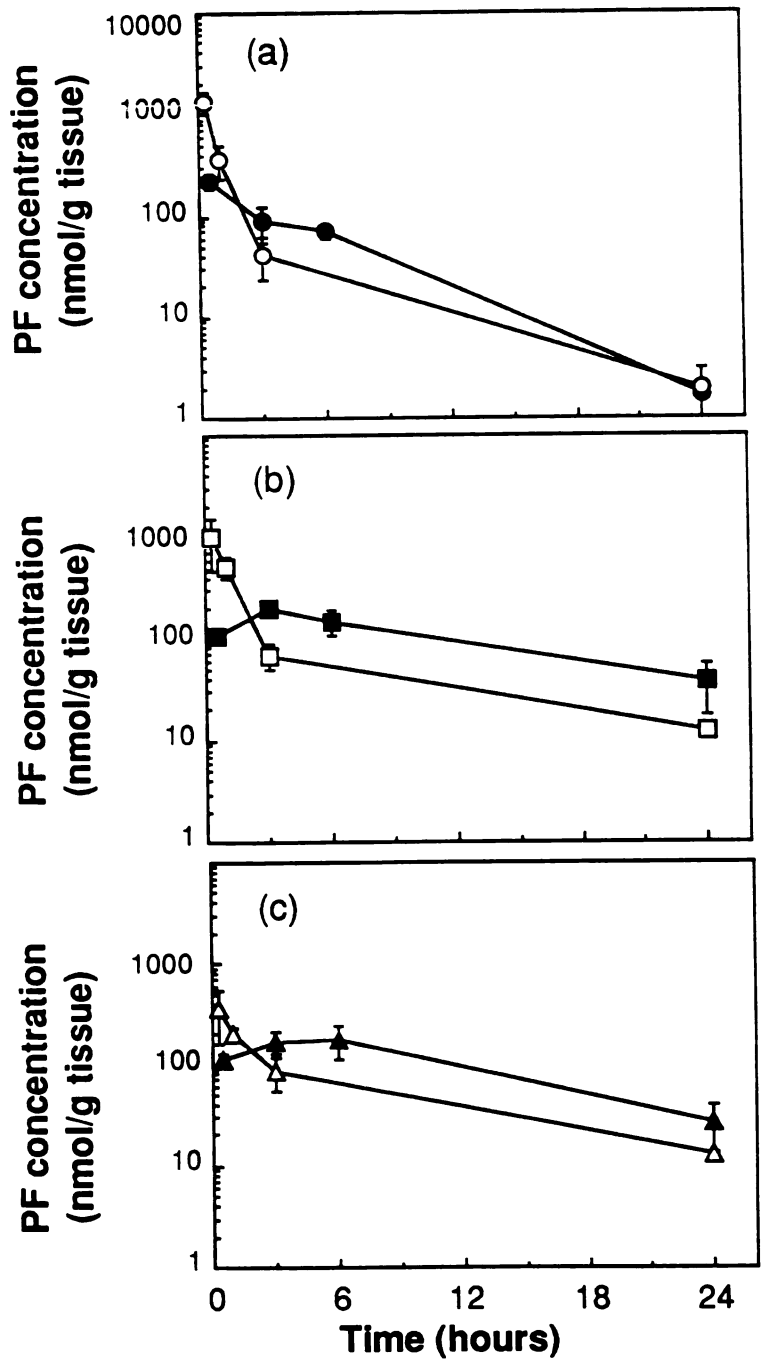


Figure 8. Tissue levels of ^{14}C-PF in RMLV infected mice receiving an i.v. bolus of ^{14}C-PF containing liposomes or an i.p. administration of nonencapsulated ^{14}C-PF. A single dose of 13 mg/kg of ^{14}C-PF containing liposomes was given intravenously (solid symbols) or 491 mg/kg of ^{14}C-PF was administered intraperitoneally (open symbols) into RMLV infected mice. At each time point, three animals were used to determine the radioactivity associated with the (a) blood, (b) liver, and (c) spleen. Values are the mean ± the SD.

later phase after the dosing (Figure 8). Therefore, liposomal PF gave higher tissue levels in the RMLV target organs than a 38 times larger dose of the nonencapsulated drug.

