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TARGETING OF TLCK-COUPLED LIPOSOME TO SIMIAN VIRUS 40 TRANSFORMED MOUSE FIBROBLASTS

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ABSTRACT

N-α-tosyl-L-lysyl-chloromethyl ketone (TLCK) has been coupled to ara-C or colchicine encapsulated liposomes via glutaraldehyde treated liposomal phosphatidylethanolamine (Lp-G-TLCK) or sodium metaperiodate oxidized oligosaccharide side chain of liposomal digoxin (Lp-D-TLCK). TLCK has been shown to react with specific histidine residue at the active site of such protein as trypsin and has high binding affinity for simian virus 40 and polyoma virus transformed fibroblasts. A 20-minute exposure of cells to Lp-G-TLCK containing ara-C impaired the growth of tumor cells (SVT_2) for up to two days, but did not impair the growth of normal cells (Balb/C 3T3). However, at 50-minute exposure, the Lp-G-TLCK containing ara-C impaired the growth of Balb/C 3T3 cells at day one but recovered by the second day. The SVT_2 cells, on the other hand, did not recover from the cytotoxic effect of Lp-G-TLCK, containing ara-C during the second day. The underivatized liposome (Lp-PE) containing ara-C was cytotoxic to both Balb/C 3T3 and SVT_2 cells at 20 or 50 minute exposure periods.

The Lp-D containing ara-C, at 20 to 60-minute exposure, slightly impaired the growth of SVT_2 and Balb/C 3T3 cells at day one. Such cytotoxic effect was, however,

not observed by the second and third days of growth. In contrast, Lp-D-TLCK containing ara-C impaired the growth of SVT_2 cells while the growth of Balb/C 3T3 cells was nearly indistinguishable from control cells over a three-day period. Thus TLCK selectively enhanced ara-C encapsulated Lp-D-TLCK SVT_2 cell cytotoxicity. Lp-D-TLCK containing colchicine, while impairing the growth of Balb/C 3T3 cells, was particularly cytotoxic to SVT_2 cells, indicating that colchicine may be an unsuitable anti-tumor drug for use in a culture system with normal cells in their exponential growth phase.

The uptake of Lp-D-TLCK by SVT_2 cells was higher than the uptake by Balb/C 3T3 cells. With respect to underivatized liposome (Lp-D) the uptake by Balb/C 3T3 cells was higher than the uptake by SVT_2 cells, indicating that TLCK enhanced liposome reaction with SVT_2 cells and may retard reaction with the Balb/C 3T3 cells.

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LIST OF ABBREVIATIONS

ara-C	arabinofuranosyl cytosine (also known as Cytosar-U)
BCG	Bacillus Calmette-Guerin
Cells:	
Balb/C 3T3	Normal Swiss mouse fibroblasts (clone Balb/C 3T3)
SVT_2	Simian Virus 40 transformed Balb/C 3T3
DNA	Deoxyribonucleic acid
HRP	Horseradish peroxidase
Lp-D	Digoxin-incorporated liposome
Lp-D-TLCK	Digoxin-incorporated liposome derivatized with N-α-tosyl-L-lysyl-chloromethyl ketone
Lp-PE	Phosphatidylethanolamine-incorporated liposome
Lp-G	Phosphatidylethanolamine-incorporated liposome treated with glutaraldehyde
Lp-G-TLCK	Phosphatidylethanolamine-incorporated liposome derivatized with N-α-tosyl-L-lysyl-chloromethyl ketone
$NaIO_4$	Sodium metaperiodate
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
RNA	Ribonucleic acid

SEM	Standard error of the mean
6-CF	6-Carboxyfluorescein

CHAPTER I

INTRODUCTION

Biological cell membranes separate cells or may separate organelles into various compartments, and are composed of varying proportions of carbohydrates, proteins, enzymes, and lipids (phospholipids, sphingolipids and cholesterol). Cell membranes are dynamic and perform diverse functions: communication of signals between cells, maintenance of discrete internal environment for biochemical reactions and provision of a structural base for transport systems. The structural and functional complexity of the biologic cell membranes have not easily lent themselves to experimental manipulation for investigating their roles at the molecular level. This led to the construction of simpler membrane models. The major structural components common to all mammalian biological membranes are phospholipids and these have been used to construct model cell membranes. Over the years three model membranes have been described; lipid monolayers, black lipid membranes and liposomes.

A. Membrane Models

1. Lipid Monolayers

The earliest membrane model was a lipid monolayer

produced by layering amphiphilic lipids on the surface of water where the hydrophilic ends of the lipids remained in contact with water and the hydrophobic tails oriented away from the aqueous environment (1). The monolayer model was used to show that the membrane has no preference in binding lithium (Li^+), sodium (Na^+) and potassium (K^+) ions but has affinity for calcium (Ca^{++}) and magnesium (Mg^{++}) ions suggesting that transport of monovalent cations across the membrane appears to be independent of lipid solubility. The model membrane was also used to elucidate the packing (condensing) characteristics of phospholipids. Phospholipids containing two long, fully saturated fatty acids (e.g. distearoyl, $C_{18:0}$) assumed dense packing conformation while a looser configuration occurred when one or both of the fatty acids was shorter (e.g. lauric acid, $C_{12:0}$) or unsaturated (e.g. oleic acid, $C_{18:1}$). This type of model, although useful in studying interfacial phenomena, is of limited value because unlike natural bilayer biomembrane, water is localized on only one side.

2. Black Lipid Membrane

Mueller and co-workers (2) later described a preparation of the black lipid membrane (BLM). The BLM is bilayer, separating two aqueous compartments and turns black when it attains a stable configuration. It was initially utilized to study transmembrane electrical events (3). BLM, when prepared from purified sphingomye-

lin or lecithin, whether singly or as a mixture, failed to show any electrical activity. However an addition of excitability inducing material (EIM), obtained from Aerobacter cloacae derived from rotted egg white, on one side of the BLM (KCl or NaCl on both sides) caused lowering of the membrane resistance and development of a steady resting potential. Furthermore, upon addition of protamine to the compartment containing EIM, the BLM produced rhythmic electrical spikes in response to an applied impulse, resembling the action potential observed in neurons (3).

3. Liposome

a. General Properties

A third type of membrane model is the liposome which displays properties that resemble natural membrane structures, as were initially described by Bangham et al (4). A liposome is an artificial microscopic vesicle composed of phospholipids. Its formation is dependent upon the capacity of the phospholipids to swell in aqueous solutions to form hydrated liquid crystals. Phospholipids are amphiphilic macromolecules possessing both a polar group and non-polar fatty acid residues, but are heterogenous with respect to fatty acid composition and the polar groups. Various phospholipids of biological membranes show varying degree of fluidity. Phospholipids may change from a crystalline gel (rigid) to liquid crystal (flexible) at a specific temperature, termed the transition temperature. Chapman and Fluck (5) determined that

the transition temperature can be raised from 25°C to 80°C by substituting dioleyl ($C_{18:1}$) phosphatidylethanolamine with distearoyl ($C_{18:0}$) phosphatidylethanolamine. Conversely, the transition temperature of distearoyl lecithin (60°C) can be lowered to below 0°C when the fatty acids are replaced with dimyristoyl ($C_{14:0}$) residues. In the presence of cholesterol, phospholipids are maintained in a liquid crystalline state irrespective of the length and saturation of the fatty acid residues. An explanation for this phenomenon is that cholesterol prevents crystallization of the fatty acids and promotes closer interaction between phospholipids (6, 7).

Phospholipids with smaller polar groups are unable to maintain interpolar group interactions without destabilizing conformation of the fatty acids. Thus such phospholipids are forced into formation of hexagonal rather than bilayer structure (8). Conversely phospholipids with larger polar groups promote interaction of fatty acids with enhanced stabilization of the bilayer structure, thus prevent formation of hexagonal configuration. Phospholipids that are capable of forming hydrated liquid crystals in aqueous solutions (liposomes) include phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cardiolipin and phosphatidic acid. Such lipids as phosphatidylethanolamine, stearylamine or cholesterol, on the other hand, form liposomes only when mixed with one or more of the self-forming lipids mentioned above (9).

b. Preparation

Preparation of liposomes involves dissolving phospholipid with or without other lipid components in an organic solvent (e.g. chloroform), removal of solvent, and mechanically shaking the dried lipid mixture in an aqueous solution which may or may not contain substances to be entrapped. The polar groups of the lipid interact with water molecules and the non-polar fatty acid residues are forced into close proximity by orienting towards the interior of the liposomes, a phenomenon referred to as swelling. The polar groups thus occupy the exterior surface of the liposomes with formation of an internal cavity occupied by aqueous medium.

The extent of swelling and the general configuration of liposomes depend on the nature of phospholipid, ionic strength, valency of ions, as well as pH of the aqueous medium (9-11). Swelling of liposomes is suppressed when the pH of the swelling solution falls below about 3 or in the presence of such divalent cations as Ca^{++}, Mn^{++}, Ba^{++}, Sr^{++}, or Mg^{++} (9, 10). The pH effect is probably due to suppressed ionization of the lipid phosphate groups and the inhibition by the cations due to their binding to phospholipid polar groups. Liposomes formed with phospholipids with no net charge at physiological pH (e.g. phosphatidylcholine) result in a reduced interlamellar spacing. Introduction of a charged phospholipid, e.g. phosphatidic acid or stearylamine, on the

other hand, causes electrostatic repulsion between the intermembrane polar groups, thus increasing the aqueous intermembraneous compartment (9, 11). On the other hand, high ionic strength of the swelling solution promotes reduction in the level of aqueous compartment.

Evidence derived from x-ray diffraction, electron microscopy and optical birefringence studies indicates that the swelled phospholipids (liposomes) are bilayers separated by aqueous spaces (9, 11). Depending on the procedure utilized, liposomes are unilamellar (single bilayer) or multilamellar. The multilamellar liposomes range in size from 50 to 1000 nm in diameter. Ultrasonic vibration of the multilamellar liposomes results in smaller unilamellar liposomes with 25 to 200 nm diameter and a membrane thickness of about 5 to 6 nm (4, 9, 12).

Another method for preparing liposome involves injecting phospholipids dissolved in an organic solution (e.g. ether) into a warm aqueous solution (about 60°C). Evaporation of the solvent then effects formation of large unilamellar liposomes of 1000 nm diameter (13). The capacity of such a liposome to entrap water soluble substances was found to be ten times that of a small unilamellar liposome. However the temperature of the aqueous solution may impose limitations when the entrapped molecules are heat sensitive. This problem can, however, be avoided when the solvent is allowed to evaporate slowly at ambient temperature (14). Entrapment of water-insoluble species is

achieved by dissolving them in the solvent along with the phospholipids. Evaporation of the solvent and subsequent dispersion of the dried lipid in an aqueous medium incorporates the compounds into the membranes.

Separation of liposome entrapped molecules from the unentrapped molecules represents the next step, and is achieved by either centrifugation, gel filtration or dialysis. Centrifugation is useful when asceptic conditions are required. However, some of the liposomes, depending on size, may be lost in the supernate. Gel filtration, while rapid and simple to perform, tends to partially adsorb liposome onto the fractionation gels (12). Alternatively dialysis removes unentrapped small molecular weight substances and avoids dilution of liposomes.

B. Interaction of Liposomes with Cells *In Vitro* and *In Vivo*

Much is known about interaction of liposomes with cells in culture and in whole animals. The manner in which a particular type of liposome interacts with cells depends largely on the composition of liposome, particularly on the state of charge. For instance, positively charged liposomes bind more avidly to monolayers of fibroblasts and HeLa cells than the negatively charged liposomes suggesting that an initial binding to the cells is predominantly electrostatic (15, 16). By use of radiolabeled liposomes, the latter with their contents are taken up by the cells either by fusion, endocytosis, lipid ex-

change or by combination of these mechanisms depending on the charge and/or fluidity of liposomes as well as the cell types (17, 18). In a fluid state, negatively charged liposomes are taken up by Balb/C 3T3 cells via fusion (17), but neutral fluid liposomes or negatively charged solid liposomes are taken up largely by endocytosis. However, uptake of neutral liposomes by Chinese hamster fibroblasts occurs by fusion and to a lesser extent by lipid exchange (18). Irrespective of the mode of liposome uptake by cells, in the majority of cases, the liposomes have shown to be an efficient vehicle for introducing agents to cells *in vitro* and *in vivo* (16-24).

Kinetics of distribution, intracellular localization and the rate of elimination of liposomes from the circulation of laboratory animals are shown to be related to the route of administration, size, lipid composition and surface charge of the liposomes. The intravenous injection of male albino rats or C3H mice with liposomes results in a rapid removal of intact liposomes from the circulation and are mainly taken up by the reticuloendothelial cells, particularly of the liver and spleen. Hence only small amounts are found in other tissues (21-24). The intraperitoneal, subcutaneous or oral route of administering liposomes, by comparison, results in a slower rate of uptake of the liposomes in hepatic and extrahepatic tissues. With respect to liposome size, mice tissues with discontinuous capillaries (e.g. liver or

spleen) are permeable to liposomes irrespective of their size (25). In contrast, tissues with continuous capillaries (e.g. lung) are not readily permeable even to unilamellar liposome with a diameter as small as 30 to 80 nm. In the lung, the unilamellar liposomes reached alveoli by passive transfer inside the migrating blood monocytes or polymorphonuclear cells. The intracellular distribution pattern of liposomes has received limited attention thus such phenomena is not well understood. However, it was shown that 60 minutes following intravenous injection of C3H mice, seventy-five percent of entrapped β-glucoronidase in anionic liposomes (phosphatidic acid incorporated) were localized in the lyosomes of hepatocytes. On the other hand, at 12 to 72 hours post injection, seventy percent of cationic liposomes (stearylamine incorporated) were localized in the cytosol of the C3H mice hepatocytes (26); suggesting that cationic liposomes may be useful for delivering agents to the cytosol of hepatic cells.

In whole animals the size of liposomes affect their rate of elimination from the circulation. For instance, multilamellar liposomes (phosphatidylcholine incorporated) were eliminated much faster from the circulation of male rats (half-life, 10 minutes) than their unilamellar counterpart (half-life, 30 minutes) (27). Kimelberg (28) reported similar findings with respect to liposome size. However the half-life of the unilamellar liposome (phosphatidylcholine incorporated) in a cynomolgous monkey was 14

hours (28), suggesting that the type of host is also important in determining the half-life of liposomes in the circulation. Other experiments showed that in Balb/C 3T3 mouse unilamellar liposomes, dipalmitoyl phosphatidylcholine or sphingomyelin incorporated, have half-lifes of 10 and 17 hours, respectively (29). The longer half-life of the liposomes in circulation may be an indication of minimal liposome uptake by hepatic cells which maximize the chance of such liposomes reaching other tissues.

A major potential limitation in the use of liposomes as a vehicle for delivery of therapeutic agents is the premature release of the entrapped material from the liposomes either spontaneously or following interaction with cellular macromolecules. Human, monkey or rat serum lipoproteins and proteins can promote egression of water soluble molecules from liposomes (30-32). Such problem can be minimized by increasing the liposomal cholesterol level (4, 6, 7). Alternatively, binding the entrapped species to the phospholipid components of the liposome can prevent loss of the entrapped molecule.

Another concern in the use of liposomes as carrier of molecules to cells is, the lipid toxicity. Liposomes containing stearylamine disrupted C3H mice lyosomal organelles (26). Similar results were reported for liposomes prepared with phospholipids from bovine brain (33). In contrast, liposomes prepared from chicken egg phospholipids did not reveal any histologic abnormalities in mice (33) or

in humans (34-36). Thus liposomes prepared with certain lipids (particularly chicken egg phospholipids) are likely to be safe.

C. Applications of Liposomes

1. In Biological Research

Liposomes have been applied to elucidate certain metabolic functions in cells. Liposome encapsulated polyinosinic or polycytosinic acids (37), simian virus 40 deoxyribonucleic acid (38) and retinol (39), unlike the free form, respectively potentiated interferon production in female albino swiss mice, enhanced infectility of Green monkey cells and stabilized the retinol, an unstable molecule in an in vitro environment. The simplicity of liposomes, in contrast to biologic cell membranes, has also been used to investigate certain membrane associated reactions. With respect to gout, monosodium urate lysed liposomes containing cholesterol or testosterone but the monosodium urate did not affect liposomes containing estradiol (40). This supports the theory that gout occurs usually in males and post-menopausal females and a diet rich in cholesterol enhances the disease. The study of complement-mediated lysis of cells has also benefited from liposomes. Liposomes prepared from normal sheep red cell components were lysed in a complement-mediated reaction (41, 42) and suggested that complement induced cell lysis does not involve enzyme(s) which disrupt cell membrane constituents.

2. Carrier of Enzymes

The genetic deficiency of certain specific enzyme leads to intracellular accumulation and storage of undegraded product(s) in the affected tissue(s), known as "storage" disease. Replacement of the deficient enzyme could potentially hydrolyze the accumulated product in host. However the exogeneous enzyme may cause adverse immunological reactions in some hosts. Enzymes can be protected from biodegradation and therefore adverse immunological reactions are minimized when entrapped in semipermeable microcapsules or liposomes. Acatalasemic mice were successfully treated by the use of a cellulose semipermeable microcapsule containing catalase (43). However, the inability of most mammalian species to degrade the cellulose can effect toxicity. On the other hand, liposomes are biodegradable and therefore should not cause long-term accumulation and toxicity.

Enzymes have either been encapsulated or incorporated into liposomes without the loss of their activity (16, 26, 44). The application of an enzyme bearing liposome in patients with storage diseases has produced variable results. For instance, liposomal β-glucosidase reduced the amount of accumulated glucocerebroside in the liver of a patient with Gaucher's disease (35). In another study, liposomes containing amyloglucosidase hydrolysed accumulated glycogen in liver but not in the muscle of a patient with type II glycogenosis known as Pompe's

disease (36). Unlike muscle tissue, liposomes are readily taken up by liver tissue. Thus the failure of the amyloglucosidase bearing liposomes to hydrolyse muscle glycogen is likely due to the low affinity of liposomes for muscle tissue.

3. Carrier of Drugs

Certain pharmacological agents lose potency because they are either unable to penetrate biological membranes or become degraded before reaching their desired sites. Both tissue culture and laboratory animal studies have shown that the use of liposomes can obviate such problems since the drugs entrapped in liposomes are not only protected from enzymatic inactivation, but their contact with serum components is minimized (7, 11, 17, 44). For instance, liposomes encapsulating such anti-tumor agents as adriamycin (19), arabinoside cytosine triphosphate (45), lymphokines (46), muramyl dipeptide (47), illudin S (48), and methotrexate (28, 49), were shown to be superior to free drug in treating various types of tumors both *in vitro* and *in vivo*. In another study, free diethylene-triamine-pentaacetic acid (DTPA), which cannot permeate membranes, was effective in removing plutonium from the liver of rats when it was encapsulated in liposomes (50). A similar result was reported for meglumine antimoniate encapsulated liposome against mice bearing Leishmania parasites (51).

4. Directing Liposomes to Target Organs

Drugs encapsulated in unmodified liposomes have been used to treat diseases of the liver and spleen in experimental animals because the liposomes localize primarily in those tissues (19, 21-26, 50, 51), but the accumulation of liposomes containing cytotoxic drugs can be detrimental to the normal cells in these organs. In order for liposomes to be effective as a drug carrier, they must be directed to diseased cells. Attempts, thus have been made to design more specific liposomes by altering the size, the composition of liposomes, and covalent modification with specific molecules.

With respect to liposome size, livers of rodents bearing Novikoff hepatoma, incorporated smaller liposomes at the expense of larger ones (52). Modification of liposomal composition has also shown some promise for concentrating liposomes in specific cells. Swiss albino rat parenchymal cells showed preference for liposomes having β-galactoside while liver non-parenchymal cells concentrated liposomes with α-mannoside (53). Swiss Webster mice polymorphonuclear leukocytes have affinity for liposomes with 6-amino mannose (54) and liposomes with ganglioside adsorbed Sendai virus and prevented the latter from agglutinizing erythrocytes (55). It should be pointed out that in spite of the promising results, targeting liposomes by modifying liposome size and composition may show varying effects since the experimental conditions, with respect to species, organs or cells tested, are widely

variable.

Liposomes with specific ligands (specifically antibodies) have shown affinity for their respective cells (52, 56, 57). Liposomes bearing anti-Meth A immunoglobulin localized primarily in Meth A subcutaneous tumor in Balb/C 3T3 mice (52). However the liposome bearing antibodies reacted with non-target cells as well. For instance, liposomes bearing antibody against human skin fibroblast reacted primarily with the fibroblasts and to a lesser extent with HeLa cells (56). To reduce such cross reactivity, monoclonal antibody has been used (58, 59). However, after a cell binds with liposome bearing antibody, the cell's surface antigen may disappear, and any further targeting of such liposome will be less effective. In addition, liposome bearing antibody may bind circulating antigens and prevent them from reaching their targets.

Drugs attached to specific antibodies or monoclonal antibody have been used to direct therapeutic agents to their respective cells (60, 61). Erythrocyte sacs (62), cellulose microcapsules (43) and magnetic microspheres (63) have also been used for drug or enzyme delivery. The effectiveness, with respect to drug-antibody conjugate, is limited by the amount of the drug which can be complexed with antibodies without inducing physico-chemical changes in the immunoglobulin linkage. The carrier potential of cellulose microcapsules and erythrocytes is also limited by their inability to be targeted to specific cells in

vivo. Although the magnetic microspheres could be targeted intracellularly, toxicity from the possible accumulation of magnetite (Fe_3O_4) makes such device less attractive.