Chemotherapy

In the following studies, the maximum tolerated dose of 500 mg/kg PF was administered by daily i.p. injection, starting 24 hr after the virus inoculation for 3 days, and then administered every other day throughout the course of the treatment. This regimen resulted in a reduced liver ($p<0.05$) and spleen ($p<0.05$) weight when compared with the nontreated virus control group (Table 2). On the other hand, PF encapsulated in liposomes (20 mg PF/0.56 mmole lipid/kg) administered at a similar schedule, via i.v. route for the first 3 doses and via i.p. route for the rest of the doses caused an 80% reduction of the RMLV infected liver weight when compared with the virus control group ($p<0.05$). This demonstrates that the liposome encapsulated drug is more potent than nonencapsulated PF in reducing the liver weight of the infected mice. In three repeated trials, liposomal drug did not have a similar effect in decreasing the infected spleen weight (Table 2). The pharmacokinetic studies reveal that the liposome uptake capacity is reduced by the RMLV infection to a greater extent in the spleen than in the liver (Figure 6). Thus the fact that effective antiviral activity was achieved in the liver by the liposomal drug but not attained in the spleen can be partially attributed to the less efficient uptake of liposomes by the infected spleen.

Since the dose of liposomal PF was limited by the volume which could be injected intravenously, the dose was increased in the second chemotherapy trial by employing an i.p. injection of a larger injection volume of liposomal drug. The mice received a daily dose of 100 mg PF/2 mmole lipid/kg for the first 6 days followed by half of the initial dose given every other day for the rest of the course of the treatment. The viral infection was enhanced by this regimen using high doses of liposomal PF (Table 3). The animals in this experimental group lost weight and were lethargic during the daily treatment period but then appeared to recover when the dose was reduced by half. These symptoms may be an adverse effect of the high dose of liposome-encapsulated drug. The noninfected mice receiving the same treatment exhibited similar symptoms and had an increased spleen weight when compared with control mice receiving PBS (Table 3). Since there was no difference in spleen weight of RMLV infected mice treated with non-drug containing liposomes when compared to that of RMLV infected mice treated with PBS (Table 3), the adverse effects displayed using high dosages of liposomal PF are not a result of the lipid alone. The drug may also contribute to the observed side effects, because the animals

Table 2. Spleen and liver weights of RMLV infected mice treated with liposomal PF, non-drug containing liposomes or nonencapsulated PF. Mice, except the control group, were infected on day 0 and all mice were sacrificed on day 20. Nonencapsulated PF was given by i.p. injection on day 1, 2, 3, 5, 7, 9, 11, 13, 15, & 17. Liposomal PF or empty Lipo. was given by i.v. injection on day 1, 2, & 3 and followed by i.p. injection on day 5, 7, 9, 11, 13, 15, & 17.

Treatment	# of mice	Spleen weight (g)		Liver weight (g)	
Control	4	0.16 ± 0.03		1.04 ± 0.12	
Virus control	8	1.31 ± 0.41		1.24 ± 0.16	
PF (500 mg/kg)	7	0.90 ± 0.35	$(p<0.05)^a$	1.05 ± 0.09	$(p<0.05)^a$
Lipo-PF (20 mg/kg)	8	1.48 ± 0.41		1.08 ± 0.12	$(p<0.05)^a$
Empty Lipo. (0.56 mmole lipid/kg)	8	1.47 ± 0.41		1.17 ± 0.17	

a : Unpaired Student's t test between treatment group and virus control group.

Table 3. Spleen and liver weights of RMLV infected mice treated with nonencapsulated PF, high dose of liposomal PF or non-drug containing liposomes intraperitoneally. Mice, except the control group and control + Lipo-PF treated group, were infected on day 0 and all mice were sacrificed on day 20. Drugs were administered by i.p. injection on day 1, 2, 3, 4, 5, 6, 8, 10, 13, 15, & 17 after virus inoculation. Dose of Lipo-PF was reduced to 50 mg/kg after the first 6 doses of 100 mg/kg. Dosage of empty Lipo. was adjusted accordingly.