D. Surface Membrane Characteristics of Normal and Transformed Cancer Cells

Cancer refers to the disease state characterized by an uncontrolled growth of normal cells that have been transformed. The mechanisms of transformation are quite complex and not clearly understood. However, a cancerous state can be induced by chemicals or viruses (see carcinogenesis below). Normal cells, compared to their corresponding cancer cells, show decreased movement and growth (contact inhibition) when they reach saturation density (64, 65). The transformed cells, on the other hand, continue their movement and growth. In has been suggested, however, that both normal and transformed cells show contact inhibition and the continuous growth of transformed cells is due to loss of inhibition of cell division (66, 67). The significance of the differences in the proposed growth response of normal and transformed cells remain uncertain. However, structural alteration of transformed cell membrane is implicated in most cancer cells. Swiss Balb/C 3T3 mouse fibroblasts deposited more glycoaminoglycan than their simian virus 40 transformed derivative (SVT_2), and may mediate cell to cell contact (68). In addition, unlike normal cells, the plasma membrane of

simian virus 40 (SV 40) transformed human fibroblasts lacked SF antigen, a protease sensitive glycosylated glycoprotein (69, 70).

Differences have also been reported with respect to receptors found on the plasma membrane of normal and transformed cells. Chemically (dimethylnitrosamine) or virally (SV 40) transformed hamster or mouse fibroblasts have comparable receptor population on their surfaces, but for the normal cells eighty-five percent of such receptors are cryptic. It was proposed that increase in the number of receptors of the transformed cells is due to removal of certain cell surface components (71). According to Burger (72) trypsin-like activity is present on transformed cell surface and suggested that receptors of a parent cell line are in cryptic form, but become exposed when it is transformed. Häyry and Defendi (73) reported similar observation. Studies by Schnebli and Burger (74) using protease inhibitors (halomethyl ketones, e.g. 1-chloro-3-toslyamido-7-amino-2-heptanone commonly known as tosyl-L-lysyl-chloromethyl ketone, TLCK) support the protease concept. At non-toxic doses, these inhibitors inhibited the growth of various cell lines transformed by either simian virus 40 or polyoma virus without affecting the growth of normal cells. In addition, TLCK reacted with transformed cell surface molecules with trypsin-like activity. Other studies have shown that TLCK reduced adhesion of Ehrlich ascites tumor cells to plastic surfaces (75), delayed

appearance of 7, 12-dimethylbenzanthracene induced mouse skin tumor (76), and at high doses induced contact inhibition of cells bearing rhabdomyosarcoma (77).

E. Carcinogenesis

Certain chemicals and viruses induce cancer. Although the mechanism for transforming a normal cell to a cancerous state is not well understood, it is known that chemicals and viruses induce cancer differently. Chemicals which are carcinogenic cause random genetic changes in cells, whereas viral carcinogenesis involves the addition of specific viral genome(s) to cells.

1. Chemical Carcinogenesis

Carcinogenesis is inducible in experimental animals by exposure to certain chemicals including 7, 12-dimethylbenzanthracene in combination with croton oil of which the active agent is 12-o-tetradecanoyl-phorbol-13-acetate (phorbol ester) (78, 79). It is thought that once phorbol ester enters cells previously exposed to initiator carcinogens, it becomes incorporated into the cellular membranes thereby inducing several biochemical changes and promotes tumor. However, such chemical carcinogens as aflatoxin B_1 (80) and benzopyrene (81) can also induce tumor in the absence of promoters.

Several chemical carcinogens are metabolised by the liver microsomal mixed function oxidases (MFO) and similar oxidases in other tissues to active intermediates, most of which are positively charged though some intermediates are

sulfated or epoxides (82-84). These intermediates are subsequently converted to chemically stable metabolites that are readily excreted into urine and bile or exhaled. Such detoxification reactions are mediated by MFO inducers, e.g. NADPH, vitamin A, selenium and cruxiferous vegetables. In addition adduct formation between the carcinogenic intermediate and conjugating substances, e.g. glutathione, glucoronic acid or by the scavenging effect of antioxidants, e.g. vitamin E and butylated hydroxytoluene are capable of detoxification (85-88). Thus the carcinogenicity of a chemical agent depends on the rate of formation and detoxification of carcinogenic intermediates.

2. Viral Carcinogenesis

Viral agents can, as with chemicals, induce carcinogenic transformation in vitro and in vivo. There are evidence that genetic alteration of normal cells by viral genome(s) (effectors) induce tumor formation, e.g. large T antigen of simian virus 40 (89, 90). Briefly, viral effector(s) stimulate DNA synthesis in the host cell as a result of the failure of the host cell to control or deactivate the persisting effector(s). For instance, the large T antigen associates with a non-glycosylated polypeptide (55 K phosphoprotein) to form large T antigen-55 K phosphoprotein complex. Continuous expression of the complex phosphorylates tyrosine residues of specific proteins that in turn induce tumor formation (89, 90).

Certain viruses have been associated with human

cancer. Herpes simplex virus type 2 is implicated in human cervical carcinoma (91); Epstein-Barr virus with Burkitt's lymphoma (92); and Shope papilloma with genital tumor (93). However these viruses are thought to be mutagenic (initiators) and require cocarcinogen(s) (chemicals, radiation, etc.) to fully express tumor activity. It is suggested that the role of the cocarcinogens is to depress host cell immune response (94).

3. Cancer and Immunity

There is evidence that tumor cells contain cell surface molecules that are recognized as being foreign to the host, and raise the possibility of treating cancer immunologically. For instance, active immunotheraphy with Bacillus Calmette-Guerin (BCG), a product of Tubercle bacillus, enhanced the rejection of various types of tumors in vivo, particularly when the size of the tumor is small (95). The limitation of BCG immunotheraphy with respect to tumor size is not clearly understood. It is thought, however, that BCG increases lymphocyte or macrophage cytotoxicity against tumor cells and induces reticuloendothelial cells to remove tumor cell-antibody complexes from the host. Other substances known to activate macrophages have shown similar results (46, 47).

Immunization against selected types of tumor is also possible. When C3H mice were injected with small doses of tumor cells (derived from methylcholanthrene bearing C3H mouse) and reinoculated with the same type of tumor cells,

none of the mice developed tumors (96). Similar results were obtained for mice immunized with adenovirus 3 and 14 (97). Immunization against specific type of tumor is attractive but since cancer consists of multiple diseases, non-specific immunization may be prohibitive with respect to the number of tumors, even if practical. However, since stimulation of the host immune response can protect against a variety of tumors, avoiding the use of substances that reduce the effectiveness of the immune defense system may lower the incidence of cancer in the host.

F. Objectives

The objectives of this research are as follows: To covalently couple TLCK to liposomes and demonstrate the preferential uptake of the liposome bearing TLCK by simian virus 40 transformed Swiss mouse fibroblasts (SVT_2) at the expense of the unmodified liposomes and normal cells (Balb/C 3T3) in culture. In addition experiments will be performed to determine if the liposome bearing TLCK, 1-β-, arabino-furanosyl cytosine (ara-C) or colchicine encapsu+ lated, will selectively inhibit the growth of SVT_2 cells without cytotoxicity to Balb/C 3T3 cells in culture.

The TLCK, when compared with antibodies or monoclonal antibodies, is more attractive as a homing molecule for targeting liposomes to tumor cells since the TLCK is known to have affinity for a variety of tumor cells (74-77). In addition, antibodies and monoclonal antibodies raised against each type of tumor is both time consuming and impractical.

CHAPTER II

EXPERIMENTAL

A. Materials

L-α-phosphatidylcholine dipalmitoyl (synthetic), L-α-phosphatidylethanolamine (egg yolk), Cholesterol, Dicetylphosphate, Digoxin, Cerebroside, Sodium metaperiodate, N-α-tosyl-L-lysylchloromethyl ketone (TLCK), α-N-benzoyl-DL-arginine-p-nitroanilide-HCl, Crystalline trypsin (bovine pancreas type I), Triton X-100, Horseradish peroxidase type VI, Glutaraldehyde (25%), Colchicine, Sodium chloride, Sodium phosphate (mono and dibasic), Picryl sulfonic acid (2,4,6-Trinitrobenzene sulfonic acid), Trizma Base Tris(hydroxymethyl-aminomethane), Sodium borohydride, Calcium chloride, 4-Amino antipyrine, Phenol and Ethanolamine were purchased from Sigma Chemical Company, St. Louis, MO, 63178.

Hydrogen peroxide (30%), Scintillation solution (Scintiverse), Scintigest were purchased from Fisher Scientific Company, Fair Lawn, NJ, 07410.

(4 - ^{14}C) Cholesterol was purchased from Research Products International Corporation, Mount Prospect, IL, 60056.

Sephadex G-200 superfine and chromatographic columns were purchased from Pharmacia Fine Chemicals, Piscata-

way, NJ, 08854.

Fluorescamine (fluram) was purchased from Roche Diagnostics, Nutley, NJ, 07110.

6-Carboxyfluorescein and Sodium borate were purchased from Kodak Company, Rochester, NY, 14650.

Tissue Culture Plates and Flasks were purchased from Lux Scientific Company, Newbury Park, CA, 91320.

Cell Culture Medium was purchased from GIBCO Laboratories, Grand Island, NY, 14072.

Calf Serum was purchased from Kansas City Biological Inc., Lenexa, KS, 66215.

Chloroform and Methanol were purchased from J.T. Baker Chemical Company, Phillipsburg, NJ, 08865.

Cytosine Arabinoside (Cytosar-U) was purchased from Upjohn Company, Kalamazoo, MI, 49001.

B. Equipment

Aminco-Bowman Spectrofluorometer, Model J48960 was purchased from American Instrument Company, Silver Springs, MD, 20910.

Sonicator Cell Disruptor, Model W-375 was purchased from Heat Systems-Ultrasonic, Inc., Plainview, NY, 11803.

Micro Fractionator was purchased from Gibson Medical Electronics, Middleton, WI, 53562.

Spectronic 21 UVD Spectrophotometer was purchased from Bausch and Lomb, Rochester, NY, 14625.

Sorvall RC-5B Refrigerated Superspeed Centrifuge was purchased from Dupont Instruments, Newton, CT, 06470.

Astra-8 was purchased from Beckman Instruments, Inc., Brea, CA, 92621.

Beckman LS 7500 Liquid Scintillation System was purchased from Beckman Instruments Inc., Fullerton, CA, 92634.

Cary 1115 was purchased from Cary Instruments, Monrovia, CA, 91016.

Conical Flask was purchased from SGA Scientific Inc., Bloomfield, NJ, 07003.

Dialysis Tubing was purchased from Arthur H. Thomas Company, Philadelphia, PA, 19105.

C. Reagents

1. Sodium phosphate (50 mM) supplemented with 145 mM sodium chloride (PBS), pH 7.2 was prepared by dissolving 3.00 g monosodium phosphate, 3.55 g disodium phosphate and 8.47 g sodium chloride in 1000 mL deionized water.

2. Sodium phosphate (50 mM), pH 6.0 was prepared by dissolving 4.25 g monosodium phosphate and 2.73 g disodium phosphate in 1000 mL deionized water.

3. Sodium borate (0.1 M), pH 8.9 was prepared by dissolving 20.2 g disodium borate in 1000 mL deionized water.

4. Substrate for Horseradish Peroxidase (HRP)

(i) 4-amino-antipyrine (0.4 mM) was prepared by dissolving 0.08 g 4-amino-antipyrine in 1000 mL of 50 mM sodium phosphate buffer, pH 6.0.

(ii) Phenol (5.3 mM) was prepared by dissolving 0.50 g Phenol in 1000 Ml of 50 mM sodium phosphate buffer,

pH 6.0

(iii) Solution for HRP analysis was freshly prepared by mixing 25 mL 4-amino-antipyrine, 25 mL phenol and 0.05 mL of 3% hydrogen peroxide.

5. Substrate for Trypsin

(i) Trizma Base Tris (hydroxymethyl-amino-methane), 50 mM supplemented with 20 mM calcium chloride, pH 8.1 was prepared by dissolving 6.06 g trizma and 2.2 g calcium chloride in 1000 mL deionized water.

(ii) Solution for trypsin analysis was freshly prepared by dissolving 0.0218 g α-N-benzoyl-DL-arginine-p-nitroanilide-HCl in 0.5 mL dimethyl sulfoxide. The resultant solution was diluted to 50 mL with the 50 mM trizma-20 mM calcium chloride buffer, pH 8.1.

D. Preparation of Liposomes

Two types of liposomes were prepared. The liposome incorporate digoxin or phosphatidylethanolamine and were designated as Lp-D and Lp-PE respectively. The Lp-D and Lp-PE were treated with sodium metaperiodate and glutaraldehyde, respectively to generate aldehyde groups on the surface of the liposomes and were subsequently coupled with TLCK.

1. Preparation of Lp-D

Liposome incorporating digoxin (Lp-D) was prepared in the following manner. In a conical flask, 29.4 mg L-α-phosphatidylcholine dipalmitoyl, 15.5 mg cholesterol, 7.0 mg digoxin and 3.1 mg dicetylphosphate were dissolved

in 20 mL chloroform-methanol (6:1 v/v) mixture. The solvent was removed with a rotary evaporator at 37°C and further dried under a stream of nitrogen. A 2.1 mL aliquot of PBS with glass beads was added to the dried lipid film and agitated on a vortex for 3 to 5 minutes to form a suspension of liposomes (45.1 mM total lipids). The suspension was kept at room temperature for 2 hours, followed by sonication for 30 minutes in an ice bath using the sonicator cell disruptor equipped with a micro probe. The sonicated suspension was kept at room temperature for 60 minutes to form largely unilamellar liposomes referred to as type A. To obtain more uniform population of liposomes referred to as type B, the type A suspension was centrifuged at 160 x g for 15 minutes, using the Sorvall Centrifuge equipped with an SE-12 rotor, and the supernatant recovered.

2. Preparation of Lp-PE

Liposome incorporating phosphatidylethanolamine (Lp-PE) was prepared as described for the Lp-D except that 29.4 mg L-α-phosphatidylcholine dipalmitoyl, 10.0 mg L-α-phosphatidylethanolamine, 4.0 mg cholesterol and 3.1 mg dicetylphosphate were dissolved in the chloroform-methanol solvent and the suspension of liposomes (33.6 mM total lipids) was sonicated for 15 minutes in an ice bath. The liposomes without L-α-phosphatidylethanolamine were prepared in a similar manner.

E. Entrapment of Drugs and Markers

1. In Lp-D

To entrap drugs (ara-C or colchicine) or the marker 6-carboxyfluorescein (6-CF), the dried lipid film was suspended in 2.1 mL PBS containing either 208.3 µmoles ara-C, 175.0 µmoles Colchicine or 256.0 µmoles 6-CF. The suspension was vibrated and sonicated as described earlier. This was followed by dialysis against isotonic PBS for 4 hours at room temperature with two changes to remove unentrapped ara-C, colchicine or 6-CF.

2. In Lp-PE

To entrap ara-C or the markers (glucose or HRP), the dried lipid film was suspended in 2.1 mL PBS containing either 208.3 µmoles ara-C, 221.0 µmoles glucose or 4.15 mg HRP. The suspension was vibrated and sonicated as described earlier. Unentrapped ara-C or glucose was removed by dialysis against isotonic PBS and unentrapped HRP was partially removed by recentrifugation after washing the liposomes with isotonic PBS.

F. Coupling Liposome with TLCK

1. Covalent Coupling of TLCK with Lp-D

The oligosaccharide side chain of digoxin was oxidized by reacting 1.0 mL Lp-D, type A (4.2 µmoles digoxin) or type B (2.0 µmoles digoxin) with 16 or 8 µmoles sodium metaperiodate ($NaIO_4$), respectively, for 2 hours in the dark at room temperature. A 0.1 mL glucose solution (6 to 12 µmoles) was added to the reaction mixture

for 60 minutes to quench the excess periodate and it was dialyzed against isotonic PBS for 4 hours. The oxidized Lp-D type A was reacted with 11.4 µmoles TLCK for 24 hours at room temperature. Residual aldehyde groups, if any, were quenched by reacting it with 5.0 µmoles ethanolamine for 60 minutes. A similar reaction was performed for oxidized Lp-D type B with 6.8 µmoles TLCK. The extent of non-covalent binding of TLCK to Lp-D type B was determined by incubating the TLCK with unoxidized liposome.

The TLCK coupled liposome (Lp-D-TLCK) was separated from unreacted (free) TLCK by Sephadex G-200 chromatography (0.9 x 15 cm, flow rate: 0.1 mL per minute) and the concentration of liposomal digoxin was determined as follows:

A volume of 0.010 mL each of Lp-D, Lp-D-TLCK type A and 0.050 mL each of Lp-D, Lp-D-TLCK type B were first dissolved in 1.0 mL methanol. A 0.010 mL of the suspension was brought to 1.0 mL with albumin diluent and the digoxin content measured by a radioimmunoassay method, using a commercial kit (Micromedic Systems, Horsham, PA, 19044).

2. Covalent Coupling of TLCK with Lp-PE

L-α-phosphatidylethanolamine (13.8 µmoles) was reacted with 0.020 mL of 25% glutaraldehyde (50.0 µmoles) for 7 minutes at room temperature. Unreacted glutaraldehyde was removed by a 12-hour dialysis at 4°C against isotonic PBS. A 1.0 mL of the glutaraldehyde treated liposome (Lp-G, 9.6 µmoles) was incubated with 10.1 µmoles

TLCK for 25 minutes at room temperature. Residual aldehyde groups were quenched with 5.0 µmoles ethanolamine. In a similar manner, lysine (10.3 µmoles) was coupled with Lp-G instead of TLCK. The extent of non-covalent binding of the TLCK with Lp-PE was determined by incubating the TLCK with unoxidized liposome.

The TLCK coupled liposome (Lp-G-TLCK) or the lysine coupled liposome (Lp-G-Lysine) was separated from the unreacted TLCK by Sephadex G-200 chromatography. The concentration of liposomal L-α-phosphatidylethanolamine was determined either by spectrophotometric or by fluorometric analysis as follows:

A volume of 0.2 mL each of Lp-PE or Lp-G was incubated with 3.0 mL picryl sulfonic acid (0.030 g per 100 mL of 0.1 M disodium borate, pH 8.9) for 90 minutes at 37°C. The absorbance of the reaction mixture was measured at 410 nm (98). With respect to the fluorometric assay, 0.1 mL solution of Lp-PE and Lp-G were incubated with 0.5 mL fluorescamine (20 mg per 100 mL acetone) at room temperature for 30 minutes and the fluorescence was measured at the excitation wavelength of 390 nm and the emission wavelength of 482 nm (99). In order to investigate the effect of reducing the Schiff's base (imine bond, C = N) formed from reacting liposomal aldehyde groups and the free amino group of TLCK, on the activity of TLCK-bound liposome, 0.02 mL cold sodium borohydride (11.0 nmoles) was added to 1.0 mL cold TLCK-bound liposome and incubated at 4°C for 5 minutes.

The reaction mixture was dialyzed in isotonic PBS at 4°C for 2 hours to remove unreacted sodium borohydride.

3. Spectral Analysis of TLCK-bound Liposome

In 5.95 mL methanol, 0.05 mL each of TLCK, Lp-G-TLCK, Lp-G and Lp-PE were dissolved. The suspensions containing the TLCK and Lp-G-TLCK were analyzed by using the Cary 1115 spectrophotometer for their absorbance at 229 to 232 nm and the disappearance of absorbance for Lp-G and Lp-PE. Methanol containing 0.05 mL PBS served as blank.

4. Quantitation of Coupled TLCK

The relative amount of TLCK coupled with liposomes was determined indirectly by measuring the unreacted TLCK. A 0.1 mL aliquot of eluant (containing unreacted TLCK) was incubated with 1.5 mL fluorescamine (3.0 mg) for 20 minutes at room temperature. The fluorescence of the reaction mixture was measured at the excitation and emission wavelengths of 390 and 482 nm respectively. The amount of TLCK that bound ($TLCK_B$) to the liposomes was calculated as the difference between the amount of TLCK added ($TLCK_A$) and the amount of the unreacted TLCK ($TLCK_U$).

$$TLCK_B = TLCK_A - TLCK_U$$

The percent TLCK coupled to the liposomes was expressed as:

$$\frac{TLCK_B}{TLCK_A} \times 100$$

5. Activity of TLCK

The activity of TLCK and TLCK-bound liposomes was

assessed by its inhibition of trypsin. First, 0.2 mL trypsin solution (12.9 nmoles) was reacted with 0.2 mL of TLCK, TLCK-bound liposomes, TLCK-free liposomes and PBS (served as control) for 2 hours at room temperature. Second, the trypsin activity of the reaction mixtures was determined by a modification of the method of Erlanger et al (100). To 0.05 mL of the suspension 2.95 mL of trypsin substrate (see reagents) was added. After 30 minutes, termination of the reaction was achieved by adding 0.5 mL of 33% acetic acid and the absorbance measured at 410 nm. An increase in the TLCK activity against trypsin (inhibition of trypsin) was related to lower absorbance reading (decreased hydrolysis of α-N-benzoyl-DL-arginine-p-nitroanilide-hydrochloride by trypsin) when compared to that of the uninhibited trypsin. The percent inhibition of trypsin was calculated as:

$$100 - \frac{(\text{Absorbance of Trypsin-TLCK Mixture})}{(\text{Absorbance of Uninhibited Trypsin})} \times 100$$

and the percent relative activity of the TLCK was expressed as:

$$\frac{(\%\ \text{Inhibition of Bound TLCK})}{(\%\ \text{Inhibition of Free TLCK})} \times \frac{(\text{Amount of Free TLCK})}{(\text{Amount of Bound TLCK})} \times 100$$

G. Liposome Entrapment

Entrapped molecules in the aqueous volume of liposome can be released and quantitated after rupturing the liposome with the detergent, Triton X-100 at 37°C. Another technique for releasing entrapped molecules, involves dissolving the liposome in an organic solvent, e.g. chloroform,

and followed by the extraction of the released molecules with the appropriate solvent.

1. Triton X-100 Treatment

To 0.4 mL liposome preparation 0.020 mL Triton X-100 (respective final concentration of Triton X-100 were 5%, 1% and 0.05%) was added and each mixture was incubated for 45 minutes at 37°C to facilitate the disruption of the liposome. The aqueous phase was then separated from the lipids of the disrupted liposomes by centrifugation at 25,000 x g for 3 hours in a Sorvall centrifuge equipped with an SE-12 rotor. The aqueous phase was analyzed for the released molecule (ara-C, colchicine, 6-CF, glucose and HRP) as follows:

To 0.1 mL of the aqueous phase containing ara-C or colchicine 1.9 mL PBS was added, vortexed and the absorbance measured at 272 and 245 nm, respectively. With respect to the markers, 6-CF was measured at excitation and emission wavelengths of 490 and 520 nm respectively; glucose was measured by the glucose oxidase method (101) using the Astra-8. Horseradish peroxidase (HRP) was measured by a modification of the method of Trinder (101). To 0.1 mL of the aqueous phase containing HRP 3.0 mL HRP substrate (see reagents) was added and vortexed. The reaction mixture was incubated for 30 minutes at room temperature and the absorbance measured at 500 nm. The standard preparations of ara-C, colchicine, 6-CF, glucose and HRP were prepared in a similar manner and served as control. The

amount of molecule entrapped by the liposome was calculated as the difference between the amount of molecule present after Triton X-100 (post-Triton X-100) and before Triton X-100 (pre-Triton X-100) treatment and expressed as:

Amount of Molecule Entrapped = (Post-Triton X-100) - (Pre-Triton X-100). The percent of molecule entrapped in liposome was calculated as:

$$\frac{\text{Amount of Molecule Entrapped in X mL of Liposome}}{\text{Amount of Molecule Added}} \times 100$$

2. Chloroform Extraction

In 1.0 mL chloroform, 0.1 mL liposomal preparation was dissolved. Then, 1.9 mL PBS was added, vortexed and the aqueous phase was allowed to separate from the organic phase. The aqueous phase was analyzed for the released molecule, e.g. ara-C. Standard preparations of the ara-C were treated with chloroform-PBS and served as control.

H. Miscellaneous Procedures

1. Studies of Liposome Entrapment as a Function of the Concentration of Entrapped Molecule or Lipid

The extent of entrapment of molecules within the aqueous volume of liposome may vary with the concentrations of the entrapped molecule or liposomal lipid. Thus the effect of varying concentration of ara-C or lipid on the efficiency of liposome entrapment was investigated as follows:

a. Entrapment as a Function of ara-C Concentration

Lp-D (type B) was prepared as described earlier, except that the concentration of ara-C in the swelling buffer was varied from 49.6 mM to 198.4 mM with liposomal lipid remaining constant (45.1mM). The rate at which ara-C is entrapped was measured at 272 nm following its release from the liposomes with either Triton X-100 (0.05% final concentration) or chloroform.

b. Entrapment as a Function of Lipid Concentration

Lp-D (type B) was prepared as described earlier except that the liposomal lipid concentration was 45.1 mM and 113.2 mM, while the concentration of ara-C in the swelling buffer remained constant (99.2 mM). The rate at which ara-C is entrapped was measured at 272 nm.

2. Studies of ara-C, Colchicine and Horseradish Peroxidase (HRP) Stability

The effect of pH and temperature on the stability of ara-C and colchicine were investigated as follows:

Colchicine (1.2, 6.0, 12.0 µm) and ara-C (12.5, 37.4, 74.8 µm) were dissolved in PBS (pH 5.2, 7.2 or 9.2) and incubated at 37°C for varying periods of up to 48 hours. Drug solutions kept at room temperature served as controls. At the end of each incubation period, the deterioration of ara-C or colchicine, if any, was monitored by the disappearance of absorbance peak at 272 and 245 nm, respectively.

The effect of sonication, temperature and Triton X-100 on the activity of HRP was also investigated. Vary-

ing amounts of HRP (1.4, 0.72 and 0.36 µg) in PBS, pH 7.2 were sonicated for 3 minutes in an ice bath followed by incubation at 37°C for 45 minutes in the presence of Triton X-100 (0.05% final concentration). The HRP activity was determined as described earlier.

I. Cell Culture

Swiss mouse fibroblasts, clone Balb/C 3T3 (untransformed) and its simian virus 40 transformed derivative, SVT_2 cells were grown in minimum essential growth medium supplemented with 10% calf serum, 250 units per mL penicillin and 0.25 mg streptomycin (MEM x 4) in plastic flasks at 37°C in a humidified chamber equilibrated with 5% CO_2 and 95% air. These cell lines were kindly provided by Dr. L.A. Culp of Case Western Reserve University Medical School, Department of Microbiology, Cleveland, OH 44106. The cells were routinely passaged once a week with trypsin solution (2.5 mg per mL) at 37°C and were checked for possible mycoplasma contamination (102). In order to remove the trypsin, the trypsinized cells were suspended in MEM x 4, centrifuged at 1,000 rpm for 5 minutes and resuspended in fresh MEM x 4. The cells were used for uptake and growth experiments between their 15th and 25th passages.

1. Preparation of Liposome-Cell Culture Media

Lp-D and Lp-D-TLCK containing either ara-C, colchicine or 6-CF; Lp-PE and Lp-G-TLCK containing either ara-C or HRP were diluted with the MEM x 4 to form liposome-

MEM x 4 suspension and was used for liposome uptake and cell growth studies. The amount of liposomes, drugs and markers exposed with the cells are indicated in the legends of the tables. Control cells were grown in MEM x 4 with or without ara-C, colchicine, 6-CF or HRP.

2. Studies of Cell Uptake of Liposomes

Balb/C 3T3 and SVT_2 cells were separately inoculated in 100 x 15 mm plastic petri dishes and incubated in 6.0 mL of MEM x 4 for 24 hours at 37°C in a humidified chamber, 5% CO_2 and 95% air. The MEM x 4 was aspirated and the cells were washed twice with PBS. Some of the cells were detached by incubating in trypsin solution and counted on a hemocytometer which indicated Balb/C 3T3 and SVT_2 cells of an average density of 5.2×10^6 and 9.7×10^6, respectively, per each cell culture dish. The rest of the cells were incubated in 4.0 mL of 6-CF entrapped in Lp-D and Lp-D-TLCK-MEM x 4 or 6-CF-MEM x 4 for varying periods of up to 4 hours under cell culture conditions. Balb/C 3T3 (5.8×10^6) and SVT_2 (7.1×10^6) cells were incubated in a similar manner in the presence of HRP-MEM x 4 or HRP entrapped Lp-PE/Lp-G-TLCK-MEM x 4 for varying periods of up to 90 minutes. Control cells were incubated in MEM x 4.

At regular intervals the cells were washed five times with PBS and dispersed with trypsin. The dispersed cells were centrifuged at 1,000 rpm for 5 minutes at 4°C and the cell precipitate was washed once with PBS and re-centrifuged. The cells were resuspended in 1.0 mL PBS

containing Triton X-100 (0.05% final concentration) and sonicated for 3 minutes in an ice bath using an ultrasonic cell disruptor equipped with a micro probe. The suspension was then incubated for 45 minutes at 37°C to facilitate the release of 6-CF or HRP from the liposomes. Fragments of the disrupted cells were packed by centrifugation at 5,000 rpm for 10 minutes. To measure the extent of liposome uptake by the cells, the supernatant was collected and 6-CF or HRP levels were determined as described earlier. Cells treated in a similar manner but incubated in MEM x 4 served as controls. The amount of 6-CF or HRP (X), taken up by 1×10^6 cells, was calculated as:

$$X = \frac{\text{6-CF or HRP Uptake by Total Number of Cells}}{\text{Total Number of Cells}} \times 10^6$$

The percent of 6-CF or HRP (Z), taken up by 1×10^6 cells was expressed as:

$$Z = \frac{X}{\text{6-CF or HRP Initially Added to Cells}} \times 10^6$$

3. Studies of Effect of Liposomes on Cell Growth

Balb/C 3T3 and SVT_2 cells were seeded separately in 60 x 15 mm plastic petri dishes. After 24 hours of incubation at 37°C in a humidified chamber containing 5% CO_2 and 95% air, the cells were washed with PBS and the cell density was determined. The cells were then incubated in 2.0 mL of ara-C entrapped liposome-MEM x 4 or colchicine entrapped liposome-MEM x 4 for varying periods up to 60 minutes under cell culture conditions. Cells incubated in MEM x 4 with or without ara-C, colchicine, TLCK and empty liposomes served as controls.

At regular intervals the cells were washed once with PBS and incubated further in 4.0 mL MEM x 4 for up to two or three days. At the end of every 24-hour period the cells which remained attached to the substratum (petri dish) were washed twice with PBS, dispersed with trypsin solution and counted on a hemocytometer. The number of cells, N that remained attached to the substratum was calculated as:

$$N = \text{Number of cells counted} \times 10^4 \times \text{Volume of dispersed cells}$$

The percent of cells that remained attached to the substratum after exposure to liposomes, ara-C, colchicine and TLCK was expressed as:

$$\% \text{ Cells Attached} = \frac{\text{Number of Attached Cells (Exposed)}}{\text{Number of Attached Cells at Day 0 (Unexposed)}} \times 100$$

Unexposed cells refer to cells incubated in MEM x 4 and was taken as 100% cells attached.

CHAPTER III

RESULTS

A. Assessment of Procedures for Determining Liposome Components

The liposome was prepared with lipid mixture containing phosphatidylcholine dipalmitoyl, cholesterol, dicetylphosphate and phosphatidylethanolamine or digoxin. The liposome has a net negative charge at pH 7.2 owing to the fact that the dicetylphosphate group is negatively charged. Phosphatidylcholine dipalmitoyl and cholesterol contributes to the stability of the liposomes. The oligosaccharide moiety of digoxin and the amino group of phosphatidylethanolamine served as attachment site for TLCK. The individual components of the liposomes were quantitated by use of the respective standards as detailed in the Methods. The standard curves for these components are shown in Appendices A, B and C. As shown in Appendix D, the pH (5.2 to 9.2), the temperature (37°C), sonication (3 minutes) and 0.05% Triton X-100 did not have measurable effects on HRP activity, ara-C or colchicine.

B. Covalent Coupling of Liposomes with TLCK

Covalent coupling of liposomes with TLCK consists of two steps. The first was generating aldehyde groups on the

surface of liposomes. In the second step, liposomal aldehyde groups were coupled with the free amino group of TLCK as illustrated in Appendix E.

1. Sodium Metaperiodate Oxidation of Digoxin Incorporated Liposome (Lp-D)

The Lp-D (type A) or Lp-D (type B) was treated with 4-fold excess sodium metaperiodate for 2 hours at room temperature (in the dark) followed by incubation with 3 to 4-fold excess of TLCK for 24 hours. The concentration of the sodium metaperiodate was sufficiently low to effect structural impairment of liposomes. The TLCK-bound liposome was separated from free TLCK on Sephadex G-200 chromatograph. As shown in Table I and Figure 1, the TLCK-bound liposome eluted in the void volume and was separated from free TLCK.

2. Glutaraldehyde Derivatization of Phosphatidylethanolamine Incorporated Liposome (Lp-PE)

The Lp-PE was treated with about 5-fold excess of glutaraldehyde for 7 minutes at room temperature followed by incubation with 1 to 2-fold excess of TLCK for 25 minutes. The TLCK-bound liposome was separated from free TLCK on a Sephadex G-200 column. As shown in Table II and Figure 2, the TLCK-bound liposome eluted in the void volume and the free TLCK eluted in subsequent column fractions.

In order to determine the rate of derivatization of the amino groups on Lp-PE or Lp-G with glutaraldehyde the amino groups were labelled with picryl sulfonic acid or

TABLE I

REMOVAL OF FREE TLCK FROM Lp-D-TLCK ON SEPHADEX G-200[a]

Fraction[b] Number	TYPE A			TYPE B		
	Relative Intensity of Fluor-escence	TLCK nmoles	Absorb-ance (410nm)	Relative Intensity of Fluor-escence	TLCK nmoles	Absorb-ance (410nm)
1	-	-	-	-	-	-
2	-	-	0.02	-	-	0.01
3	-	-	0.26	-	-	0.16
4	-	-	0.08	-	-	0.06
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	14	140	-	42	410	-
8	54	530	-	190	1870	-
9	24	230	-	8	80	-
10	2	20	-	4	40	-
11	-	-	-	-	-	-

[a]Following the reaction between TLCK and sodium metaperiodate oxidized Lp-D (types A and B) a 0.5 mL of the mixture was chromatographed on Sephadex G-200 (0.9 x 15 cm, flow rate: 0.10 mL per minute) to separate unreacted TLCK from TLCK-bound liposome. The TLCK-bound liposome eluted in the void volume and the absorbance was measured at 410 nm. The unreacted TLCK was derivatized with fluorescamine and the relative intensity of fluorescence was determined at excitation, 390 nm and emission, 482 nm.

[b]Each fraction number contains 0.5 mL eluant.

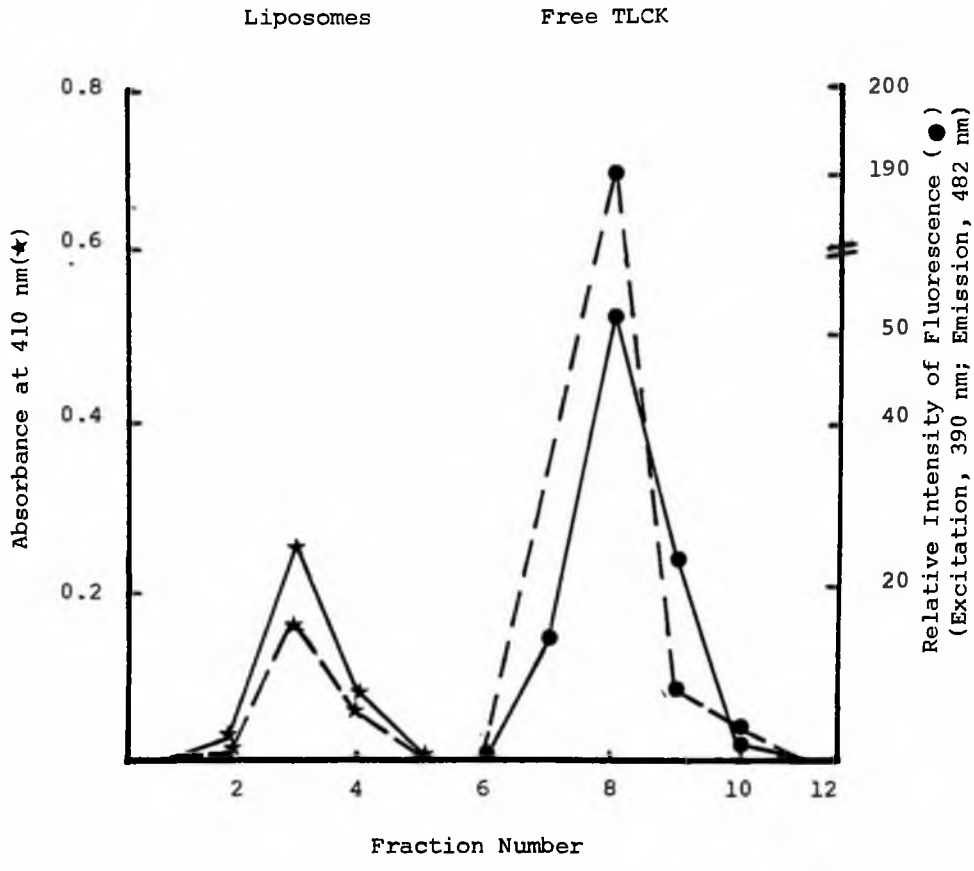

Figure 1. Sephadex G-200 chromatography of the mixture following eaction of sodium metaperiodate treated Lp-D type A (—) and Lp-D type B (- - -) with TLCK. The experimental conditions are as described in the legend for Table I.

TABLE II

REMOVAL OF FREE TLCK FROM Lp-G-TLCK ON SEPHADEX G-200[a]

Fraction Number[b]	Relative Intensity of Fluor-escence	TLCK nmoles	Absorb-ance (410 nm)
1	-	-	-
2	-	-	0.02
3	-	-	0.45
4	-	-	0.10
5	-	-	0.01
6	-	-	-
7	-	-	-
8	20	200	-
9	120	180	-
10	210	2080	-
11	38	380	-
12	4	40	-
13	-	-	-

[a] Following the reaction between TLCK and glutaraldehyde treated Lp-PE (Lp-G), a 0.5 mL of the mixture was chromatographed on Sephadex G-200 (0.9 x 15 cm, flow rate: 0.10 mL per minute) to separate unreacted TLCK from liposome-bound TLCK. The latter eluted in the void volume and the absorbance was measured at 410 nm. The unreacted TLCK was derivatized with fluorescamine and the relative intensity of fluor-escence was determined at excitation, 390 nm and emission, 482 nm.

[b] Each fraction number contains 0.5 mL eluant.

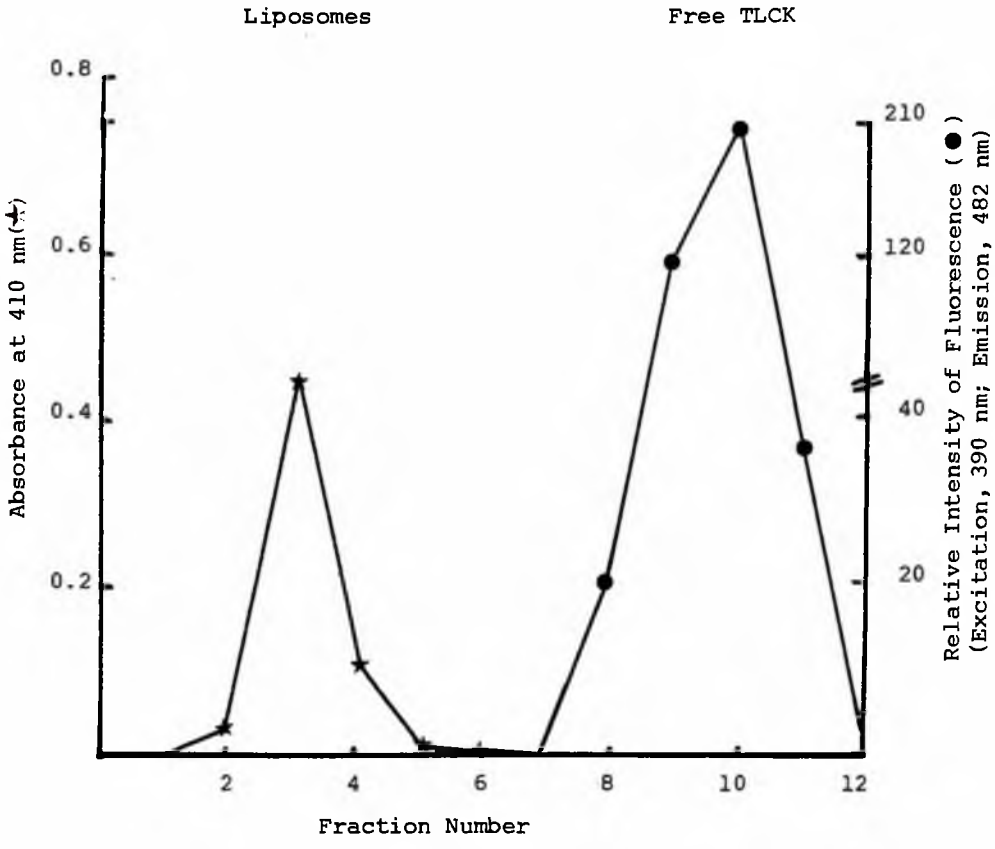

Figure 2. Sephadex G-200 chromatography of the mixture following reaction of glutaraldehyde-treated Lp-PE with TLCK. The experimental conditions are as described in the legend for Table II.

fluorescamine. Liposomes are impermeable to picryl sulfonic acid thus the latter reacts only with the amino groups located on the surface of liposomes. Fluorescamine on the other hand is capable of labelling all available amino groups since its solvent (acetone) renders liposomes permeable to fluorescamine. The picryl sulfonic acid labelled 20% of the amino groups on Lp-G (Table III) suggesting that the glutaraldehyde derivatized 80% of the amino groups on Lp-PE. This was confirmed by the fluorescamine test (Table III) which showed that 85% of the amino groups were derivatized by glutaraldehyde. Comparison of the picryl sulfonic acid and fluorescamine labelling results suggests that most of the amino groups were located on the surface of the liposomes.

3. Determination of TLCK on Liposomes

a. Quantitation of TLCK-bound Liposome

The amount of TLCK that covalently coupled with liposomes was determined indirectly by measuring the unreacted TLCK following chromatography on Sephadex G-200. As shown in Table IV Lp-G, Lp-D (type B) and Lp-D (type A) coupled TLCK at different rate and varied from 62.4% to 91.9%.

b. Spectral Analysis of TLCK-bound Liposome

As shown in Figure 3, free TLCK and Lp-G-TLCK possess maximum absorption peaks at 229 and 232 nm, respectively, while Lp-PE and Lp-G liposomes did not display similar absorption characteristic, suggesting that the observed

TABLE III

GLUTARALDEHYDE DERIVATIZATION OF Lp-PE[a]

	Picryl Sulphonic Acid Test		Fluorescamine Test	
	Absorbance (410 nm)	Lp-G %[b]	Relative Intensity of Fluorescence	Lp-G %
Lp[c]	0	-	21	-
Lp-PE	0.20	-	1000	-
Lp-G	0.04	80	150	85

[a] Following reaction of Lp-PE and Lp-G with picryl sulphonic acid at 37°C for 90 minutes the rate of labelling of liposomal amino groups was determined by the absorbance of the reaction mixture at 410 nm. The liposomes (Lp-PE and Lp-G) were also labelled with fluorescamine at room temperature for 30 minutes and were measured fluorometrically at excitation, 390 nm and emission, 482 nm.