Treatment	# of mice	Spleen weight (g)		Liver weight (g)
Control	4	0.11 ± 0.03		0.95 ± 0.14
Control + Lipo-PF (50-100 mg/kg)	6	0.22 ± 0.05	($p<0.01$)[a]	0.97 ± 0.14
Virus control	8	0.95 ± 0.58		0.94 ± 0.23
PF (500 mg/kg) [c]	8	1.10 ± 0.09		1.10 ± 0.09
Lipo-PF (100-50 mg/kg) [c]	7	1.86 ± 0.70	($p<0.02$)[b]	1.10 ± 0.09
Empty Lipo. (2.0-1.1 mmole lipid/kg)	8	1.29 ± 0.52		1.17 ± 0.22

a : Unpaired Student's t test between control group and control mice receiving Lipo-PF.
b : Unpaired Student's t test between virus control group and treatment group.
c : Animals were lethargic, lost weight and seemed to be affected by the increased dosing frequency at 500 mg/kg of nonencapsulated PF or by the high dose of 100 mg/kg of Lipo-PF.

receiving the maximum tolerated dose of nonencapsulated PF (500 mg/kg) for 6 consecutive days also showed symptoms of lethargy and loss of body weight.

The pharmacokinetic data obtained after an i.p. administration of a high dose of liposomal PF to RMLV infected mice suggest that the RES was saturated by this large dose of liposomes. This saturation of the RES renders macrophages in the RES less efficient for defense against the RMLV infection, as described previously by Knysynski and Danon (1977) using colloidal carbon as an RES saturating agent. Consequently, the disease progression was enhanced by the high dose of liposomal PF.

The pathology of RMLV infection originates from the transformation of erythroblasts by the virus to cancerous erythroleukemia cells (Rauscher, 1962). Given that the primary target of the antiviral action of PF is RMLV reverse transcriptase (Öberg, 1989), arrest of reverse transcriptase activity in target cells before the beginning of transformation will ensure the maximum effect of the drug, provided an efficacious concentration of drug is present within these cells. Chemotherapy employing either nonencapsulated drug (500 mg/kg by i.p. injection) or encapsulated drug (25 mg/kg by i.v. injection) at 3 hr before and at 24 hr after the virus inoculation resulted in a reduced liver weight, while a reduction of spleen weight was not achieved by either treatment. Thus, liposomal PF is 20 times more potent in reducing the liver weight than nonencapsulated drug using this regimen. Empty liposomes (0.6 mmole/kg) showed no therapeutic effect, nor did they interfere with the antiviral effect of nonencapsulated PF (Table 4). The failure to control the development of disease by prophylactic chemotherapy suggests that the efficacious drug concentration in the target cells was not attained at the time of virus inoculation using the treatment schedule outlined here.

Uptake of Liposomes by RMLV Infected Splenocytes in Culture

The inability of liposomal PF to produce a reduction in the size of the RMLV infected spleens prompted us to evaluate the liposome uptake capacity of these splenocytes, both in vivo and in vitro. The hypothesis that liposomes accumulate in the target organ of RMLV infection was validated by the result showing that about 10-25% of injected ^{125}I-labeled liposomes were present in the spleen at 3 hr after dosing, shown as the percentage of injected dose (Figure 9a). Liposomes uptake per gram spleen was 90% of injected dose in the uninfected animals. However, the liposome uptake capacity of the infected spleen diminishes as the infection advances, as shown by the inverse correlation between the liposome uptake per unit spleen weight and the enlarged spleen at different stages after the viral infection (Figure 9b). The same result is obtained when ^{14}C-PF containing liposomes were used (data not shown).

Table 4. Spleen and liver weights of RMLV infected mice receiving prophylactic dose of nonencapsulated PF, non-drug containing liposomes, and liposomal PF. Nonencapsulated drug was given by i.p. injection and liposomal preparations were given via the i.v. route. Mice received one dose at 3 hr before and one dose at 24 hr after virus inoculation. Mice, except the control group, were infected on day 0 and all mice were sacrificed on day 20.