[b] Percent liposomal amino groups that were derivatized with glutaraldehyde was calculated from the formula:

$$100 - \frac{(\text{Absorbance of Labelled Lp-G})}{(\text{Absorbance of Labelled Lp-PE})} \times 100$$

The absorbance of labelled Lp-PE was taken as 100%.

[c] The liposome was without phosphatidylethanolamine.

TABLE IV

QUANTITATION OF TLCK BOUNDED TO LIPOSOME[a]

	TLCK Added µmoles	TLCK Recovered µmoles	TLCK Bound µmoles	TLCK Bound %[b]
Lp-G	10.1	3.88	6.22	62.4
Lp-D (Type B)	6.8	2.40	4.40	64.7
Lp-D (Type A)	11.4	0.92	10.48	91.9

[a] Following the reaction of TLCK with sodium metaperiodate oxidized Lp-D type A (4.23 µmoles digoxin), Lp-D type B (2.14 µmoles digoxin) at room temperature for 24 hours and with glutaraldehyde treated Lp-PE (9.6 µmoles phosphatidylethanolamine) at room temperature for 25 minutes, unreacted TLCK was separated from TLCK-bound liposome on Sephadex G-200 (0.9 x 15 cm; flow rate: 0.10 mL per minute). The unreacted TLCK was derivatized with fluorescamine and the relative intensity of fluorescence was determined at excitation 390 nm and emission, 482 nm.

[b] Calculated from the formula:

$$\frac{\text{TLCK Added} - \text{TLCK Recovered}}{\text{TLCK Added}} \times 100$$

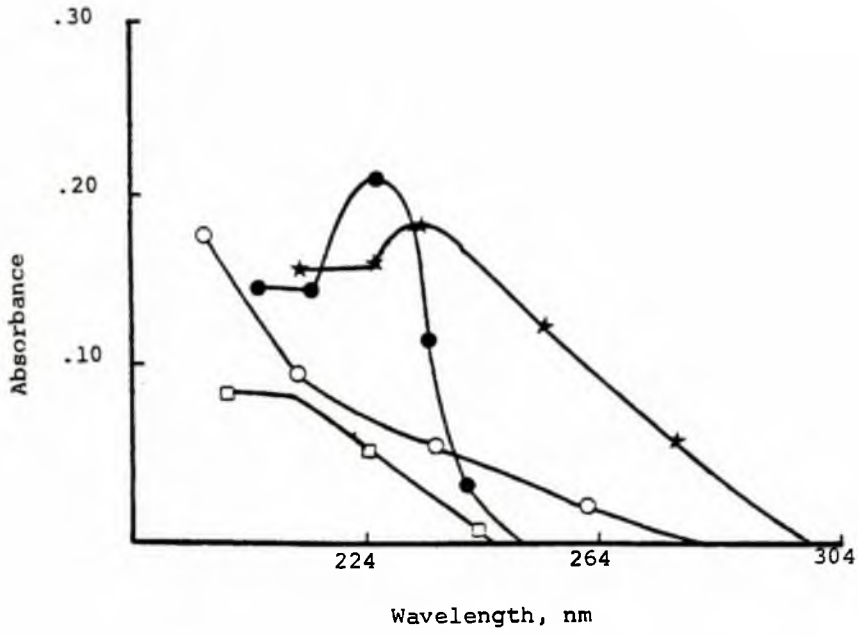

Figure 3. UV spectra of free and TLCK-bound liposomes. TLCK (15.3 nmoles, ●—●), Lp-PE (36.7 µmoles phosphatidylethanolamine, □—□), Lp-G (○—○) and Lp-G-TLCK (★—★) in methanol were scanned on Cary 1115 UV-VIS spectrophometer.

absorbance pattern of Lp-G-TLCK was attributable to the TLCK and not liposomal components. The presence of TLCK on the liposomes was further assessed by measuring anti-trypsin activity of TLCK-bound liposome.

c. Antitrypsin Activity of TLCK-bound Liposome

The antitrypsin activity of TLCK-bound liposome was determined by measuring trypsin activity using α-benzoyl-arginine-p-nitroanilide hydrochloride as substrate. Although TLCK exhibits maximum antitrypsin activity at pH 7.5, its stability begins to decline at pH greater than 7.5 (103). Thus the pH of the reaction mixture containing TLCK (or TLCK-bound liposome) and trypsin, was carefully adjusted to 7.2. The antitrypsin activity of free TLCK is shown in Table V. Antitrypsin activities were also obtained for Lp-G-TLCK (Table VI) and Lp-D-TLCK (Table VII). When the percent trypsin inhibition of bound TLCK was compared to that of free TLCK, the relative antitrypsin activity of TLCK bound to Lp-G (Lp-G-TLCK) was about 7% of the free TLCK (Table VI). The relative antitrypsin activity of Lp-D-TLCK types A and B was 20 and 53%, respectively, of the free TLCK indicating that there were different activities of TLCK bound to different liposomes. The loss of antitrypsin activity of the bound TLCK may be explained by the coupling of the side chain ($H_2N-CH_2-R_1$) of TLCK since the amino group is required for an efficient inhibition of trypsin (103). At incubation period, less than 24 hours (18 hours), the antitrypsin activity of TLCK-

TABLE V

ANTITRYPSIN ACTIVITY OF FREE TLCK[a]

	Absorbance (410 nm)	Inhibition of Trypsin, %[b]
Trypsin (12.9 nmoles)	0.36	-
Trypsin plus TLCK (nmoles)		
1.7	0.37	0
3.4	0.33	8
6.8	0.30	17
13.5	0.28	22
40.6	0.14	61
101.4	0	100
202.8	0	100

[a] A 0.2 mL PBS, pH 7.2 containing the stated concentrations of TLCK was incubated with trypsin (12.9 nmoles) in a final volume of 0.4 mL at room temperature for 2 hours. A 0.05 mL aliquot of the incubation mixture was assayed for trypsin activity using α-benzoyl-arginine-p-nitroanilide hydrochloride (BAPNA) as substrate. The rate of hydrolysis of the BAPNA was measured at 410 nm.

[b] Calculated from the formula:

$$100 - \frac{(\text{Absorbance of Trypsin - TLCK Mixture})}{(\text{Absorbance of Uninhibited Trypsin})} \times 100$$

TABLE VI

ANTITRYPSIN ACTIVITY OF Lp-G-TLCK[a]

	Absorbance (410 nm)	Inhibition of Trypsin, %	Relative Activity of TLCK, %[b]
Trypsin (12.9 nmoles)	0.36	-	-
Trypsin plus			
TLCK (40.6 nmoles)	0.14	61	-
Lp-G[c]	0.36	0	-
Lp-G-TLCK[d]	0.13	64	7.0
Lp-G-TLCK[e]	0.37	0	-
Lp-PE+TLCK[f]	0.36	0	-

[a]Following the reaction of TLCK with Lp-G at room temperature for 25 minutes, unreacted TLCK was separated from TLCK-bound liposome (Lp-G-TLCK) on Sephadex G-200 column. The Lp-G-TLCK (and Lp-PE) eluting in the void volume was pooled and the liposomal phosphatidylethanolamine redetermined. A 0.2 mL of the liposomal suspension (0.88 nmoles phosphatidylethanolamine) was incubated with 12.9 nmoles trypsin and the antitrypsin activity was determined as described in the legend for Table V.

[b]Calculated from the formula:

$$\left(\frac{\%\ \text{Inhibition B}}{\%\ \text{Inhibition F}} \times \frac{\text{Concentration of TLCK}_F}{\text{Concentration of TLCK}_B}\right) \times 100$$

B = Bound F = Free

[c]Glutaraldehyde treated liposome.

[d]The amount of TLCK bound to the liposome was 606 nmoles.

[e]Sodium borohydride treated liposome.

[f]A mixture of Lp-PE and TLCK at their concentrations comparable to those utilized for preparing Lp-G-TLCK was chromatographed on Sephadex G-200. The purpose was to determine the extent to which free TLCK binds non-covalently to Lp-PE.

TABLE VII

ANTITRYPSIN ACTIVITY OF Lp-D-TLCK[a]

	Absorbance (410 nm)	Inhibition of Trypsin, %	Relative Activity of TLCK, %
1. TYPE A			
Trypsin (12.9 nmoles)	0.36	-	-
Trypsin plus			
TLCK (40.6 nmoles)	0.14	61	-
Lp-D	0.38	0	-
Lp-D-TLCK	0.05	86	20.4
2. TYPE B			
Lp-D	0.37	0	-
Lp-D-TLCK	0.06	83	52.7
Lp-D-TLCK[b]	0.22	39	24.6
Lp-D-TLCK[c]	0.36	0	-
Lp-D + TLCK	0.28	22	-

[a] TLCK was incubated with sodium metaperiodate oxidized Lp-D at room temperature for 24 hours and the unreacted TLCK was separated from TLCK-bound liposome (Lp-D-TLCK) on Sephadex G-200 column. The Lp-D-TLCK (and Lp-D) eluting in the void volume was pooled and the liposomal digoxin redetermined. A 0.2 mL of the liposomal suspension was incubated with 12.9 nmoles trypsin and the antitrypsin activity was determined as described in the legend for Table V.

The digoxin content of the liposomes were as follows: Lp-D type A 122.2 nmoles, Lp-D-TLCK type A 112.8 nmoles (and 280 nmoles TLCK), Lp-D type B 57.4 nmoles, Lp-D-TLCK type B 51.0 nmoles (and 105 nmoles TLCK).

[b] TLCK was incubated with the sodium metaperiodate oxidized Lp-D for 18 hours at room temperature.

[c] Sodium borohydride treated liposome.

bound liposome was lower (Table VII) suggesting that less TLCK coupled to the liposomes. The results, however, indicate that the active site, chloromethyl ketone (R_2-C(=O)-CH_2-Cl) portion of the TLCK was preserved and reacted with the histidine residue on the active site of trypsin.

When the Schiff's base of the TLCK-bound liposome was reduced with sodium borohydride the TLCK-bound liposome lost its antitrypsin activity (Tables VI and VII). This suggests that the sodium borohydride reduced the chloromethyl ketone group as well. The results (Tables VI and VII) further showed that the TLCK-free liposomes did not show antitrypsin activity. In addition, when the unreacted liposomes (Lp-D or Lp-PE) were incubated with TLCK, little antitrypsin activity was displayed, indicating that non-covalent binding of TLCK to the liposomes is negligible.

C. Release of Entrapped Molecules from Liposome

1. Triton X-100 Treatment

In order to determine the efficiency of Triton X-100 for releasing ara-C the liposomes were incubated with Triton X-100 with final concentrations ranging from 0.05 to 5% at 37°C for 45 minutes. The ruptured liposomes were precipitated by centrifugation and ara-C in the supernate was measured as described in the Methods. As shown in Table VIII the absorbance peak of ara-C from 0.05% Triton X-100 was comparable to that of ara-C extracted by chloroform. Thus 0.05% Triton X-100 is unlikely to interfere with the determination of ara-C and is sufficient to re-

TABLE VIII

EFFECTS OF TRITON X-100 AND CHLOROFORM ON THE DETERMINATION OF LIPOSOME ENTRAPPED ara-C[a]

	Triton X-100, %				Chloroform
	0	0.05	1.0	5.0	Absorbance (272 nm)
	Absorbance (272 nm)				
PBS	0	0.02	0.70	1.50	0.01
ara-C[b]	0.40	0.41	1.06	-	0.39
Lp-D	0.01	0.47	1.07	1.56	0.49
Lp-D[c]	0.02	0.02	0.57	1.48	0.03

[a]Liposome (Lp-D type B) was prepared in PBS containing 99.2 µmoles ara-C followed by dialysis in isotonic buffer for 4 hours at room temperature to remove unentrapped ara-C. In order to effect the release of entrapped ara-C, a 0.4 mL of the liposomal suspension was incubated for 45 minutes at 37°C in the presence of Triton X-100 (final concentration of 0.05, 1.0 and 5.0%), centrifuged at 25,000 x g for 3 hours and the ara-C in the supernatant was measured at 272 nm. Release of entrapped ara-C was also effected by chloroform as follows: A 0.1 mL fraction of the liposomal suspension was mixed with 1.0 mL chloroform on a vortex. A 1.9 mL PBS was added to the mixture and the ara-C recovered in the aqueous phase.

[b]The ara-C in the swelling buffer was treated as described for the liposome.

[c]Liposome is devoid of ara-C.

cover the latter from liposomes. The 1 to 5% Triton X-100, on the other hand, increased the background with no increase in recovery of ara-C. The results also showed that liposome components did not interfere with the ara-C assay.

2. Chloroform Extraction

Entrapped ara-C in Lp-D (type B) was extracted by dissolving the liposome in chloroform and then PBS as described in the Methods. As shown in Table VIII the absorbance of ara-C was comparable to that obtained in the presence of 0.05% Triton X-100. Although the chloroform extraction method was faster and easier to perform for recovering such water soluble compounds as ara-C, the denaturing effect of the chloroform on enzymes and the poor recovery of water insoluble drugs limit the usefulness of this method.

D. Entrapment of Drugs and Markers

1. Effects of Concentration of ara-C and Lipids on Liposome Entrapment of ara-C

The extent to which a molecule is entrapped by liposome has been shown to depend on the concentration of the molecule to be entrapped and of liposomal lipids. To assess this phenomena Lp-D (type B) was prepared by varying concentrations of lipids as well as ara-C. As shown in Table IX on the basis of chloroform extraction, a 2.5-fold increase in the concentration of liposome lipid (from 45.1 to 113.2 µmoles) resulted in a 4.5-fold increase in entrapment of ara-C (from 2.6 to 11.8%). However, when the lipid

TABLE IX

EFFECTS OF CONCENTRATIONS OF ara-C AND LIPIDS ON LIPOSOME ENTRAPMENT OF ara-C[a]

Liposomes	ara-C added (µmoles)	Triton X-100, 0.05% Absorbance (272 nm)	Triton X-100, 0.05% % Entrapped[b]	Chloroform Absorbance (272 nm)	Chloroform % Entrapped
	0.0496	-	-	0.25	-
	0.0992	0.40	-	0.49	-
Lp-D	-	0.02	-	0.03	-
Lp-D[c]	99.2	-	-	5.12	11.8
Lp-D[d]	49.6	0.34	3.1	0.32	3.2
	99.2	0.47	2.2	0.49	2.6
	198.4	1.18	2.9	1.12	2.8

[a] Liposome (Lp-D type B) was prepared in PBS, pH 7.2 containing the stated concentrations of ara-C and dialyzed in isotonic PBS to remove unentrapped ara-C. Release of entrapped ara-C is effected with 0.05% Triton X-100, or chloroform and measured at 272 nm as described in the legend for Table VIII.

[b] Calculated from the formula:

$$\frac{\text{Amount of ara-C Entrapped}}{\text{Amount of ara-c Added}} \times 100$$

[c] Total liposomal lipids: 113.2 µmoles.

[d] Total liposomal lipids: 45.1 µmoles.

concentration is maintained at 45.1 µmoles and the ara-C in the swelling buffer was progressively increased to 198.4 µmoles, the extent of ara-C entrapment remained essentially unchanged, suggesting that the concentration of liposome lipid is critical in determining the degree of liposome entrapment.

While the entrapment capacity of ara-C at 45.1 µmoles of liposomal lipid was less than optimal, the amount of the lipid had to be compromised in order to minimize the possible toxic effects of the liposomal lipid on the cultured cells.

2. Entrapment of ara-C, Colchicine, Horseradish Peroxidase (HRP), Glucose and 6-carboxyfluorescein (6-CF) in Liposomes

In addition to lipid concentration, the extent of entrapment of a substance in liposomes is also dependent on the net ionic charge of the liposome, the ionic charge and solubility of the substance to be entrapped. Entrapped ara-C, colchicine, HRP, glucose and 6-CF in Lp-D, Lp-D-TLCK, Lp-PE and Lp-G-TLCK was released when the liposomes were treated with 0.05% Triton X-100 after the removal of the unentrapped molecules by Sephadex G-200 chromatography. As shown in Table X the Lp-D (type A), Lp-D (type B) and Lp-PE entrapped 0.80, 0.40 and 1.1% ara-C, respectively, while Lp-D-TLCK (type A), Lp-D-TLCK (type B) and Lp-G-TLCK entrapped 0.75, 0.33, and 0.74% ara-C, respectively. From the absorbance readings taken before treatment with Triton

X-100, it is clear that loss of ara-C from the liposome is negligible. As expected when unentrapped ara-C was removed by dialysis, the amount of ara-C released from Lp-D (type B) was higher (2.2 to 2.6%, Table IX) than the 0.4% released from the column chromatographed Lp-D (type B). The reason for this is that the latter dilutes liposomes.

The results (Table X) showed that the solubility of entrapped colchicine effected its retention in liposomes. Compared to ara-C, the extent of entrapment of colchicine in Lp-D (0.07%) and Lp-D-TLCK (0.06%) both type B, was lower. However, the absorbance readings before Triton X-100 treatment were high, suggesting that colchicine may readily permeate liposomes. Therefore barring liposome leakage, the actual percent of colchicine entrapment would be higher.

With respect to HRP entrapment, it is not certain whether electrostatic or hydrophobic interactions between the enzyme and Lp-PE predominantly affected the extent of entrapment. However, an increase in the activity of HRP following treatment with Triton X-100 showed that 3.1% of the enzyme was entrapped in Lp-PE (Table XI). In comparison entrapment in Lp-G-TLCK (1.6%) was lower. Whether the loss of HRP activity was due to the concentration of the glutaraldehyde (50 µmoles) used to derivatize the Lp-PE at room temperature for 7 minutes, is not certain since at higher concentration (250 µmoles) glutaraldehyde did not inhibit HRP activity (104). Compared to ara-C the extent of entrapment of glucose in Lp-PE (6.6%) and Lp-G-TLCK

TABLE X

ENTRAPMENT OF ara-C AND COLCHICINE BY LIPOSOMES[a]

	Absorbance (272 nm)	Entrapped ara-C (nmoles)	% Entrapped[b]	Absorbance (245 nm)	Entrapped Colchicine (nmoles)	% Entrapped
ara-C	0.40	-	-	-	-	-
Colchicine	-	-	-	0.25	-	-
Lp-D[c]						
post-Triton	0.69	158.7	0.80	-	-	-
pre-Triton	0.05	-	-			
Lp-D-TLCK						
post-Triton	0.64	148.8	0.75	-	-	-
pre-Triton	0.04	-	-			
Lp-D[d]						
post-Triton	0.34	79.4	0.40	0.24	2.1	0.07
pre-Triton	0.02	-	-	0.14	-	-
Lp-D-TLCK						
post-Triton	0.28	64.5	0.33	0.16	1.8	0.06
pre-Triton	0.02	-	-	0.07	-	-
Lp-PE[e]						
post-Triton	0.86	210.8	1.10	-	-	-
pre-Triton	0.01	-	-			
Lp-G-TLCK						
post-Triton	0.60	146.3	0.74	-	-	-
pre-Triton	0.01	-	-			

[a]Following the reaction of sodium metaperiodate oxidized Lp-D and glutaraldehyde derivatized Lp-PE with TLCK at room temperature for 24 hours and 25 minutes, respectively, unreacted TLCK was separated from TLCK-bound liposomes (Lp-D-TLCK and Lp-G-TLCK) on Sephadex G-200 column (0.9 x 15 cm, flow rate: 0.1 mL per minute). The TLCK-bound liposome eluted in the void volume, pooled and 0.2 mL aliquots were treated with Triton X-100 (0.05% final concentration) and incubated at 37°C for 45 minutes to effect the release of ara-C and colchicine. This was followed by centrifugation at 25,000 x g for 3 hours and ara-C or colchicine in the supernate measured at 272 and 245 nm, respectively. Triton X-100 exposed liposomes are referred to as post-Triton and those unexposed to Triton X-100 as pre-Triton. The latter and PBS containing 0.05% Triton X-100 served as blanks. Standard concentration of ara-C and colchicine were respectively 99.2 and 7.8 nmoles, respectively. A 0.2 mL of the swelling buffer contained 19.9 µmoles ara-C and 3.2 µmoles colchicine.

TABLE X
(Continued)

[b]Calculated from the formula:

$$\frac{\text{Amount of ara-C or Colchicine Entrapped}}{\text{Amount of ara-C or Colchicine Added}} \times 100$$

[c]Lp-D (122.2 nmoles digoxin) and Lp-D-TLCK (112.8 nmoles digoxin) both type A.

[d]Lp-D (57.4 nmoles digoxin) and Lp-D-TLCK (51.0 nmoles digoxin) both type B.

[e]Lp-PE and Lp-G-TLCK (880 nmoles phosphatidylethanolamine).

TABLE XI

ENTRAPMENT OF HORSERADISH PEROXIDASE (HRP), GLUCOSE AND 6-CARBOXYFLUORESCEIN (6-CF) BY LIPOSOMES[a]

	Entrapped HRP (µg)	% Entrapped	Entrapped Glucose (mg %)	% Entrapped	Entrapped 6-CF (nmoles)	% Entrapped
Lp-PE[b]						
post-Triton	6.52	3.1	125.0	6.6	-	-
pre-Triton	0.65	-	-	-	-	-
Lp-G-TLCK						
post-Triton	3.65	1.6	100.0	5.3	-	-
pre-Triton	0.72	-	-	-	-	-
Lp-D[c]						
post-Triton	-	-	-	-	265.0	1.1
pre-Triton	-	-	-	-	-	-
Lp-D-TLCK						
post-Triton	-	-	-	-	212.0	0.9
pre-Triton	-	-	-	-	-	-

[a]Following the reaction of sodium metaperiodate oxidized Lp-D and glutaraldehyde derivatized Lp-PE with TLCK, unreacted TLCK was separated from TLCK-bound liposomes on Sephadex G-200 column (0.9 x 15 cm, flow rate: 0.1 mL per minute). The release of entrapped molecules was effected with 0.05% Triton X-100 as described in the legend of Table X. The HRP was measured at 500 nm using 4-amino-antipyrine, phenol and hydrogen peroxide as substrates. Glucose was determined by the glucose oxidase method using the Astra-8 and 6-CF was measured fluorometrically at excitation 490 nm and emission 520 nm. A 0.2 mL aliquot of the swelling buffer contained 187.8 µg HRP, 1895 mg% glucose and 24.4 µmoles 6-CF.