Treatment	# of mice	Spleen weight (g)	Liver weight (g)	
Control	4	0.12 ± 0.02	1.11 ± 0.11	
Virus control	6	1.26 ± 0.26	1.44 ± 0.09	
PF (500 mg/kg)	6	1.25 ± 0.19	1.24 ± 0.12	$(p<0.02)^a$
Lipo-PF (25 mg/kg)	6	1.49 ± 0.52	1.26 ± 0.14	$(p<0.02)^a$
Empty Lipo. (0.6 mmole lipid/kg)	6	1.35 ± 0.22	1.38 ± 0.09	
Empty Lipo. + PF (0.6 mmole lipid + 500 mg PF/kg)	6	1.20 ± 0.34	1.25 ± 0.08	$(p<0.02)^a$

* : Unpaired Student's t test between virus control group and treatment group

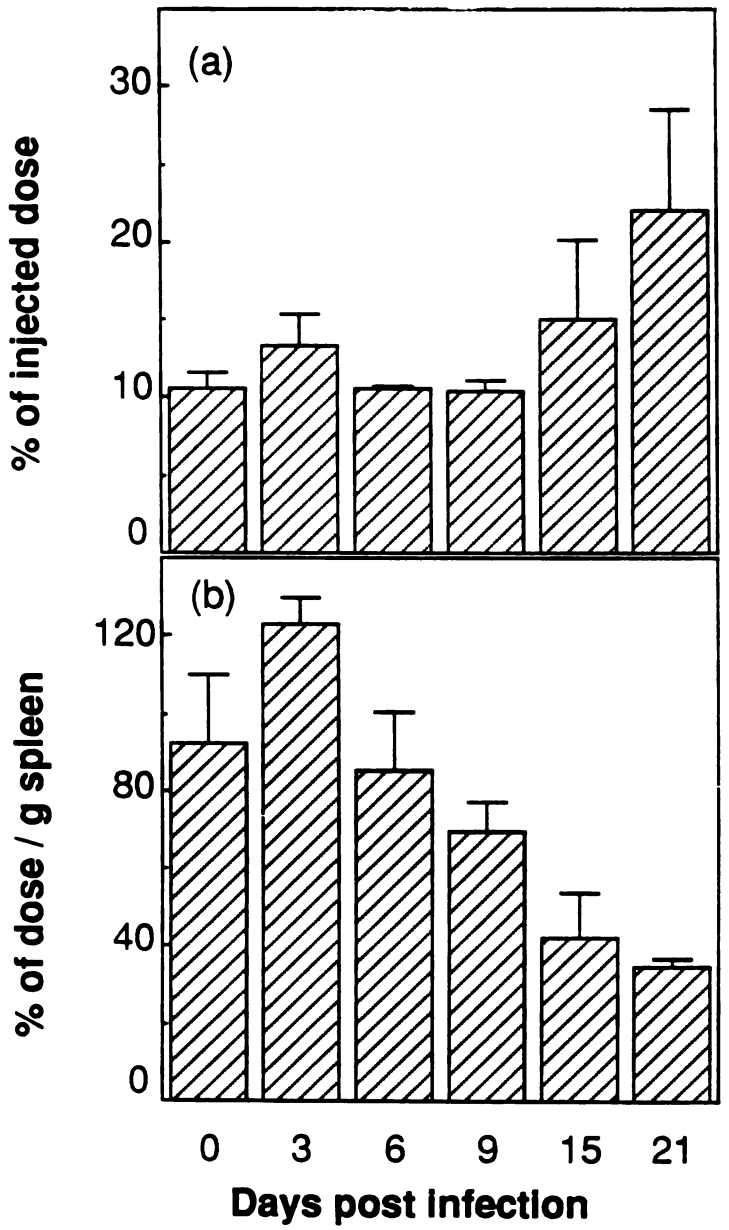


Figure 9. Liposome uptake by the spleens after RMLV infection. Eight μmoles of ^{125}I-labeled liposomes were injected intravenously into RMLV mice at 3, 6, 9, 15, & 21 days after viral infection. Spleens were weighed and radioactivity associated with spleen was measured at 3 hours after dosing. (a) ^{125}I-radioactivity associated with the spleen was plotted as percentage of total injected dose. (b) ^{125}I-radioactivity associated with the spleen expressed as percentage of injected dose per unit weight of spleen. Values represents the mean and the SD of three mice.

In the RMLV infected mice as the spleen weight increases from 0.1 gram to 1 gram, it is impossible for the % of the injected dose per gram spleen to remain as high as that observed in the uninfected animals. The observation of less uptake (liposomes per gram of tissue) in the RMLV spleen compared to the normal spleen may originate from (i) a lower blood supply to the enlarged spleen, (ii) competition of liposome uptake from other organs, or (iii) the extraction ratio of the infected spleen is decreased, possibly because the phagocytotic activity of the infected spleen is less efficient than that of the normal spleen.

The liposome uptake by the RMLV spleen was further investigated using isolated RMLV splenocytes in culture. De Both and colleagues have reported that at 3 weeks after RMLV infection, approximately 60-85% of the spleen consists of erythroleukemia cells (De Both et al., 1978; Lobue, 1984). Thus nonadherent splenocytes were easily obtained after removal of the adherent splenocytes by two consecutive platings onto culture flasks. Greater than 95% of nonadherent spleen cells collected from spleens at 6 weeks post-infection are believed to be erythroleukemia cells. The uptake of liposomes by these nonadherent RMLV spleen cells is shown in Figure 10. When compared with the uptake of the same kind of liposomes by endocytotically active Vero cells (8.7, 22.7, and 35.9 nmol lipid/mg protein were associated with Vero cells after 1, 6, and 24 hr incubation with 100 μM liposomes, respectively) (Szoka & Chu, 1988), the uptake by erythroleukemia cells is at least 10 times less efficient than that of Vero cells. Similar results were obtained with the adherent and nonadherent spleen cells isolated from RMLV mice (at 6 weeks post-infection) which had received ^{125}I-REV at 3 hr before they were sacrificed. The adherent splenocytes isolated from these mice had about an 8 fold higher ^{125}I-REV uptake capacity (1.14 nmol lipid/10^6 cells) than the nonadherent splenocytes collected from the same mice (0.148 nmol lipid/10^6 cells). In the same experiment, 13-16% of the radioactivity associated with the cells collected from the enlarged spleen at 3 hours post-dosing was removed at each washing, and greater than 30% of the total radioactivity associated with the cells were readily dissociated during the washing. Thus a significant portion of radioactivity associated with the enlarged spleen may be due to liposomes adsorbed to the surface of erythroleukemia cells in the organ.

Since liposomes are not efficiently internalized by erythroleukemia cells, nonencapsulated PF released from liposome-ingested macrophages into the circulation is the principle form of PF available for transport into erythroleukemia cells. The intracellular concentration of PF in erythroleukemia cells will be low if a high local concentration of nonencapsulated PF is not maintained. Thus the ineffective chemotherapy with liposomal PF is most likely due to the inefficient uptake of liposomes by erythroleukemia cells in the