[b]Lp-PE and Lp-G-TLCK (880 nmoles phosphatidylethanolamine).

[c]Lp-D (57.4 nmoles digoxin) and Lp-D-TLCK (51.0 nmoles digoxin) both type B.

(5.3%) was higher which is compatible with the suggestion that entrapment of glucose, a non-ionic substance, is independent of the ionic charge of liposomes. The results (Table XI) also showed that Lp-D and Lp-D-TLCK (both type B) entrapped 1.1 and 0.9% 6-CF, respectively.

E. Cell Culture Studies

Studies of directing TLCK-derivatized liposomes to transformed cells (SVT_2) consisted of two parts. First, the pattern of uptake of liposomes and TLCK-bound liposome by the SVT_2 cells were established with liposomes containing either 6-CF or HRP. Secondly, the effect of ara-C or colchicine containing liposome on the growth of SVT_2 cells was investigated. Control studies were similarly performed with untransformed cells (Balb/C 3T3).

1. Uptake of Liposomes by Balb/C 3T3 and SVT_2 Cells

a. 6-CF Containing Lp-D and Lp-D-TLCK (Type B)

The relative rate of uptake of Lp-D and Lp-D-TLCK (both type B) was followed by measuring the entrapped 6-CF following sonication of the cell suspension and incubation in 0.05% Triton X-100 at 37°C for 45 minutes. Maximum uptake of 6-CF entrapped in Lp-D-TLCK by SVT_2 cells was reached in one hour of incubation (Table XII and Figure 4). In comparison, the uptake of 6-CF entrapped in Lp-D by SVT_2 cells was much lower. The Balb/C 3T3 cells, on the other hand, showed preference for 6-CF entrapped in Lp-D whose incorporation increased with time reaching 33.8% (from 1.5%) at the end of the incubation. The uptake of 6-CF entrapped

TABLE XII

UPTAKE OF 6-CARBOXYFLUORESCEIN (6-CF) IN Lp-D AND Lp-D-TLCK (TYPE B) BY BALB/C 3T3 AND SVT_2 CELLS[a]

	Time of Incubation (Hours)	BALB/C 3T3 6-CF Uptake By $1x10^7$ Cells (p moles)	BALB/C 3T3 % 6-CF Uptake By $1x10^7$ Cells (x10)[b]	SVT2 6-CF Uptake By $1x10^7$ Cells (p moles)	SVT2 % 6-CF Uptake By $1x10^7$ Cells (x10)
None		-	-	-	-
6-CF[c]	0.25	1.3	0.3	TLD	-
Lp-D	"	1.3	1.5	3.5	4.1
Lp-D-TLCK	"	TLD	-	9.1	13.4
6-CF	0.5	2.7	0.5	2.8	0.6
Lp-D	"	5.2	6.1	4.2	5.0
Lp-D-TLCK	"	TLD	-	11.1	16.4
6 CF	1.0	2.7	0.5	5.6	1.1
Lp-D	"	18.3	21.6	6.3	7.4
Lp-D-TLCK	"	3.8	5.6	24.4	36.0
6-CF	2.0	7.9	1.6	11.1	2.2
Lp-D	"	20.8	24.5	7.0	8.3
Lp-D-TLCK	"	5.2	7.7	0.1	13.4
6-CF	3.0	20.8	4.2	13.9	2.8
Lp-D	"	22.1	26.1	9.1	10.7
Lp-D-TLCK	"	7.9	11.6	11.8	17.4
6-CF	4.0	26.0	5.2	15.3	3.1
Lp-D	"	28.7	33.8	9.1	10.7
Lp-D-TLCK	"	10.4	15.3	11.1	16.4

[a] Balb/C 3T3 (0.50 to 0.54 x 10^7) and SVT_2 (0.96 to 0.98 x 10^7) cells were incubated in MEM x 4 containing 92 and 82 nmoles digoxin for Lp-D and Lp-D-TLCK (both type B) entrapping 8.5 and 6.8 nmoles 6-CF respectively at 37°C for periods up to 4 hours. At each time interval the cells were washed with PBS and removed with trypsin. The cells were rewashed and ruptured with sonication in an ice bath for 3 minutes followed by incubation at 37°C for 45 minutes in the presence of 0.05% Triton X-100. Cellular fragments were precipitated by centrifugation and the 6-CF in the supernate was measured fluorometrically at excitation 490 nm and emission 520 nm. The experiment was performed two times.

[b] Calculated from the formula: $\frac{\text{Amount of 6-CF Taken by } 1x10^7 \text{ Cells}}{\text{Amount of 6-CF Added}} \times 100$

[c] Free 6-CF, 49.6 nmoles.

TLD, Too Low to Determine

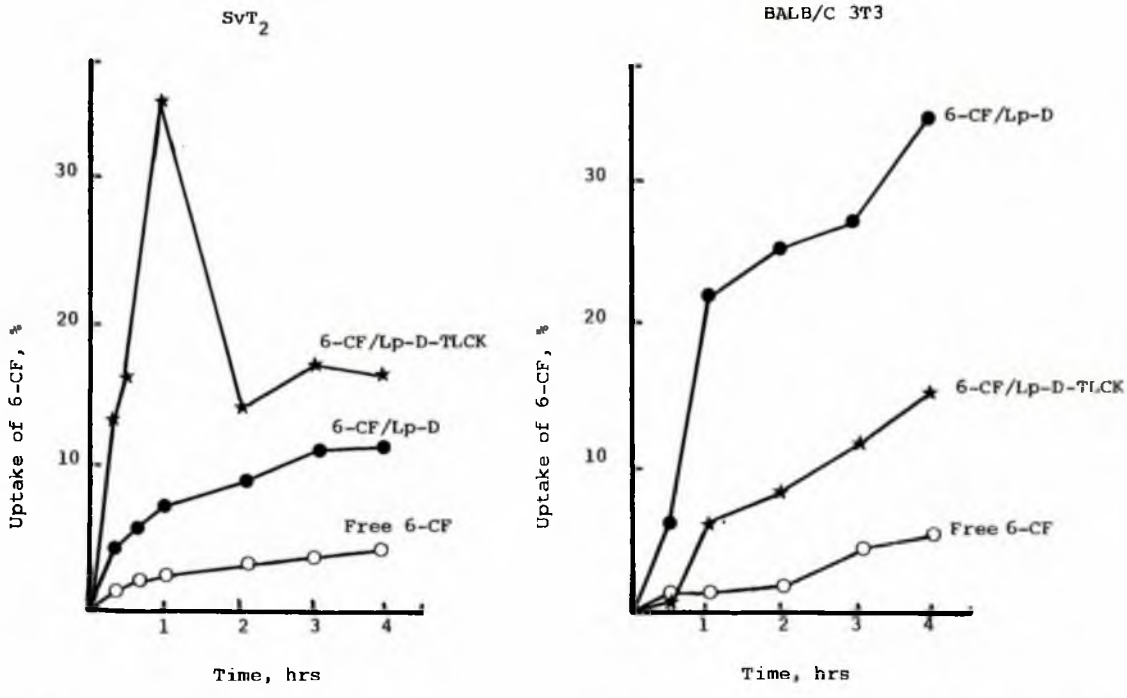

Figure 4. Uptakes of 6-CF by Balb/C 3T3 and SVT_2 cells in culture. The cells were grown in MEM x 4 in the presence of free or liposome-entrapped 6-CF. At each time interval the cells were removed and their 6-CF content was measured fluorometrically at excitation 490 nm and emission 520 nm.

in Lp-D-TLCK by Balb/C 3T3 cells was 15.3% during the same period. The results indicate that TLCK enhanced uptake of 6-CF entrapped in Lp-D-TLCK by SVT_2 cells while reducing the uptake of the Lp-D-TLCK by Balb/C 3T3 cells.

b. HRP Containing Lp-PE and Lp-G-TLCK

As with the Lp-D-TLCK, the relative uptake of HRP entrapped in Lp-G-TLCK by SVT_2 cells exceeded that of HRP in Lp-PE (Table XIII and Figure 5). The uptake of HRP in Lp-G-TLCK by the SVT_2 cells increased with time and reaching 2.68% by the end of incubation, whereas 1.63% HRP entrapped in Lp-PE was taken up. With respect to the Balb/C 3T3 cells uptake of HRP in Lp-PE was higher at 30 minutes of incubation. However with continued incubation for up to 90 minutes the uptake of HRP in Lp-G-TLCK by the Balb/C 3T3 cells exceeded that of the HRP in Lp-PE.

2. Effects of Liposomal Components on the Growth of Cells in Culture

a. TLCK

Tosyl-L-lysyl-chloromethyl ketone (TLCK) has been shown to inhibit growth of tumor (transformed) cells and prevents the adhesion of Ehrlich ascites tumor cells to plastic plates (74-77). When SVT_2 cells were treated with TLCK at concentrations of 82 and 277 nmoles for 30 minutes at 37°C growth of the SVT_2 cells was moderately impaired at day 1 (Table XIV). The cells, however, resumed their normal growth by the second day. On the other hand the growth of Balb/C 3T3 cells was not affected by TLCK at

TABLE XIII

UPTAKE OF HORSERADISH PEROXIDASE (HRP) IN Lp-PE AND Lp-G-TLCK BY BALB/C 3T3 AND SVT_2 CELLS[a]

	Time of Incubation (min.)	BALB/C 3T3 HRP Uptake by $1x10^7$ cells (ng)	BALB/C 3T3 % HRP Uptake By $1x10^7$ Cells (x10)[b]	SVT_2 HRP Uptake By $1x10^7$ Cells (ng)	SVT_2 % HRP Uptake By $1x10^7$ Cells (x10)
HRP[c]	Up to 90	TLD	-	TLD	-
Lp-PE	30	0.47	0.59	0.74	0.93
Lp-G-TLCK	"	0.18	0.46	0.45	1.12
Lp-PE	60	0.86	1.07	0.81	1.01
Lp-G-TLCK	"	0.53	1.33	0.63	1.57
Lp-PE	90	1.28	1.61	1.30	1.63
Lp-G-TLCK	"	1.12	2.81	1.07	2.68

[a] Balb/C 3T3 (0.57 to 0.59 x 10^7) and SVT_2 (0.70 to 0.72 x 10^7) cells were incubated in MEM x 4 containing 1.2 µmoles phosphatidylethanolamine for Lp-PE or Lp-G-TLCK entrapping 7.98 and 3.99 µg HRP, respectively, at 37°C for periods up to 90 minutes. At each time interval HRP taken up by the cells was released as described in the legend for Table XII and measured at 500 nm using 4-amino antipyrine, phenol and 3% hydrogen peroxide as substrates. The experiment was performed twice.

[b] Calculated from the formula:

$$\frac{\text{Amount of HRP Taken by 1 x } 10^7 \text{ Cells}}{\text{Amount of HRP Added}} \times 100$$

[c] Free HRP, 8.0 ug.

TLD, Two Low to Determine.

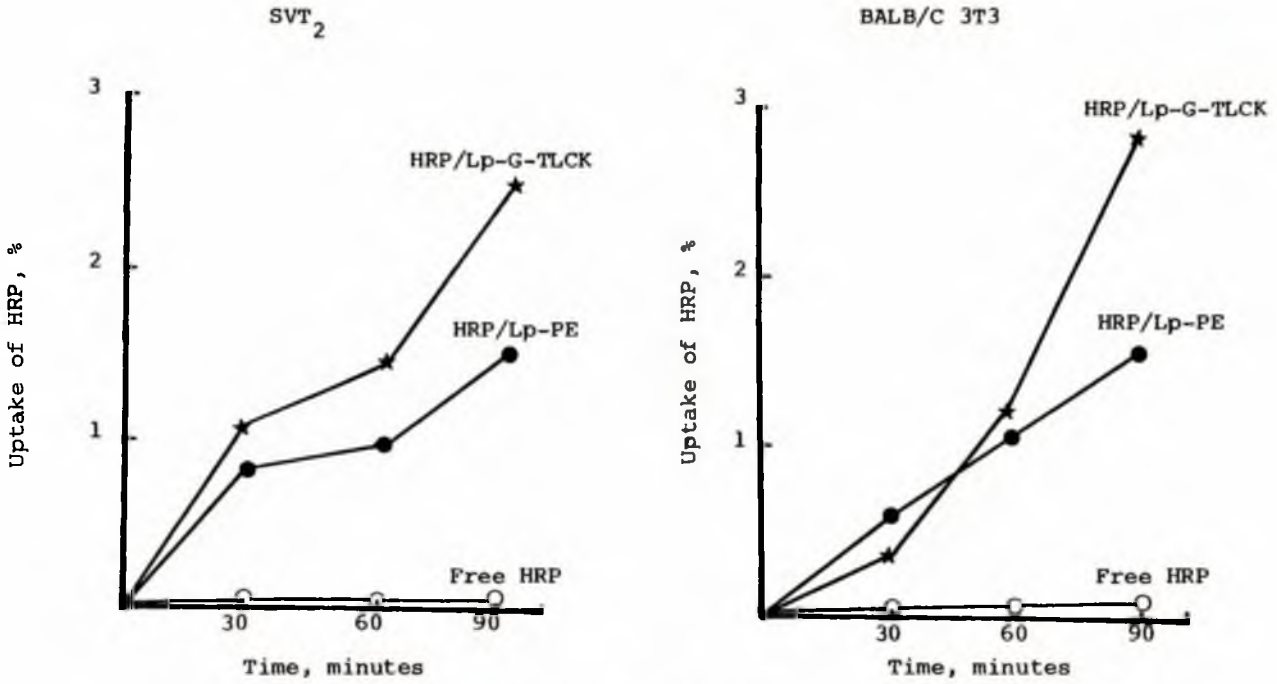

Figure 5. Uptakes of HRP by Balb/C 3T3 and SVT_2 cells in culture. The cells were grown in MEM x 4 in the presence of free or liposome-entrapped HRP. At each time interval the cells were removed and their HRP content was measured at 500 nm using 4-amino antipyrine, phenol and 3% hydrogen peroxide as substrates.

TABLE XIV

CYTOTOXIC EFFECT OF FREE TLCK ON THE GROWTH OF BALB/C 3T3 AND SVT_2 CELLS IN CULTURE[a]

TLCK (nmoles)	% Cells Attached[b] BALB/C 3T3 Day 1	Day 2	Day 3	SVT_2 Day 1	Day 2	Day 3
None[c]	214.3	304.8	477.8	185.7	325.7	862.9
82	234.9	303.2	488.9	174.3	320.0	860.0
277	228.6	312.7	484.1	157.1	322.9	894.3

[a] Balb/C 3T3 (3.5 to 3.7×10^5) and SVT_2 (2.0 to 2.2×10^5) cells were inoculated into 60 x 15 mm plastic petri dishes and were incubated in MEM x 4 for 24 hours at 37°C. The cells were then exposed to the stated concentrations of TLCK in MEM x 4 for 30 min. at 37°C; washed once with PBS and were incubated in 4.0 mL of fresh MEM x 4 at 37°C for up to three days. After each time period, the cells were removed with trypsin, washed twice with PBS and counted on a hemocytometer. At Day 0, the Balb/C 3T3 and SVT_2 cell densities were 6.3×10^5 and 3.5×10^5 respectively. The data is expressed as mean percent cell counts ± SEM of four similar experiments.

[b] Calculated from the formula:

$$\frac{\text{No. of Cells Attached (Exposed)}}{\text{No. of Cells Attached At Day 0 (Unexposed)}} \times 100$$

No. of cells attached ($\times 10^{-5}$):

TLCK (nmoles)	BALB/C 3T3 Day 1	Day 2	Day 3	SVT_2 Day 1	Day 2	Day 3
None[c]	13.5 ± 1.6	19.2 ± 1.4	30.1 ± 2.7	6.5 ± 1.3	11.4 ± 1.5	30.2 ± 3.4
82	14.8 ± 1.5	19.1 ± 1.9	30.8 ± 3.1	6.1 ± 1.2	11.2 ± 1.7	30.1 ± 2.1
277	14.4 ± 1.6	19.7 ± 1.8	30.5 ± 3.1	5.5 ± 1.1	11.3 ± 1.5	31.3 ± 4.1

[c] These cells were not exposed to TLCK.

these concentrations.

b. Arabinofuranosyl Cytosine (ara-C)

The ara-C differs in its sugar moiety from the normal metabolites, cytidine and deoxycytidine and inhibits DNA synthesis of mammalian cells and viruses (105, 106). The cytotoxicity of ara-C is mediated by deoxycytidine kinase by converting ara-C to ara-C-5'-triphosphate (ara-CTP) which competes with cell uptake of deoxycytidine triphosphate (dCTP). Deoxycytidine deaminase on the other hand inactivates ara-C by converting it to ara-uracil (107). Mayhew et al (45) showed that ara-C entrapped in liposomes had antitumor activity against SV 40 transformed 3T3 cells and Ehrlich-Lettre ascites carcinoma cells both in vitro and in vivo.

To investigate the effect of ara-C on the growth of both SVT_2 and Balb/C 3T3 cells, the exposure period was either 20 or 50 minutes since uptakes of HRP in Lp-G-TLCK and 6-CF in Lp-D-TLCK by the Balb/C 3T3 cell was lower for incubation periods of up to 30 minutes when compared with the uptakes of Lp-PE and Lp-D. On the other hand, uptake of 6-CF in Lp-D-TLCK by SVT_2 cells was maximum in one hour of incubation. As shown in Table XV ara-C impaired the growth of both Balb/C 3T3 and SVT_2 cells in a dose dependent fashion. However, the growth of the SVT_2 cells did not differ significantly whether the cells were exposed to the ara-C for either 20 or 50 minutes.

TABLE XV

CYTOTOXIC EFFECT OF ara-C ON THE GROWTH OF BALB/C 3T3 AND SVT_2 CELLS IN CULTURE[a]

ara-C (nmoles)	% Cells Attached[b] BALB/C 3T3 Day 1	BALB/C 3T3 Day 2	SVT_2 Day 1	SVT_2 Day 2
None[c]	214.3	304.8	185.7	325.7
11.6	212.7	238.1	180.0	254.3
14.4[d]	---	---	174.3	265.7
25.5	161.9	234.9	131.4	245.7
283.0	---	---	91.4	68.6
283.0[d]	---	---	62.9	65.7

[a] Balb/C 3T3 (3.5 to 3.7 x 10^5) and SVT_2 (2.0 to 2.2 x 10^5) cells were inoculated into 60 x 15 mm plastic petri dishes and were incubated in MEM x 4 for 24 hours at 37°C. The cells were then exposed to the stated concentration of ara-C in MEM x 4 for 20 or 50 minutes at 37°C. The cells were washed once with PBS and were incubated in 4.0 mL fresh MEM x 4 for up to two days. At Day 0 the Balb/C 3T3 and SVT_2 cell densities were 6.3 x 10^5 and 3.5 x 10^5 respectively. The data is expressed as mean percent cell counts ± SEM of three similar experiments.

[b] Calculated from the formula: $\frac{\text{No. of Cells Attached (Exposed)}}{\text{No. of Cells Attached at Day 0 (Unexposed)}} \times 100$

TABLE XV (cont'd)

No. of cells attached ($x10^{-5}$):

ara-C (nmoles)	BALB/C 3T3		SVT_2	
	Day 1	Day 2	Day 1	Day 2
None[c]	13.5 ± 1.60	19.2 ± 1.40	6.5 ± 1.3	11.4 ± 1.5
11.6	13.4 ± 2.80	15.0 ± 1.70	6.3 ± 1.60	8.9 ± 1.34
14.4[d]	---	---	6.1 ± 0.70	9.3 ± 0.58
25.5	10.2 ± 2.20	14.8 ± 1.50	4.6 ± 1.50	8.6 ± 1.45
283.0	---	---	3.2 ± 1.60	2.4 ± 0.02
283.0[d]	---	---	2.2 ± 0.90	2.3 ± 0.29

[c]These cells were not exposed to ara-C.

[d]50-minute exposure to ara-C.

c. Colchicine

Colchicine (N-(5, 6, 7, 9-tetrahydro-1, 2, 3, 10-tetramethoxy-9-oxobenzo-[a]-heptalen-7-yl) acetamide) readily enters cells, binds with tubulin and prevents the formation of microtubules which are responsible for intracellular motion. Thus cell division is inhibited in the presence of colchicine (108, 109). As shown in Table XVI colchicine was cytotoxic to both Balb/C 3T3 and SVT_2 cells.

d. PBS-loaded Lp-D and Lp-D-TLCK (Type B)

The effect of Lp-D and Lp-D-TLCK (type B) without drug on cell growth was also studied. As shown in Table XVII neither Lp-D nor Lp-D-TLCK (type B) had any effect on the cells, an indication that the liposomal components of these liposomes, at the concentrations indicated, were not toxic to the cells.