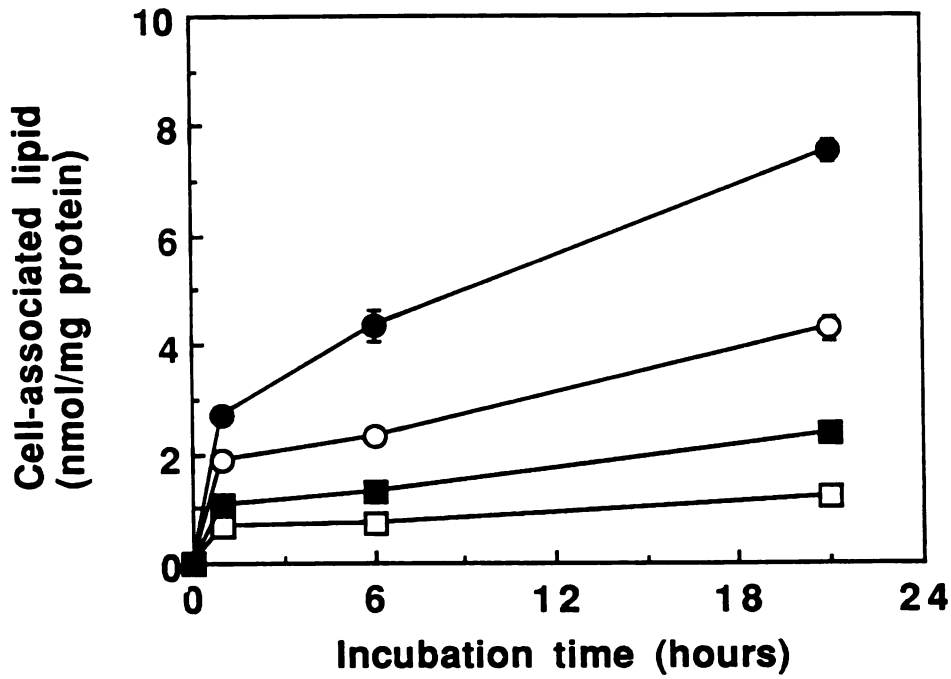


Figure 10. In vitro uptake of liposomes by nonadherent RMLV infected spleen cells. Nonadherent splenocytes were isolated after two consecutive plating of RMLV spleen cells at 42 days post infection as described in the Materials & Methods. ^{125}I-labelled liposomes at various concentrations, 100 μM (□), 250 μM (■), 500 μM (○), and 1000 μM (●), were incubated with 10 x 10^6 nonadherent splenocytes for the times indicated. After two washing, radioactivity associated with cells was measured and the cellular protein was determined. Values are the mean and the range of duplicate measurements.

spleen and by analogy in other organs, together with an insufficient amount of nonencapsulated drug available for transport in the vicinity of target cells.

4. Discussion

We hypothesized that liposomes can passively target encapsulated drug to the RMLV infected spleen and liver which would subsequently result in a high local drug concentration and an increase in the drug potency. The results from biodistribution and pharmacokinetic studies demonstrate that PF is delivered to the target organs of RMLV infection and the residence time of PF in these organs is extended when PF is delivered by liposomes. However, the postulated enhancement of potency of PF in reducing the spleen weight of RMLV mice was not observed using liposomal delivery. The discrepancy between the chemotherapy results and pharmacokinetic data mainly reflects the adverse distribution of the drug at the cellular level. This is the consequence of the low liposome uptake capacity of the target cells of RMLV infection. A significant portion of the liposomes containing PF associated with the target organ is reversibly adsorbed to the cell surface.

The efflux of the drug from macrophages, subsequent to liposome uptake and degradation, is also a source of nonencapsulated drug in the target organ. Therefore a sustained high local concentration of the drug may be attainable when macrophages are loaded with large amounts of liposome-encapsulated drug (Mayhew et al., 1982; Storm et al., 1988). However, saturation of the RES with liposomes diminishes its role in defense against pathogens. In this study a high dose of liposomal PF resulted in aggravation of RMLV infection. This reinforces the idea that the ratio of encapsulated drug to lipid is a critical factor in determining the outcome of the targeted delivery using liposomes. The optimal effect of liposomal PF may be realized if the encapsulation ratio (M drug/M lipid) can be significantly increased.

The biodistribution of liposomes, the stability of liposomes in vivo, the release of the drug from liposomes, and the slow efflux of drug from liposome-ingested cells make the pharmacokinetics of liposomal drug more complicated than that of nonencapsulated drug (Hwang, 1987). The pharmacokinetic parameters of liposomal drugs are determined by the size, surface characteristics, and lipid composition of the liposomes (Senior, 1986). Therefore, formulation of liposome preparations for targeted delivery must be tailored for each drug and for its potential use. For instance, in the case of an intracellularly active drug used for a non-RES disease, small sized, long circulating liposomes with minimal RES

uptake capacity (e.g. stealth liposomes) (Allen & Chonn, 1987; Gabizon & Papahadjopoulos, 1988) might be suitable. On the other hand, for intracellularly active drugs to be used against a RES disease, large liposomes which carry negative charges or ligands with high affinity for the RES (Senior, 1986) would be preferable. Physicochemical properties of the drug also play a critical role in the outcome of the targeted delivery (Stella & Himmelstein, 1980; Hunt et al., 1986). For instance when the drug is delivered to the target organ, but not to the target cells, by a carrier, the partition coefficient of the drug will determine the extent and rate of the permeation of the drug into the target cells. Physicochemical properties will also determine the kinetics of the drug redistribution to the extracellular site, when the drug is delivered into the target cells by a carrier.