3. Effects of ara-C Containing Lp-D and Lp-D-TLCK

a. Lp-D and Lp-D-TLCK (Type B)

Lp-D and Lp-D-TLCK (type B) containing 14.2 and 11.6 nmoles ara-C respectively were incubated with Balb/C 3T3 and SVT_2 cells for periods of up to 60 minutes. Both Lp-D and Lp-D-TLCK (type B) moderately inhibited growth of Balb/C 3T3 cells on Day 1. The cells, however, resumed their normal growth by the second day (Table XVIII and Figure 6). With respect to SVT_2 cells,on the other hand, their growth were significantly impaired by Lp-D-TLCK. Furthermore the rate of SVT_2 cell growth was slightly affected by the duration of exposure of the cells to the liposomes for

TABLE XVI

CYTOTOXIC EFFECT OF COLCHICINE ON THE GROWTH OF BALB/C 3T3 AND SVT_2 CELLS IN CULTURE[a]

Colchicine (nmoles)	% Cells Attached[b]			
	BALB/C 3T3		SVT_2	
	Day 1	Day 2	Day 1	Day 2
None[c]	196.7	385.7	207.2	396.9
3.0	175.2	316.2	163.6	316.2
3.0[d]	110.9	259.1	137.9	257.7
5.0	110.5	300.9	108.2	208.5

[a] Balb/C 3T3 (1.6 to 1.8×10^5) and SVT_2 (1.4 to 1.8×10^5) cells were incubated in MEM x 4 for 24 hours at 37°C. The cells were then exposed to the stated concentrations of colchicine in MEM x 4 for 15 or 30 minutes at 37°C. The cells were washed once with PBS and were incubated in 4.0 mL of fresh MEM x 4 for up to two days. At Day 0, the Balb/C 3T3 and SVT_2 cell densities were 2.10×10^5 and 3.90×10^5 respectively. The data is expressed as mean percent cell counts ± SEM of three similar experiments.

[b] Calculated from the formula: $\frac{\text{No. of Cells Attached (Exposed)}}{\text{No. of Cells Attached at Day 0 (Unexposed)}} \times 100$

TABLE XVI (cont'd)

No. of Cells Attached (x 10^{-5}):

Colchicine (nmoles)	BALB/C 3T3		SVT_2	
	Day 1	Day 2	Day 1	Day 2
None	4.13 ± 0.7	8.10 ± 1.7	8.08 ± 1.0	15.48 ± 1.1
3.0	3.68 ± 0.4	6.64 ± 0.8	6.38 ± 0.7	12.33 ± 1.3
3.0	2.33 ± 0.3	5.44 ± 1.0	5.38 ± 0.8	10.05 ± 0.3
5.0	2.32 ± 0.50	6.32 ± 1.5	4.22 ± 0.5	8.13 ± 0.8

[c] These cells were not exposed to colchicine.

[d] Thirty-minute exposure to colchicine.

TABLE XVII

EFFECT OF PBS-LOADED Lp-D and Lp-D-TLCK (TYPE B)
ON THE GROWTH OF
BALB/C 3T3 AND SVT_2 CELLS IN CULTURE[a]

	% Cells Attached[b]			
ara-C	BALB/C 3T3		SVT_2	
(nmoles)	Day 1	Day 2	Day 1	Day 2
None[c]	196.7	385.7	207.2	396.4
Lp-D	190.5	376.2	210.3	423.1
Lp-D-TLCK	195.2	466.7	205.1	405.1

[a] Balb/C 3T3 (1.6 to 1.8 x 10^5) and SVT_2 (1.4 to 1.8 x 10^5) cells were incubated in MEM x 4 for 24 hours at 37°C. The cells were then exposed to PBS-loaded Lp-D (13.1 nmoles digoxin) and Lp-D-TLCK (11.7 nmoles digoxin) both type B in MEM x 4 for 60 minutes at 37°C. The cells were washed once with PBS and were incubated in 4.0 mL of fresh MEM x 4 for up to two days. At Day 0, the Balb/C 3T3 and SVT_2 cell densities were 2.10 x 10^5 and 3.90 x 10^5 respectively. The data is expressed as mean percent cell counts ± SEM of two similar experiments.

[b] Calculated from the formula: $\frac{\text{No. of Cells Attached (Exposed)}}{\text{No. of Cells Attached at Day 0 (Unexposed)}} \times 100$

TABLE XVII (cont'd)

No. of cells attached (x 10^{-5}):

	BALB/C 3T3		SVT_2	
	Day 1	Day 2	Day 1	Day 2
None[c]	4.13 ± 0.7	8.08 ± 1.7	8.08 ± 1.0	15.48 ± 1.1
Lp-D	4.00 ± 0.6	7.90 ± 1.4	8.20 ± 1.3	16.50 ± 1.2
Lp-D-TLCK	4.10 ± 0.5	9.80 ± 2.4	8.00 ± 0.9	15.80 ± 1.2

[c]These cells were not exposed to the liposomes.

TABLE XVIII

CYTOTOXIC EFFECT OF ara-C ENCAPSULATED Lp-D AND Lp-D-TLCK (TYPE B) ON THE GROWTH OF BALB/C 3T3 AND SVT_2 CELLS IN CULTURE[a]

Exposure (min.)		% Cells Attached[b] BALB/C 3T3 Day 1	BALB/C 3T3 Day 2	BALB/C 3T3 Day 3	SVT_2 Day 1	SVT_2 Day 2	SVT_2 Day 3
	None	203.7	387.9	--	193.3	403.4	--
20	Lp-D	189.8	386.1	--	189.9	360.7	--
	Lp-D-TLCK	184.3	396.3	--	132.6	298.9	--
	None	205.7	365.7	622.9	216.1	454.2	1000.0
30	Lp-D	191.4	402.9	637.1	187.1	480.7	877.4
	Lp-D-TLCK	197.1	377.1	602.9	158.1	341.9*	664.5*
	None	203.7	387.9	--	193.3	403.4	--
50	Lp-D	176.9	400.0	--	176.4	349.4	--
	Lp-D-TLCK	173.2	394.4	--	133.7*	268.5*	--

TABLE XVIII(cont'd)

Exposure (min.)		% Cells Attached[b]					
		BALB/C 3T3			SVT_2		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
60	None	210.9	354.4	--	209.1	422.4	--
	Lp-D	206.5	356.5	--	193.9	407.1	--
	Lp-D-TLCK	208.7	371.7	--	139.4*	310.0*	--

[a]Balb/C 3T3 and SVT_2 cells were exposed to Lp-D (13.1 nmoles digoxin containing 14.2 nmoles ara-C) and Lp-D-TLCK (11.7 nmoles digoxin containing 11.6 nmoels ara-C) in MEM x 4 for the stated periods at 37°C. The cells were washed once with PBS and were incubated in 4.0 mL of fresh MEM x 4 for two or three days. At Day 0, the Balb/C 3T3 and SVT_2 cell densities were 10.8×10^5 and 8.9×10^5 for 20 and 50 min. exposure respectively; 3.5×10^5 and 3.1×10^5 for 30 min. exposure; 4.6×10^5 and 10.1×10^5 for 60 min. exposure. The data is expressed as mean percent cell counts ± SEM of four similar experiments.

[b]Calculated from the formula: $\frac{\text{No. of Cells Attached (Exposed)}}{\text{No. of Cells Attached at Day 0 (Unexposed)}} \times 100$

(continued)

TABLE XVIII (cont'd)

No. of Cells attached ($x10^{-5}$):

Exposure (Min.)		BALB/C 3T3 Day 1	BALB/C 3T3 Day 2	BALB/C 3T3 Day 3	SVT_2 Day 1	SVT_2 Day 2	SVT_2 Day 3
20	None[C]	22.02 ± 1.9	41.9 ± 4.0	--	17.2 ± 2.6	35.9 ± 3.6	--
	Lp-D	20.5 ± 2.4	41.7 ± 3.3	--	16.9 ± 2.2	32.1 ± 3.0	--
	Lp-D-TLCK	19.9 ± 1.8	42.8 ± 3.1	--	11.8 ± 1.7	26.6 ± 3.5	--
30	None[C]	7.1 ± 0.8	12.8 ± 1.3	21.8 ± 7.1	6.7 ± 0.8	16.9 ± 1.4	31.0 ± 1.9
	Lp-D	6.7 ± 0.9	14.1 ± 1.2	22.3 ± 2.2	5.8 ± 1.0	14.9 ± 1.7	27.2 ± 2.7
	Lp-D-TLCK	6.9 ± 1.2	13.1 ± 1.4	21.1 ± 2.3	4.9 ± 1.4	10.6 ± 1.2	20.6 ± 2.1
50	None[C]	22.0 ± 1.9	41.9 ± 4.0	--	17.2 ± 2.6	35.9 ± 3.6	--
	Lp-D	19.1 ± 2.3	43.2 ± 3.0	--	15.7 ± 2.2	31.1 ± 2.9	--
	Lp-D-TLCK	18.7 ± 2.2	42.6 ± 2.8	--	11.9 ± 1.9	23.9 ± 3.4	--
60	None[C]	9.7 ± 1.4	16.3 ± 2.0	--	20.7 ± 1.9	43.8 ± 1.4	--
	Lp-D	9.5 ± 1.7	16.4 ± 2.2	--	19.2 ± 2.3	40.3 ± 2.1	--
	Lp-D-TLCK	9.6 ± 1.3	17.1 ± 1.8	--	13.8 ± 1.6	29.8 ± 1.8	--

[C]These cells were not exposed to the liposomes.

*Significant when compared to control (unexposed) cells ($P \gg 0.001$)

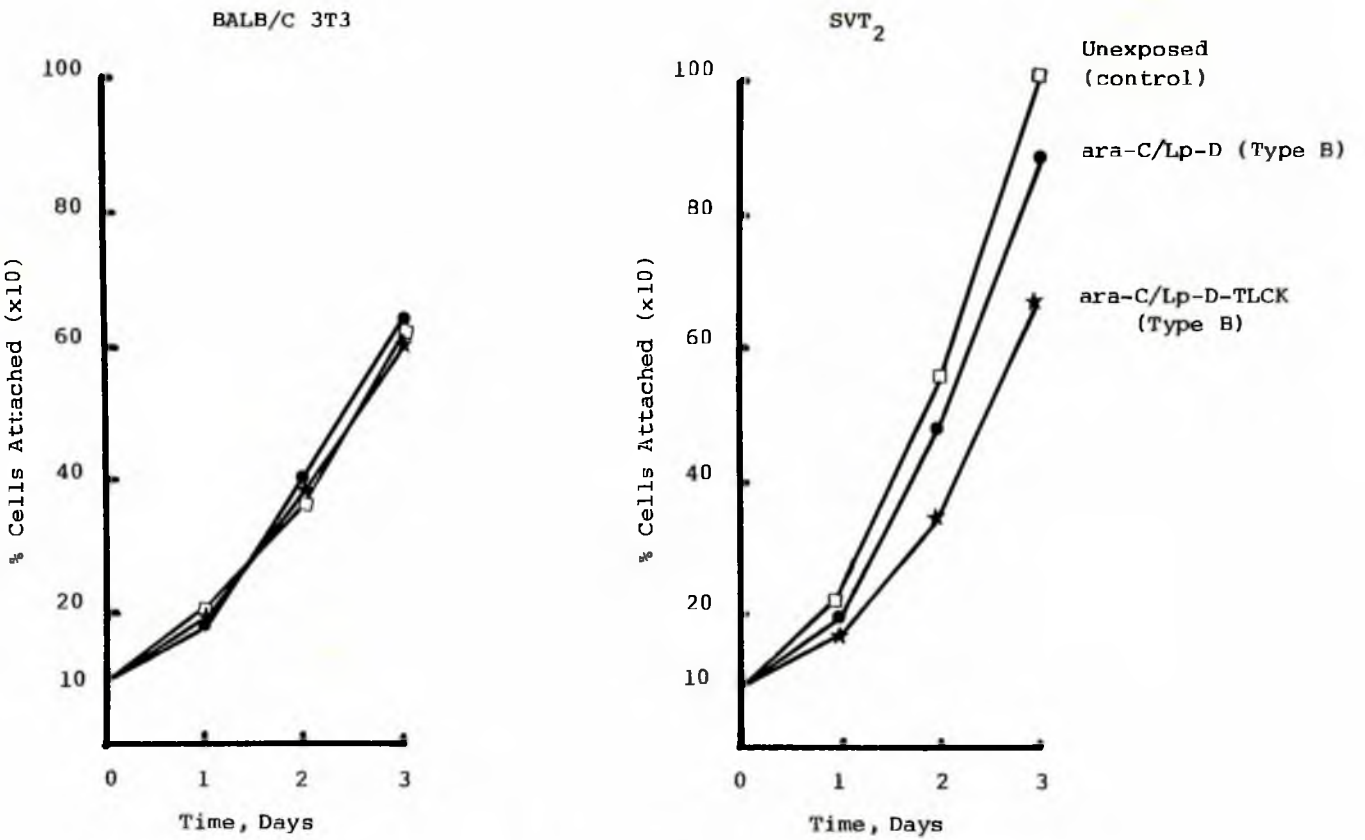

Figure 6. Growth of Balb/C 3T3 and SVT_2 cells following 30-minute exposure to ara-C encapsulated Lp-D and Lp-D-TLCK (Type B). The experimental conditions are as described in the legend for Table XVIII.

up to 60 minutes. In contrast the Lp-D containing ara-C exhibited much less cytotoxicity to the SVT_2 cells though the amount of ara-C in Lp-D-TLCK (type B) was about 18% lower than that entrapped in Lp-D (type B). Thus TLCK on the Lp-D-TLCK potentiated the effect of ara-C toward the SVT_2 cells. Furthermore, since the PBS-loaded Lp-D-TLCK (type B) was without cytotoxic effect, the growth inhibition of SVT_2 cells was attributable to the ara-C entrapped in Lp-D-TLCK. These results are consistent with the observation that uptake of 6-CF entrapped in Lp-D-TLCK (type B) by the SVT_2 cells was much greater than the uptake of 6-CF entrapped in Lp-D (Table XII and Figure 4).

b. Lp-D and Lp-D-TLCK (Type A)

The effect of Lp-D and Lp-D-TLCK (both type A) containing ara-C on the growth of Balb/C 3T3 and SVT_2 cells was also investigated. Unlike the Lp-D which impaired the growth of Balb/C 3T3 cells during day 1, the Lp-D-TLCK was not toxic to the cells (Table XIX and Figure 7). With respect to the transformed cells, the Lp-D impaired the growth of SVT_2 cells at day 1 while Lp-D-TLCK did not. On the other hand, Lp-D-TLCK inhibited the growth of SVT_2 cells from day 2. The results again suggested that TLCK enhanced Lp-D-TLCK interaction with SVT_2 cells.

4. Effects of Colchicine Containing Lp-D and Lp-D-TLCK (Type B)

Both Lp-D and Lp-D-TLCK (type B) containing 5.5 and 5.0 nmoles colchicine, respectively, retarded the growth

TABLE XIX

CYTOTOXIC EFFECT OF ara-C ENCAPSULATED Lp-D AND Lp-D-TLCK (TYPE A) ON THE GROWTH OF BALB/C 3T3 AND SVT_2 CELLS IN CULTURE[a]

Exposure (min.)	% Cells Attached[b] BALB/C 3T3 Day 1	BALB/C 3T3 Day 2	BALB/C 3T3 Day 3	SVT_2 Day 1	SVT_2 Day 2	SVT_2 Day 3
None[c]	205.7	365.7	622.9	216.1	545.2	1000.0
Lp-D	168.6	362.9	637.1	180.7	522.6	987.1
Lp-D-TLCK	202.9	360.0	602.9	222.6	429.0	609.7

[a] Balb/C 3T3 (1.5 to 1.7 x 10^5) and SVT_2 (1.4 to 1.6 x 10^5) cells were inoculated into 60 x 15 mm plastic petri dishes and were incubated in MEM x 4 for 24 hours at 37°C. The cells were exposed to Lp-D (27.9 nmoles digoxin containing 32.9 nmoles ara-C) and Lp-D-TLCK (25.7 nmoles digoxin containing 31.6 nmoles ara-C) in MEM x 4 for 30 minutes at 37°C. The cells were washed once with PBS and were incubated in 4.0 mL of fresh MEM x 4 at 37°C for three days. At Day 0, the Balb/C 3T3 and SVT_2 cell densities were 3.5 x 10^5 and 3.1 x 10^5 respectively. The data is expressed as mean percent cell counts ± SEM of four similar experiments.

[b] Calculated from the formula: $\frac{\text{No. of Cells attached (Exposed)}}{\text{No. of Cells Attached at Day 0 (Unexposed)}} \times 100$

TABLE XIX (cont'd)

No. of cells attached (x 10^{-5}):

	BALB/C 3T3			SVT_2		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
None	7.2 ± 0.9	12.8 ± 1.3	21.8 ± 2.1	6.7 ± 0.8	16.9 ± 1.4	31.0 ± 1.9
Lp-D	5.9 ± 1.3	12.7 ± 1.4	22.3 ± 2.2	5.6 ± 0.9	16.2 ± 1.3	30.6 ± 2.7
Lp-D-TLCK	7.1 ± 0.8	12.6 ± 1.5	21.1 ± 2.3	6.9 ± 1.2	13.3 ± 1.7	18.9 ± 1.8

[c]These cells were not exposed to the liposomes.

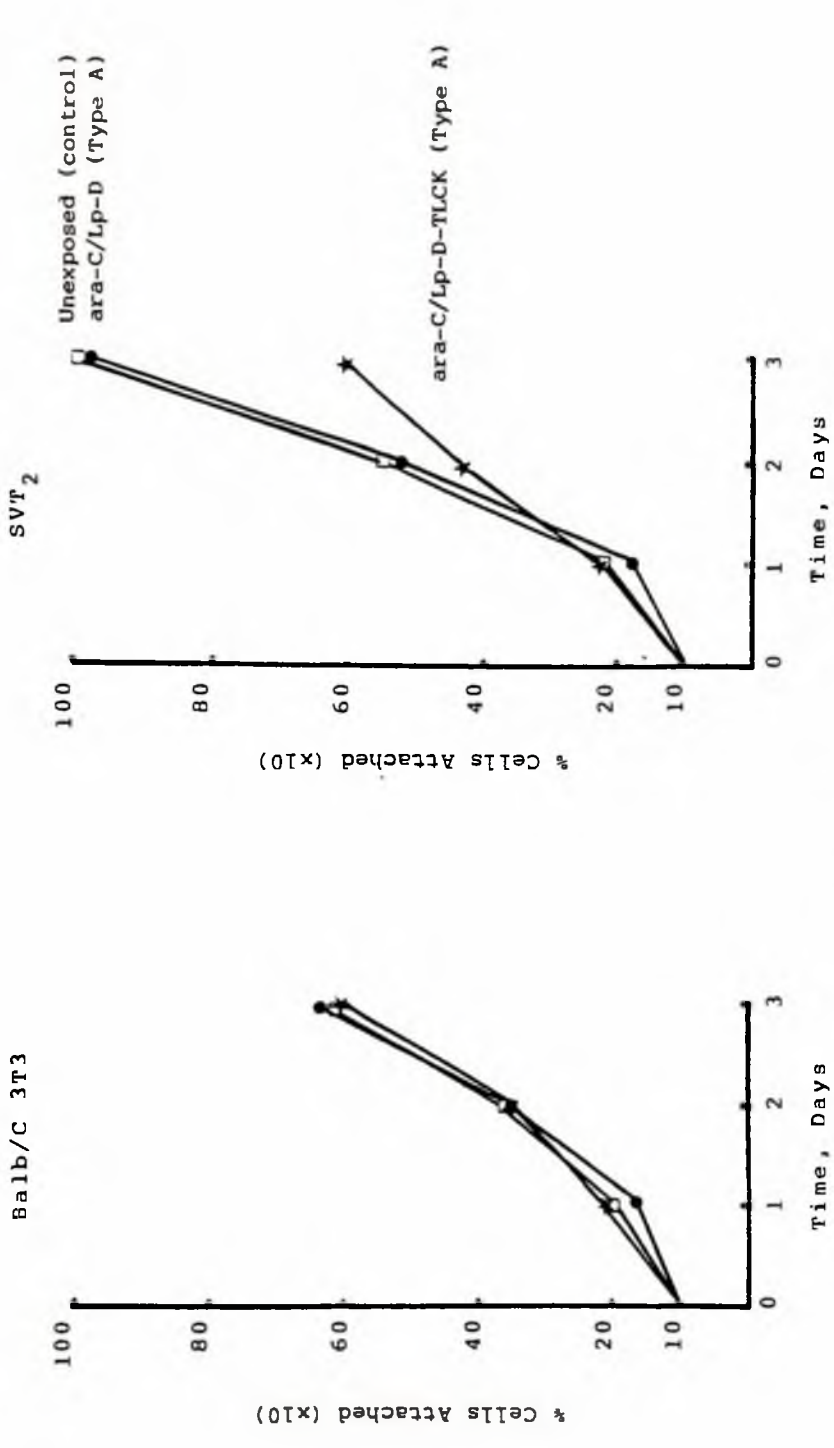

Figure 7. Growth of Balb/C 3T3 and SVT_2 cells following 30-minute exposure to ara-C encapsulated Lp-D and Lp-D-TLCK (Type A). The experimental conditions are as described in the legend for Table XIX.

TABLE XX

CYTOTOXIC EFFECT OF COLCHICINE ENCAPSULATED Lp-D AND Lp-D-TLCK (TYPE B) ON THE GROWTH OF BALB/C 3T3 AND SVT_2 CELLS IN CULTURE[a]

	% Cells Attached[b]			
	BALB/C 3T3		SVT_2	
	Day 1	Day 2	Day 1	Day 2
None[c]	187.2	338.5	194.6	462.2
Lp-D	112.8	126.4	136.8	214.9
Lp-D-TLCK	151.3	159.0	61.6	83.2

[a] Balb/C 3T3 (2.0 to 2.2 x 10^5) and SVT_2 (1.8 to 2.0 x 10^5) cells were inoculated into 60 x 15 mm plastic petri dishes and were incubated in MEM x 4 for 24 hours at 37°C. The cells were then exposed to Lp-D (13.1 nmoles digoxin containing 5.5 nmoles colchicine) and Lp-D-TLCK (11.7 nmoles digoxin containing 5.0 nmoles colchicine) both type B in MEM x 4 for 15 minutes at 37°C. At Day 0, the Balb/C 3T3 and SVT_2 cell densities were 3.9 x 10^5 and 3.7 x 10^5 respectively. The cells were washed once with PBS and were incubated in 4.0 of fresh MEM x 4 at 37°C for two days. The data is expressed as mean percent cell counts ± SEM of four similar experiments.

[b] Calculated from the formula: $\frac{\text{No. of Cells Attached (Exposed)}}{\text{No. of Cells Attached at Day 0 (Unexposed)}} \times 100$

TABLE XX (cont'd)

No. of cells attached (x 10^{-5}):

	BALB/C 3T3		SVT_2	
	Day 1	Day 2	Day 1	Day 2
None[C]	7.30 ± 1.4	13.20 ± 2.1	7.20 ± 1.3	17.10 ± 1.9
Lp-D	4.40 ± 1.2	4.93 ± 0.9	5.06 ± 0.8	7.95 ± 1.1
Lp-D-TLCK	5.90 ± 1.1	6.20 ± 1.5	2.28 ± 0.7	3.08 ± 0.5

[C]These cells were not exposed to the liposomes.

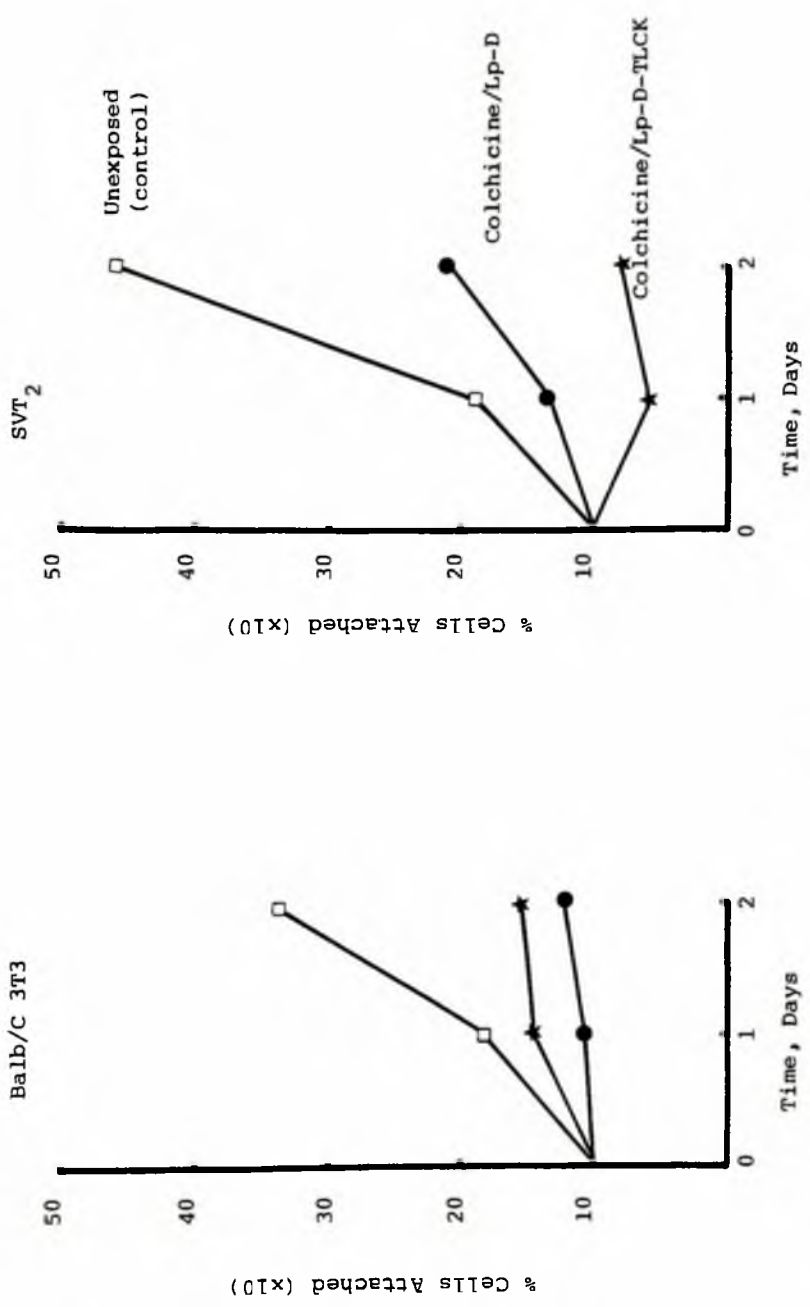

Figure 8. Growth of Balb/C 3T3 and SVT_2 cells following 15-minute exposure to colchicine encapsulated Lp-D and Lp-D-TLCK (Type B). The experimental conditions are as described in the legend for Table XX.

of Balb/C 3T3 cells following 15 minutes exposure to the liposomes (Table XX and Figure 8). However Lp-D was more effective than Lp-D-TLCK toward the cells. With respect to the transformed cells, colchicine entrapped in Lp-D-TLCK (type B) was particularly toxic compared to colchicine entrapped in Lp-D.

5. Effects of Lp-PE, Lp-G-TLCK, Lp-G-Lysine With or Without ara-C

Growth inhibition of Balb/C 3T3 cells following 20 minutes exposure to Lp-PE containing 47 nmoles ara-C was clearly seen at day 1 but again as for the Lp-D experiment, the cells resumed their normal growth (Table XXI and Figure 9). In contrast, the Lp-G-TLCK was not toxic to the cells. However, when the exposure period was increased to 50 minutes the Lp-PE as well as the Lp-G-TLCK was toxic to Balb/C 3T3 cells. For the SVT_2 cells both Lp-PE and Lp-G-TLCK were cytotoxic regardless of whether the cells were exposed for 20 or 50 minutes. The results therefore indicate that the period of liposomal exposure to the cells is critical in determining toxicity of Lp-G-TLCK. Furthermore, since Lp-G-Lysine without ara-C did not inhibit the growth of the SVT_2 cells but that of Lp-PE displayed cytotoxicity at day 1, there is an indication that the liposomal amino groups might be toxic to the cells.

TABLE XXI

CYTOTOXIC EFFECT OF ara-C ENCAPSULATED Lp-PE, Lp-G-TLCK AND PBS-LOADED LIPOSOMES ON THE GROWTH OF BALB/C 3T3 AND SVT_2 CELLS IN CULTURE[a]

Exposure (min.)		% Cells Attached[b]			
		BALB/C 3T3		SVT_2	
		Day 1	Day 2	Day 1	Day 2
20	None[c]	164.8	262.9	163.2	600.0
	Lp-PE	124.8	254.3	121.1	247.4*
	Lp-G-TLCK	154.3	261.9	121.6	244.7*
50	Lp-PE	85.2	208.1	86.8	301.1*
	Lp-G-TLCK	88.6	214.8	91.6	331.1*
	Lp-PE[d]	--	--	118.9	577.4
	Lp-G-Lysine[d]	--	--	181.1	605.8

[a] Balb/C 3T3 (1.1 to 1.3 x 10^5 and SVT_2 (1.0 x 1.2 x 10^5) cells were inoculated into 60 x 15 mm plastic petri dishes and were incubated in MEM x 4 for 24 hours at 37°C. The cells were then exposed to Lp-PE (204 nmoles phosphatidylethanolamine containing 47 nmoles ara-C) and Lp-G-TLCK (containing 33 nmoles ara-C) in MEM x 4 at 37°C. The cells were washed once with PBS and were incubated in 4.0 mL of fresh MEM x 4 at 37°C for two days. At Day 0, the Balb/C 3T3 and SVT_2 cell densities were 2.1 x 10^5 and 1.9 x 10^5 respectively. The data is expressed as mean percent cell counts ± SEM of three similar experiments.

[b] Calculated from the formula: $\frac{\text{No of Cells Attached (Exposed)}}{\text{No of Cells Attached at Day 0 (Unexposed)}} \times 100$

TABLE XXI (cont'd)

No. of cells attached (x 10^{-5}):

Exposure (Min.)		BALB/C 3T3		SVT_2	
		Day 1	Day 2	Day 1	Day 2
	None	3.46 ± 0.6	5.52 ± 0.8	3.10 ± 0.5	11.40 ± 2.3
20	Lp-PE	2.62 ± 0.9	5.34 ± 0.6	2.30 ± 0.4	4.70 ± 0.6
	Lp-G-TLCK	3.24 ± 0.4	5.50 ± 1.0	2.31 ± 0.6	4.65 ± 1.0
50	Lp-PE	1.79 ± 0.5	4.37 ± 1.0	1.65 ± 0.3	5.72 ± 1.4
	Lp-G-TLCK	1.89 ± 0.3	4.51 ± 0.9	1.74 ± 0.2	6.29 ± 1.1
	Lp-PE[d]	--	--	2.26 ± 1.5	10.97 ± 2.9
	Lp-G-Lysine[d]	--	--	3.44 ± 1.8	11.51 ± 2.7

[c] These cells were not exposed to the liposomes.

[d] Liposomes were devoid of ara-C.

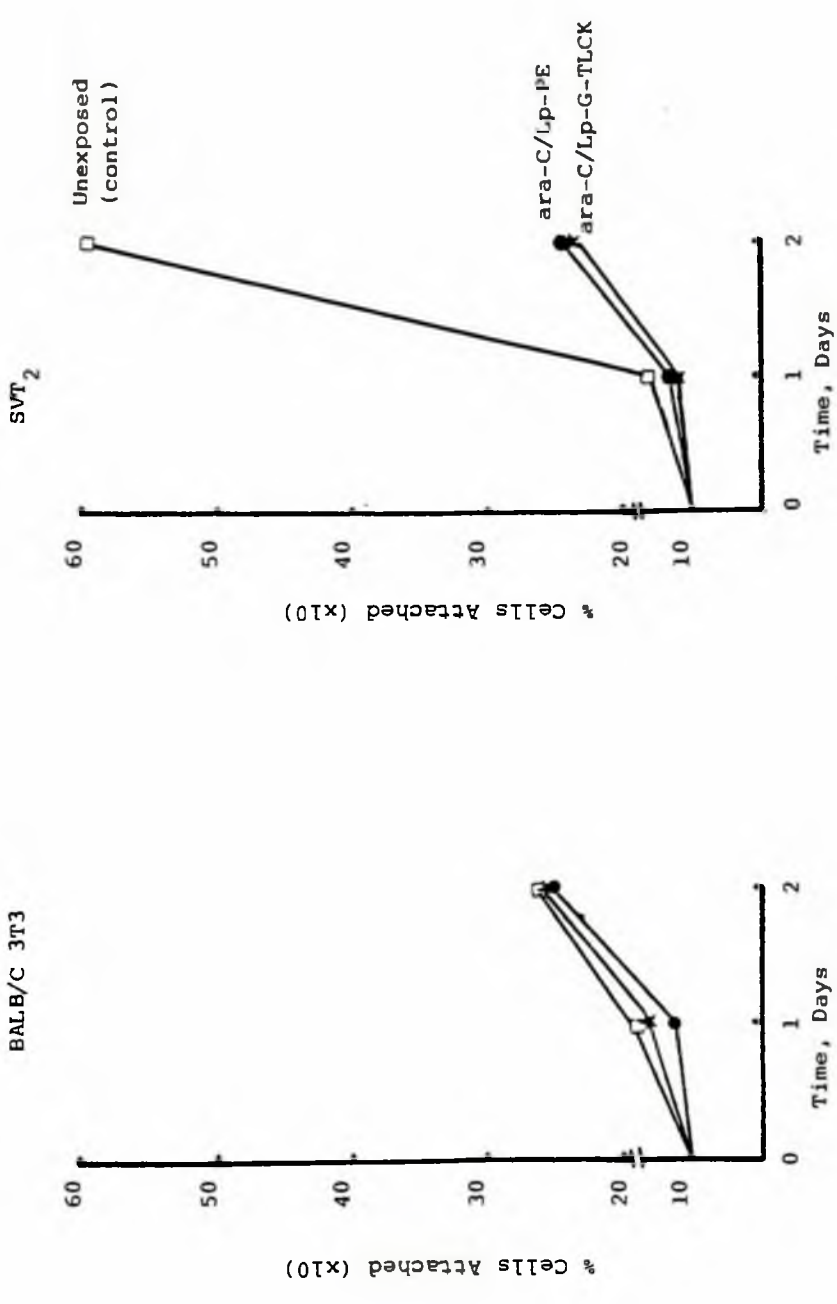

Figure 9. Growth of Balb/C 3T3 and SVT_2 cells following 20-Minute exposure to ara-C encapsulated Lp-PE and Lp-G-TLCK. The experimental conditions are as described in the legend for Table XXI.

CHAPTER IV

DISCUSSION

A. Protease-like Activity of Transformed Cells

The surface of transformed (tumor) cells is thought to have protease-like activity as evidenced by the binding and inhibition of the growth of Simian virus 40, Polyoma virus transformed hamster or mouse cells by antiproteases, e.g. TLCK (74). The binding of the protease inhibitor to the tumor cells does not necessarily effect the inhibition of such cellular processes as DNA synthesis (110) or the cell viability since the cells are capable of normal growth following removal of the inhibitor. The TLCK was also shown, in a concentration dependent manner, to reduce the adhesion of Ehrlich ascites tumor cells to plastic surfaces (75), delayed the appearance of mouse skin tumor (76) and induced contact inhibition of hamster cells bearing fibrosarcoma (77). These observations raised the possibility that the TLCK should, when attached to liposomes, enhance binding of the liposomes to transformed cells and thus making the delivery of liposome-entrapped antitumor agent considerably more efficient. To test this possibility TLCK was covalently coupled to the liposomes containing antitumor drugs and was applied to the model cell systems, SVT_2 (Simian virus 40 transformed swiss mouse

fibroblast) and Balb/C 3T3 (untransformed cells).

B. TLCK-bound Liposome

1. Preparation

In order to couple TLCK with the liposomes, two distinct types of liposomes were used. The liposomes incorporated either digoxin or phosphatidylethanolamine. The digoxin incorporated liposome was treated with sodium metaperiodate to oxidize the oligosaccharide side chain of digoxin to form aldehyde groups which then reacted with the amino group of the TLCK (H_2N-CH_2-TLCK) as illustrated in Appendix E. The digoxin was used mainly for economic reasons but it could be substituted, in *in vivo* studies, with either cerebroside or ganglioside whose carbohydrate side chain could similarly be oxidized to generate aldehyde groups. For the phosphatidylethanolamine incorporated liposome, the amino groups were first treated with glutaraldehyde to form aldehyde groups. Another method for attaching TLCK with the liposomes was also considered. For instance, liposomes bearing sulfhydryl groups could couple the TLCK since tosly group of the TLCK ($C_2H_7-\overset{O}{\overset{\|}{S}}-$TLCK) can react with the sulfhydryl groups of cysteine and glutathione (111, 112). However, the preparation of such a liposome could lead to the probable loss of the chloromethyl ketone group of the TLCK (TLCK-$\overset{O}{\overset{\|}{C}}-CH_3-Cl$) *via* nucleophilic substitution. Since the chloromethyl ketone group is of outmost importance for TLCK antiprotease activity

(103), this method for binding TLCK to the liposome could effect the coupling of an inactive portion of the TLCK to liposomes.

All the liposomes used in the present study contained phosphatidylcholine dipalmitoyl, cholesterol and dicetyl-phosphate. The two former lipids provide stability to the liposomes while the latter confer negative charge characteristics. Thus both types of liposomes employed in this study have a net negative charge. When the lipid mixture is suspended in aqueous solution, it results in the formation of multilamellar liposomes whose size vary from 50 to 1000 nm in diameter (4, 9, 11). Sonication of the multilamellar liposomes reduces their size whose diameter ranges from 25 to 200 nm. The liposomes with the diameter of 25 to 50 nm are unilamellar (9, 11, 12, 19). Therefore sonication alone does not fully effect formation of unilamellar liposomes. The large liposomes can be separated by centrifugation to form a more homogenous suspension of liposomes. The centrifugation step results in about 50% yield of unilamellar liposomes. In vivo, the unilamellar liposomes are retained in the circulation for a longer period since they are cleared less rapidly by the liver and spleen than their multilamellar counterparts (27, 28, 52). However, the latter can entrap much more substance than the unilamellar liposome.

Liposomes have been shown to entrap a wide variety of substances including drugs (19, 20, 28, 45, 49), enzymes

(35, 36, 44) and immune stimulating factors (46, 47). The lipid composition of liposomes and the solubility of entrapping molecule(s), among other factors, are important in determining the extent of entrapment as well as retention of the molecules in liposomes. Cholesterol and phosphatidylcholine dipalmitoyl are important in maintaining liposome stability and minimizing leakage of entrapped molecules (6, 7, 11). Further, the ratio of cholesterol to phosphatidylcholine dipalmitoyl influences the rate of spontaneous leakage of molecules from the liposomes. For instance, when the ratio of cholesterol to the phosphatidylcholine dipalmitoyl is greater than one, the membrane of the liposome becomes less fluid and the leakage of the entrapped molecule(s) results (6).

Horseradish peroxidase (HRP) and 6-carboxyfluorescein (6-CF) were entrapped in the liposomes to study the binding of liposomes to the Balb/C 3T3 and SVT_2 cells because the HRP and 6-CF are readily available, sensitive and they have been found to be effective as markers in studying liposome interaction with cells *in vitro* and *in vivo* (7, 16). Liposomes prepared with radiolabelled lipids or entrapping radiolabelled molecules can also be used to study the binding of liposomes to cells. For the liposome containing HRP, treatment with the glutaraldehyde (0.050 mmoles, pH 7.2) consistently resulted in the loss of HRP activity. It is unlikely that the glutaraldehyde had a direct adverse effect on the enzyme because treatment of

the free enzyme with 0.250 mmoles glutaraldehyde did not affect the HRP activity (104).

During the preparation of phosphatidylethanolamine incorporated liposome, the amount of the phosphatidyl-ethanolamine relative to phosphatidylcholine dipalmitoyl was found to be critical in producing non-aggregated lipo-some. When 0.040 mmoles of phosphatidylcholine dipalmitoyl and 0.0135 mmoles of phosphatidylethanolamine (molar ratio of approximately 3:1) were mixed with 0.0114 mmoles of cholesterol and 5.7×10^{-3} mmoles of dicetylphosphate the liposomes did not aggregate. However, the liposomes began to aggregate when the molar ratio of the phosphatidylcho-line dipalmitoyl to phosphatidylethanolamine decreased to less than one. Similar observation was made by Papahajo-poulos and Miller (9) who suggested that interaction be-tween the positively charged amino groups and negatively charged phosphate groups of the phosphatidylethanolamine is responsible for the aggregation.

2. Characteristics of TLCK-bound Liposome

The covalent binding of TLCK to the liposomes was assessed by spectrophotometric analysis and the inactiva-tion of trypsin. The results showed that both the TLCK-bound liposome and free TLCK displayed absorption peaks at 232 and 229 nm, respectively, whereas the TLCK-free liposomes did not display the characteristic absorption peak in this wavelength region. The slight shift in the absorbance wavelength of the bound TLCK is probably due to the con-

jugation of the TLCK with the liposome. To determine whether the liposome-bound TLCK remained active, antitrypsin activity of the TLCK-bound liposome was assessed. Depending on the liposome, the coupling of the TLCK under the prescribed conditions led to about 47 to 93% loss of activity. Since the amino group of TLCK is known to be essential for antitrypsin activity (103), it is likely that the loss of the antitrypsin activity of the bound TLCK is due to conversion of the amino group to Schiff's base when it reacts with the aldehyde groups. An alternative explanation is that the binding of TLCK to a relatively large liposome may sterically hinder its interaction with the active site of trypsin. However, since the bound TLCK still retained 7 to 53% activity, there is an indication that the intended role of TLCK could be demonstrated with the model cell systems.

C. Interaction of Liposomes with Cells

The interaction of liposomes with cells was assessed in two phases. First, the role of TLCK for liposome binding and uptake by the model cells was investigated. The uptake of the liposomes was followed by measuring 6-CF or HRP after incubation with the SVT_2 and Balb/C 3T3 cells. Second, the effect of the TLCK-bound liposome containing antitumor drug on the growth of SVT_2 and Balb/C 3T3 cells was investigated.

1. Uptake of Liposomes by Cells

The relative recovery of the 6-CF entrapped in Lp-D-TLCK from the SVT_2 cells was much higher, reaching maximum by 60 minutes of incubation when compared with that recovered from Balb/C 3T3 cells, although the SVT_2 cells are much smaller (0.012 vs. 0.043 mm^2 in surface area). Furthermore, the reversal of the uptake pattern of the liposomes by the Balb/C 3T3 cells indicated that the TLCK in some manner mediated the interaction of the liposomes with the Balb/C 3T3 cells. The preferential binding of the Lp-D-TLCK by the SVT_2 cells is consistent with the concept that transformed cells possess protease-like activity on their surface (71, 74). Since TLCK has affinity for various types of tumor cells, including Ehrlich ascites tumor cells (75), it can potentially enhance the binding of liposomes to other tumor cells as well. A preliminary study *in vivo* indicated that ascites tumor cells in the peritoneal cavity of mice appears to bind preferentially with the TLCK-bound liposome (Appendix F).

A similar study with the Lp-G-TLCK containing HRP also indicated that binding of the TLCK derivatized liposomes by the SVT_2 cells was higher for periods up to 45 minutes, but upon further incubation the amount of the Lp-G-TLCK taken up by the Balb/C 3T3 cells was higher. Although the reason for such observation is not clear, it seems that electrostatic attraction between the positively charged amino groups of the Lp-PE with the Balb/C 3T3 cells

play a predominant role because the Balb/C 3T3 cells have about twice as much sialic acid as SVT_2 cells (102). The electrostatic force as a mechanism of cell-liposome interaction has been indicated by the binding of stearylamine incorporated liposomes to HeLa cells (16). Thus the binding of Lp-PE to the membrane of the Balb/C 3T3 cells is expected to occur by electrostatic means. While the Lp-PE entrapped HRP is recoverable from the cytosol of the Balb/C 3T3 cells, some of the entrapping HRP will remain associated with the Balb/C 3T3 cell membrane. When compared with the Lp-PE, the electrostatic attraction between the Balb/C 3T3 cell and the Lp-G-TLCK is drastically reduced since about 80% of the positively charged amino groups of the Lp-G-TLCK was derivatized.