Many reports confirm that drug delivery via passively targeted liposomes is successful in the treatment of the diseases which reside in the RES (Alving et al., 1978; Mayhew & Papahadjopoulos, 1983; Gregoriadis, 1988). Without an active targeting scheme, liposome-mediated delivery to treat diseases outside of the RES has achieved limited success. It is instructive to examine instances of successful passive targeting, such as liposome-encapsulated adriamycin to treat metastatic lymphoma cells in the spleen and liver in a murine model (Gabizon et al., 1983). In this model both tumor development in the liver was delayed and the survival time of infected animals was increased when using a liposomal adriamycin treatment versus a nonencapsulated drug treatment. Moreover, the drug level in the tumor cells isolated from the liver of mice receiving liposomal drug was significantly higher than that of mice receiving nonencapsulated drug. Several factors contributed to this successful outcome. (i) Sonicated liposomes (SUV) were used. These small vesicles have a longer circulation lifetime in the blood and thus have a greater chance to encounter tumor cells (Gabizon & Papahadjopoulos, 1988). (ii) Lymphoma cells might internalize SUV better than larger vesicles, e.g. REV. (iii) Adriamycin released from proximal macrophages kept a high local concentration of drug in the vicinity of target cells for a prolonged time (Gabizon et al., 1983). (iv) Adriamycin could partition into adjacent cells rather efficiently.

In the case of PF, poor permeation of the nonencapsulated drug into the target cells and inefficient uptake of REV by the target cells resulted in the unsuccessful chemotherapy using liposomal PF to treat RMLV infection. Although PF is an ideal compound for targeted delivery, the poor chemotherapy from the passive targeting approach using liposomal drug in this model reinforces the necessity to insure that the carrier reaches the correct target.

In summary, the maximal therapeutic effect via passively targeted delivery of liposomal drugs can be obtained by use of carefully designed liposomes containing a properly selected drug for encapsulation. In the case of intracellularly active drugs for use in a non-RES disease, long circulating liposomes with minimal RES uptake containing a drug which readily permeates through the cell membrane would be an appropriate therapeutic combination, as illustrated by liposomal adriamycin mentioned above. In contrast, for intracellularly active drugs used against a RES disease, we would like to have liposomes with high affinity for the RES, containing an encapsulated drug with a low permeation coefficient through the cell membrane, e.g. liposomal PF described in this report.

Thus, liposomal PF would be suitable for treating viral diseases which reside in the RES, such as AIDS. Macrophages have been shown to be involved in the pathology of AIDS syndrome and found to be the reservoir of HIV in AIDS patients (Crowe et al., 1987; Gartner et al., 1986). PF has been shown to inhibit HIV replication in vitro. However, due to low permeation of the drug to the intracellular compartment and the rapid clearance of the drug, PF has to be administered in large quantities by continuous i.v. infusion to maintain the effective therapeutic blood level (110-400 μM). In this report, we show that liposomal PF is concentrated in the RES and the drug is slowly eliminated from these organs. These results suggest that liposomal PF may be a good candidate for chemotherapy of HIV infection.

5. SUMMARY

Liposomal delivery of phosphonoformate (PF), an antiviral agent with poor membrane permeability, was evaluated using a passive targeting strategy to treat Rauscher murine leukemia virus (RMLV) infection. Pharmacokinetic studies in RMLV infected mice using the nonmetabolized ^{14}C-PF reveal that nonencapsulated PF is rapidly excreted with an approximate clearance of 18.8 g blood/hour, while liposome (egg phosphatidylcholine /egg phosphatidylglycerol / cholesterol : 9/1/8)-encapsulated PF has an approximate clearance of 0.61-0.96 g blood/hour. When administered in the liposome encapsulated form, 50-80% of the dose is found in the liver, spleen and blood at 3 hours post-injection. The area under the drug level-time curve (AUC) in the liver and spleen following a single dose of encapsulated PF is 1.3-1.7 times higher than the AUC of a 25 fold larger dose of nonencapsulated PF. Biodistribution studies of ^{125}I-labeled liposomes or encapsulated ^{14}C-PF in vivo show that the specific uptake of liposomes and entrapped contents

(amount/g tissue) by RMLV infected spleen declines as the disease progresses. Uptake studies in vitro show a low level of liposomal uptake by the virally infected nonadherent spleen cells when compared to endocytically active cells. Treatment with nonencapsulated PF (500 mg/kg) or liposome-encapsulated PF (20 mg/kg) was started one day after virus infection. PF encapsulated in liposomes has an increased potency evaluated as the reduction of the RMLV infected liver weight on day 20 when compared with nonencapsulated drug. A similar enhancement by liposomal drug in decreasing the infected spleen weight is not observed. Moreover, the viral infection is enhanced when the reticuloendothelial system (RES) is saturated with high doses of liposomal PF. Thus although the pharmacokinetic data shows an elevated spleen level of PF, the chemotherapy results reflect the inability of viral target cells to take up liposomes in the spleen.