2. Effects of Liposomes on Cell Growth

a. ara-C Containing Liposomes

Liposomes containing such chemotherapeutic drug as ara-CTP had been shown to be more efficient in inhibiting the growth of tumors in vitro than the free drug (45, 107). However, entrapment of drug in liposome does not always enhance drug action. Mayhew et al (45), for instance, showed that free and liposome entrapped ara-C was equally cytotoxic to L1210 murine leukemic cells in vitro. The authors ascribed their observation to inactivation of excess 1-β-D-arabinofuranosyl cytosine (ara-C) by deoxycytidine deaminase to 1-β-D-arabinofuranosyl uracil which has no antitumor activity. The entrapment of drug in lipo-

somes also minimizes contact between the entrapped drug and blood macromolecules *in vivo* (25). Thus, if a liposome is made to deliver antitumor drugs to tumor cells, it will reduce cytotoxic side effects to the non-target cells, a major problem associated with the use of free drugs (113, 114). From the experimental results it is apparent that ara-C, when entrapped in the Lp-D-TLCK, displayed inhibitory activity against SVT_2 cells without significantly altering the growth of the Balb/C 3T3 cells. The inhibitory activity against the SVT_2 cells is due to the entrapped ara-C and not other liposomal components including TLCK, since the liposomes without ara-C did not affect the growth of the cells. In similar studies the ara-C entrapped Lp-G-TLCK displayed selective inhibitory activity against SVT_2 cells only when the exposure did not exceed 20 minutes; the growth of both the SVT_2 and Balb/C 3T3 cells was inhibited when the exposure was 50 minutes. Such loss of selectivity was, however, transitory since, unlike the SVT_2 cells, the Balb/C 3T3 cells recovered their growth by the second day. Since in the presence of ara-C entrapped Lp-PE the Balb/C 3T3 cells did not display such a recovery of growth, it can be concluded that the binding of the ara-C entrapped Lp-PE to the Balb/C 3T3 cells is higher than that of the ara-C entrapped Lp-G-TLCK. This observation supports the earlier suggestion that electrostatic attraction between the Lp-PE and Balb/C 3T3 cells played a dominant role in the binding of the Lp-PE with the

Balb/C 3T3 cells.

b. Colchicine Containing Liposomes

The inhibitory activity of colchicine entrapped Lp-D-TLCK was also investigated. Colchicine accomplished its cytotoxicity by binding and disrupting microtubular aggregation and cell mitosis (108, 109). The colchicine is also known to inhibit nucleoside transport in mammalian cells (115). From the experimental results it is clear that the colchicine entrapped Lp-D-TLCK inhibited the growth of both Balb/C 3T3 and SVT_2 cells, but the SVT_2 cells were particularly sensitive. It should be noted that both the Balb/C 3T3 and SVT_2 cells were exposed to the colchicine entrapped Lp-D-TLCK during their exponential growth phase when cellular mitotic activity and microtubular concentrations are high. Such conditions are known to increase a cell's susceptibility to colchicine (108, 109). Furthermore, since colchicine readily permeates the liposomes, it could effect Balb/C 3T3 cell cytotoxicity independent of the targeted colchicine (colchicine that remained entrapped in the Lp-D-TLCK). Thus it is apparent that colchicine is not a suitable antitumor drug for use in cell culture systems with normal cells in their exponential growth phase. It should be pointed out that the preceding observation did not materialize for ara-C which effects cell death by inhibiting DNA synthesis after the conversion of 1-ß-D-arabinofuranosyl cytosine to 1-ß-D-arabinofuranosyl cytidine triphos-

phate (ara-CTP) by deoxycytidine kinase (105,106). Thus, unlike colchicine, ara-C entrapped Lp-D-TLCK selectively displayed inhibitory activity against the SVT_2 cells without significantly inhibiting the growth of the Balb/C 3T3 cells.

D. Summary

1. The protease inhibitor, 1-chloro-3-tosyl-amido-7-amino-2-heptanone, commonly known as tosyl-L-lysyl-chloromethyl ketone (TLCK), was covalently coupled to two types of liposomes containing either digoxin (Lp-D-TLCK) or phosphatidylethanolamine (Lp-G-TLCK).

2. The TLCK-bound liposome remained active against trypsin. However, depending on the liposome, the anti-trypsin activity of the TLCK-bound liposome, varied from 7 to 53%.

3. The TLCK enhanced binding of the liposomes (particularly the Lp-D-TLCK) to SVT_2 cells.

4. The Balb/C 3T3 cells bind preferentially with the TLCK-free liposome.

5. The ara-C entrapped Lp-D-TLCK displayed inhibitory activity against the SVT_2 cells without any significant inhibition of the growth of the Balb/C 3T3 cells.

6. The ara-C entrapped Lp-G-TLCK selectively inhibited the growth of the SVT_2 cells when the exposure period was not more than 20 minutes. An increased exposure period (50 minutes), on the other hand, inhibited both the Balb/C

3T3 and SVT_2 cells. However, the Balb/C 3T3 cells recovered its growth by the second day.

7. The colchicine entrapped Lp-D-TLCK readily permeated the liposome and displayed inhibitory activity against both Balb/C 3T3 and SVT_2 cells but was particularly toxic to the SVT_2 cells.

8. Ehrlich ascites tumor cells bind preferentially to TLCK-bound liposome (Appendix F) in in vivo.

The preferential binding and uptake of the Lp-D-TLCK by the two different types of transformed cells (SVT_2 cells in vitro and Ehrlich ascites tumor cells in vivo after replacing the digoxin with cerebroside) indicate that, the TLCK-bound liposome could deliver entrapped antitumor drug(s) to a variety of transformed cells. The selective inhibition of the SVT_2 cells by the ara-C entrapped Lp-D-TLCK further indicate that the TLCK-bound liposome could prevent cytotoxicity of antitumor drug(s) to normal cells, particularly to cells of the immune systme which plays a major role in maintaining host defense against cancer.

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APPENDICES

APPENDIX A

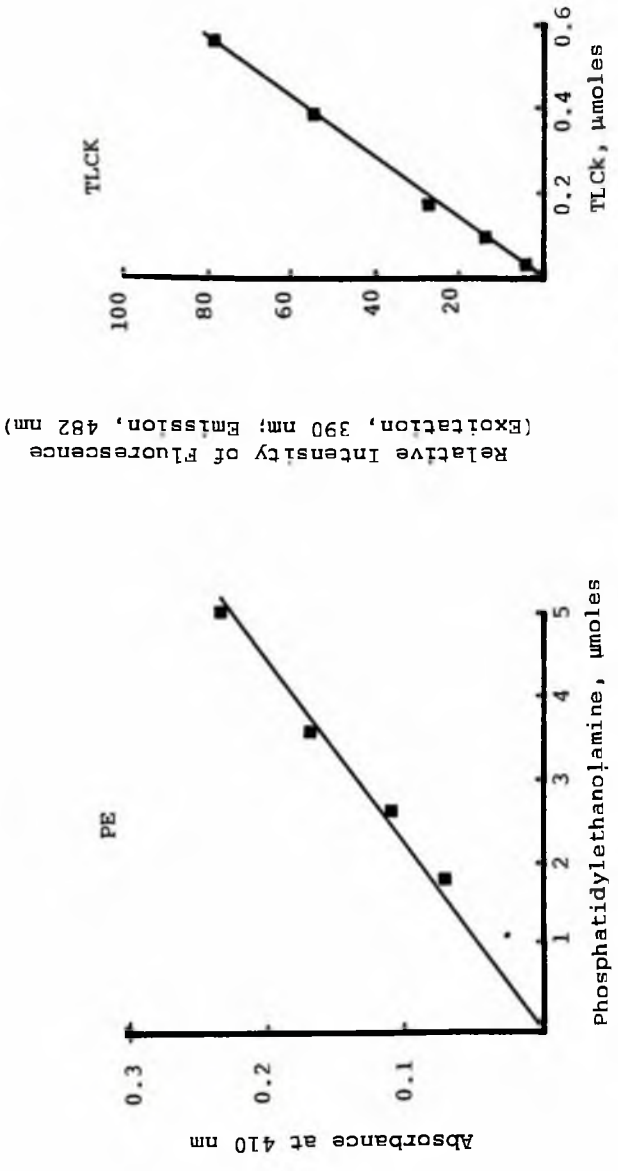

Figure 10. Standard curves for phosphatidylethanolamine (PE) and TLCK.

APPENDIX B

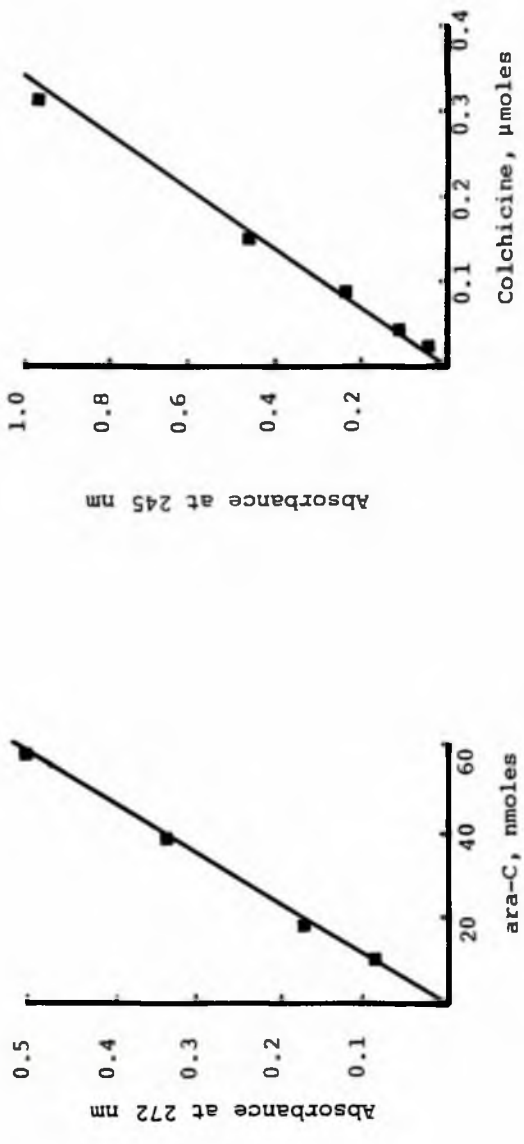

Figure 11. Standard curves for ara-C and colchicine.

APPENDIX C

Figure 12. Standard curves for 6-carboxyfluorescein (6-CF) and Horseradish peroxidase (HRP).

APPENDIX D

TABLE XXII

EFFECT OF pH, TEMPERATURE, TRITON X-100 AND SONICATION ON ara-C, COLCHICINE AND HRP STABILITY[a]

	Absorbance (272 nm)			Absorbance of HRP (500 nm)		
	pH Value					
nmoles	5.2	7.2	9.2	HRP/µg)		Control[b]
ara-C						
12.5	0.05	0.05	0.05	1.40	0.31	0.31
37.4	0.16	0.16	0.16	0.72	0.15	0.16
74.8	0.31	0.30	0.31	0.36	0.07	0.08
Control[b]						
12.5	--	0.05	--			
37.4	--	0.16	--			
Colchicine						
1.2	0.03	0.04	0.04			
6.0	0.17	0.18	0.19			
12.0	0.35	0.38	0.38			
Control						
1.2	--	0.04	--			
6.0	--	0.19	--			

[a]Varying amounts of ara-C and colchicine were incubated in PBS (pH 5.2, 7.2 or 9.2) at 37°C for two days and were measured at 272 and 245 nm respectively. With respect to HRP, the enzyme was sonicated in an ice bath for 3 minutes, incubated at 37°C for 45 minutes in the presence of Triton X-100 (0.05% final concentration) and its activity measured at 500 nm using 4-amino antipyrine, phenol and hydrogen peroxide as substrate.

[b]These were not exposed to Triton X-100, sonication or incubation at 37°C.

The results from the absorbance peaks showed that the stability of either ara-C or colchicine was not adversely affected over the stated pH range and incubation at 37°C for 48 hours. With respect to the horseradish peroxidase, sonication in a bath for 3 minutes and incubation in 0.05% Triton X-100 at 37°C for 45 minutes did not effect denaturation of the enzyme.

APPENDIX E

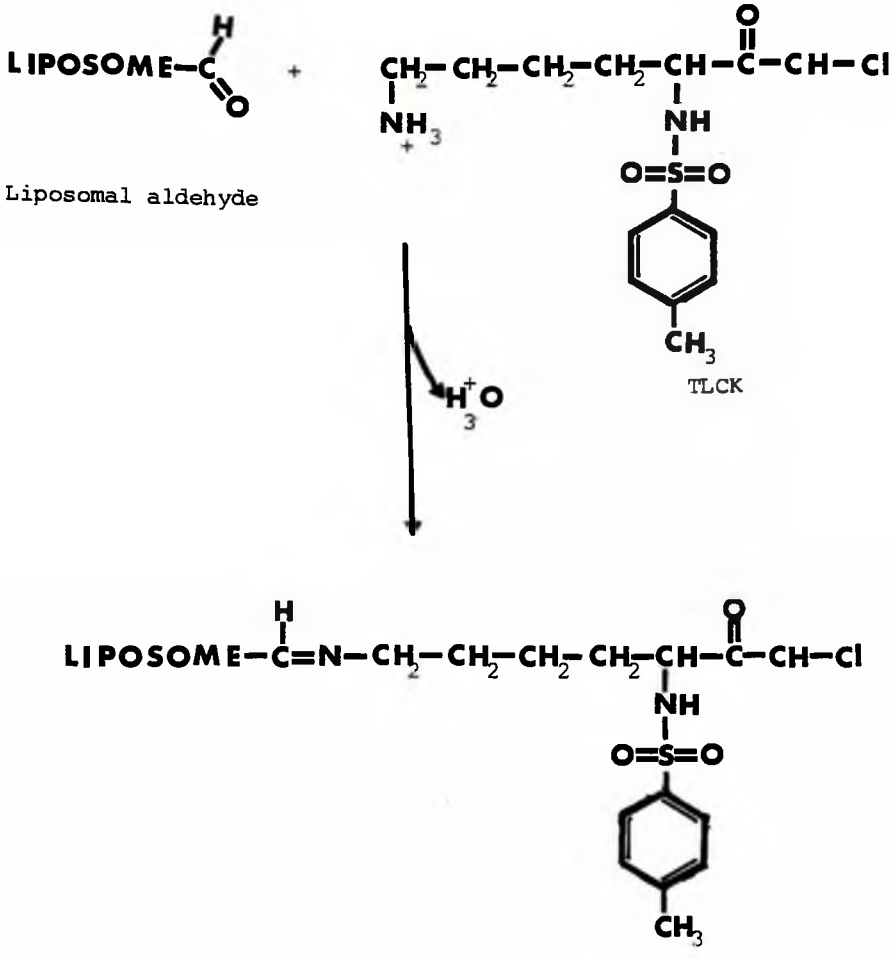

Figure 13. Covalent coupling of TLCK with liposomes.

APPENDIX F

TABLE XXIII

UPTAKE OF TLCK-BOUND LIPOSOME BY EHRLICH ASCITES TUMOR CELLS IN VIVO[a]

	Ehrlich Ascites Tumor Cells		Liver Cells[b]	
	CPM/g Protein[c]	Uptake (%)[d]	CPM/g Wet Liver	Uptake (%)[e]
^{14}C-Lp-TLCK				
5 hrs	6666 ± 572	1.07	293	0.05
10 hrs	6916 ± 1068	1.11	1192	0.19
20 hrs	5933 ± 1157	0.96	625	0.10
^{14}C-Lp				
5 hrs	34 ± 26	0.01	332	0.05
10 hrs	53 ± 14	0.01	210	0.03
20 hrs	179 ± 4	0.03	901	0.13

[a]Ehrlich ascites tumor cells were maintained by weekly passage in male and female CF #1 Swiss mice peritoneal cavities.

For the uptake study, pairs of mice (30 g average body weight) bearing tumor cells for 4 to 5 days were injected intraperitoneally with 0.5 mL liposomal suspensions containing either ^{14}C-liposome (^{14}C-Lp) or ^{14}C-liposome-TLCK (^{14}C-Lp-TLCK). It should be noted that the liposomes used for the in vivo study was different from that used for the in vitro study.

Liposomes incorporating cerebroside was prepared as follows: In 25 mL chloroform-methanol (6:1 v/v) dissolved 240.4 µmoles dipalmitoyl phosphatidylcholine, 237.8 µmoles cholesterol plus 18 µCi ^{14}C-cholesterol, 68.7 µmoles cerebroside and 34 µmoles dicetylphosphate. The solvent was evaporated over a stream of nitrogen, dispearsed in 6.0 mL PBS, pH 7.2 and cup sonicated for 2 hours at 4°C. The sugar moiety of the cerebroside was oxidized with sodium metaperiodate and then reacted with TLCK as described in Methods. Unreacted TLCK was separated from TLCK-bound liposome by dialysis in isotonic PBS for 16 to 20 hours at room temperature. At 5, 10, and 20 hour post liposome injection, mice were sacrificed by cervical dislocation. The ascites tumor cells and liver were removed immediately and prepared for radio activity counting as follows:

APPENDIX F (cont'd)

Preparation of Ehrlich Ascites Tumor Cells

A 2.0 mL of Ehrlich ascites tumor cells was collected from mouse peritoneum into plastic syringe fitted with 23g needle and was washed 4 times with cold PBS by centrifugation (85 x g for 5 minutes) at 4°C. The supernate was discarbed and the Enrlich ascites tumor cell precipitate was resuspended in 1.0 mL deionized water and the protein content was determined by the biuret method (116). A 0.3 mL of the tumor cell suspension was incubated in scintigest, 0.6 μΩ final volume, overnight at 50°C. Following the incubation, the mixture was oxidized with 0.1 mL hydrogen peroxide (30%), a 6.0 mL of scintiverse was added and the radioactivity was counted on a Beckman LS 7500 Liquid Scintillation System.

Preparation of Liver Cells

Freshly removed liver was weighed, washed and homogenized in 3 mL of cold 0.25 M sucrose-5 mM EDTA-KCl buffer per gram liver tissue. The homogenization was performed through a series of smaller bore needles (18 to 25 g) and then passed through cheese cloth to remove particulate matter. Th filtrate was washed by centrifugation and its radioacitivty was counted as described earlier.

[b]Represents non-tumor cells.

[c]Calculated from the formula: $\frac{\text{CPM of Cells per gm Protein}}{\text{CPM of Liposome Added}} \times 100$

CPM: Counts Per Minute

[d]Represents ± 2SD of four counts.

[e]Represents average of two counts.

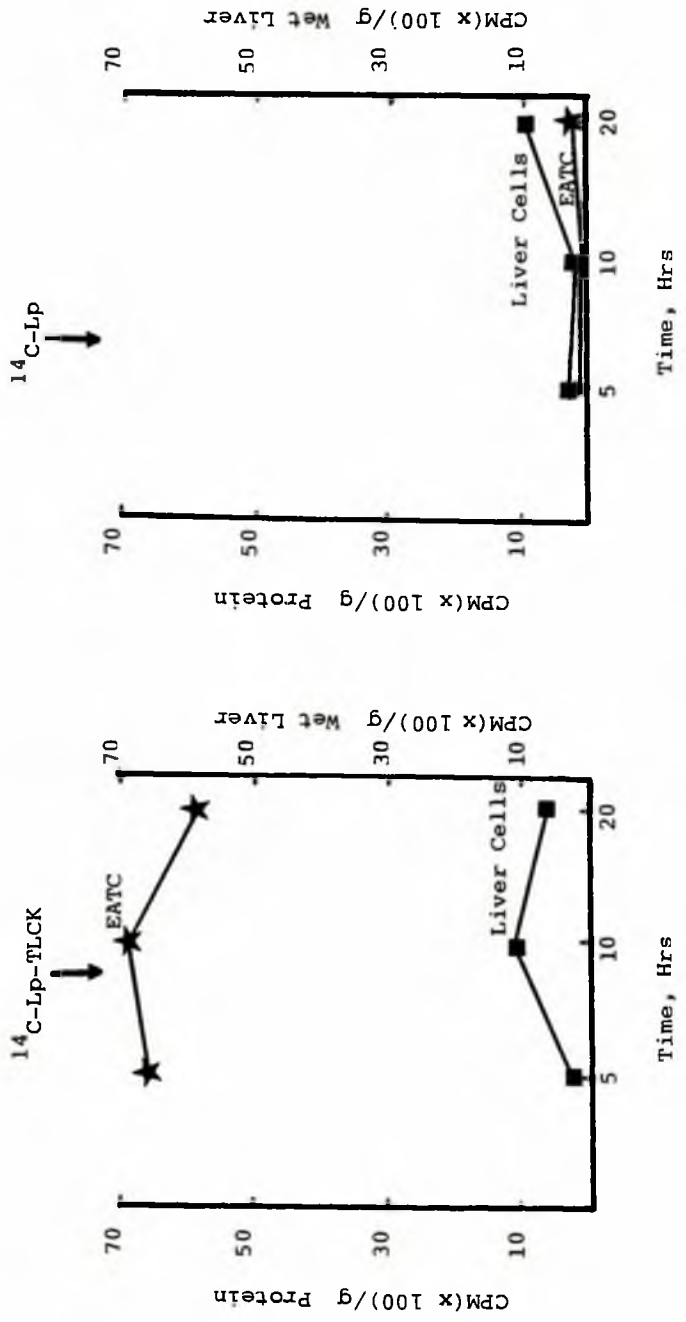

Figure 14. Uptakes of ^{14}C-liposomes with or without TLCK by Ehrlich ascites tumor cells (EATC) and liver cells In Vivo. The experimental conditions are as described in the legend for Table XXIII.