(This work will be published in Journal of Liposome Research, 1991)

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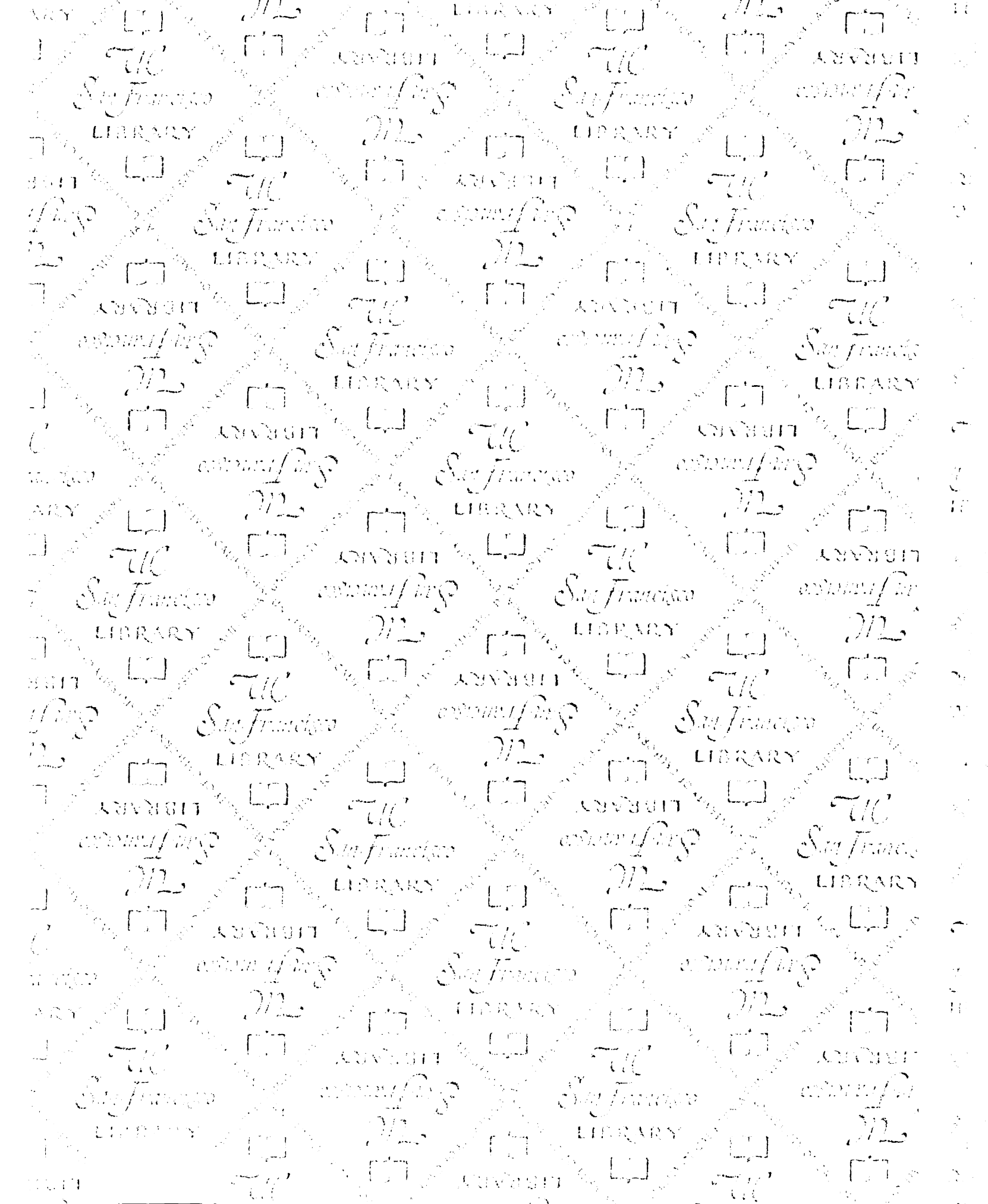
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