

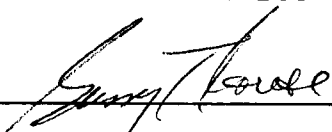
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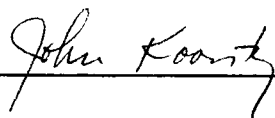


Leaf Huang, Major Professor

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Vice Provost
and Dean of The Graduate School

pH-SENSITIVE LIPOSOMES: CHARACTERIZATION
AND APPLICATION

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Jerome Connor

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ABSTRACT

It has been demonstrated that liposomes composed of dioleoylphosphatidylethanolamine (DOPE) and palmitoyl-homocysteine (PHC) have the ability to fuse with adjacent membranes upon exposure to mildly acid pH. This phenomenon was shown using resonance energy transfer, gel filtration, and electron microscopy. The ability of liposomes to fuse is absolutely dependent on the presence of DOPE and a weakly acidic amphiphile. The acid induced fusion event is a leaky process, but the leakage can be reduced by 50%, with only a small loss of fusion ability, by the inclusion of 40 mole percent cholesterol. Using an established monoclonal antibody targeting system (Huang et al. 1983 JBC 258, 14034), pH-sensitive immunoliposomes were prepared which successfully delivered entrapped calcein to the cytoplasm of target cells. The addition of chloroquine, which raises the internal pH of cellular vacuoles, blocks the cytoplasmic delivery by the pH-sensitive immunoliposomes. pH-insensitive immunoliposomes delivered calcein only to the endosome/lysosome system and not the cytoplasm. Liposomes composed of DOPE:oleic acid (OA) have also been demonstrated to be fusogenic at mildly acidic pH. Using resonance energy transfer, a synergistic effect in the ability to promote fusion was established between protons and the divalent cations Ca^{+2} and Mg^{+2} .

^{31}P -NMR and light scattering of DOPE:OA liposomes under acidic conditions demonstrate that the effect of the protons and the divalent cations is to force the DOPE to revert to the hexagonal II configuration. Using DOPE:OA, pH-sensitive immunoliposomes successfully delivered the cytotoxic drugs Ara-C and MTX to the cytoplasm of target cells. Furthermore the pH-sensitive immunoliposomes are more effective than free drug or pH-insensitive immunoliposomes. The lysosomotropic amines, chloroquine and NH_4Cl both were able to block the pH-sensitive immunoliposome delivery by raising the internal pH of endosomes/lysosomes. In vivo experiments with DOPE:OA immunoliposomes indicate that the liposomes rapidly aggregate and release their contents upon exposure to plasma. These results indicate that pH-sensitive immunoliposomes are an effective tool for in vitro cytoplasmic delivery but are ineffective for in vivo delivery at this point in development.

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CHAPTER 1

INTRODUCTION

Liposomes have become an important model system for studying the phenomenon of membrane fusion (1,2). The simplicity of form of artificial lipid membranes makes them an effective tool for elucidating the actual mechanism of fusion between opposing membranes. The information derived from the study of model liposome fusion is a crucial contribution to the understanding of biologically relevant fusion activities including endocytosis, exocytosis and viral infection.

Many studies have been done which demonstrate the ability of proteins such as: melittin (3), bovine serum albumin (4), clathrin (5), myelin basic protein (6), polymyxin B (7), tetanus toxin (8), and synexin (9) to induce fusion between liposomes. Though these studies have important implications for the mechanisms of the corresponding physiological events, the actual membrane fusion is dependent upon the presence of an exogeneous component and little information can be deduced concerning the membrane to membrane interaction during a fusion event.

Recently, several groups have demonstrated the ability to induce liposome membrane fusion solely by a decrease in the medium pH (10, 11, 12, 13, 14). These

systems are all based on the central theme of stabilizing a phosphatidylethanolamine (PE) lipid in a bilayer configuration by the addition of a second lipid component which contains a titratable acidic group.

Properties of Phosphatidylethanolamine (PE)

The propensity of PE to form non-bilayer structures; i.e. hexagonal II phase, under physiological conditions (15,16) is an important factor which contributes to its essential requirement as a lipid component of pH-sensitive liposomes. PE molecules prefer the hexagonal phase because the lipid head group is small in size compared to the acyl chains; this allows for increased interaction between adjacent lipid head group (17) including the possibility of intermolecular hydrogen bonding (18, 19). This PE head group interaction causes a decrease in the surface hydration which is compensated for by assuming the hexagonal II phase over a bilayer configuration. The transition of PE from the hexagonal II phase to the bilayer phase is easily monitored by ^{31}P -NMR (16). It is possible to form a stable bilayer conformation of PE by increasing the pH to greater than 9.0 (18). More importantly PE can be formed into stable bilayer configurations by the addition of a second lipid component. Phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylglycerol

(PG) at a mole percent of 20 or greater have the ability to stabilize PE in the bilayer phase presumably by both decreasing the intermolecular interaction between PE molecules and also by increasing the interfacial hydration level. Interestingly, the addition of Ca^{+2} to bilayers composed of PE:PS or PE:PG cause a phase separation of the negatively charged stabilizing lipid allowing the PE to revert to the hexagonal II phase (13).

An essential factor for the development of pH-sensitive liposomes is the ability of the lipid component which contains a titratable acidic head group to maintain PE in a bilayer form. For example, the acylated amino acid palmitoylhomocysteine (PHC) (10) with a titratable carboxyl group, and the fatty acids oleic acid (OA) (12,20,21) and palmitic acid (PA) (13,20) both with carboxylic head groups, can all stabilize PE into a bilayer configuration under physiological condition. In addition, cholesterol derivatized on the hydroxyl group with a succinate group yielding cholesterol hemisuccinate (CHEMS), also with a titratable carboxyl head group, can maintain PE as a bilayer at neutral pH (11).

All of these stabilizing lipids contain a negative charge on the head group which provides an electrostatic repulsion reducing the PE intermolecular interaction, which causes it to form the hexagonal II phase. Upon titration of the weakly acidic head group by lowering the

pH, the negative charge is neutralized and the PE reverts to the hexagonal II phase. Thus, the bilayer vesicle is pH dependent for its stability. The significance of this transition of PE from a bilayer to hexagonal phase is the observation that membrane fusion in biological systems may involve an inverted micelle (hexagonal II) intermediate structure during the fusion event. Therefore, PE lipid vesicles which undergo a transition to hexagonal phase at acid pH could be used to produce a model membrane system capable of membrane to membrane fusion induced by protons.

Measurement of Fusion

The fusion of liposomes can be monitored by a variety of methods. Morphologically the fusion of small unilamellar liposomes into larger vesicles can be seen using both negative stain electron microscopy or freeze fracture electron microscopy. In order to study the kinetics of pH induced leakage and fusion of pH-sensitive liposomes, several fluorometric assays have been developed. The intermixing of membrane lipids can be monitored by use of the resonance-energy transfer assay developed by Struck et al. (22). The measurement of aqueous content mixing between fusing liposomes can be determined by using either the 8-aminonaphthalene-1,3,6 trisulfonic acid (ANTS) and p-xylyl-enebis (pyridinium) bromide (DPX) (11) or the terbium-dipicolinic acid (23) assays. The ANTS/DPX

fluorescence assay has the advantage of being insensitive to pH above 4.0 which makes it an excellent method for measuring acid induced fusion. Leakage from vesicles due to proton-induced destabilization is monitored by the ANTS/DPX assay, the terbium/dipicolinic acid assay, or the dequenching of entrapped calcein.

In order to study the biophysical phenomenon occurring during proton induced destabilization and fusion of liposomes both ^{31}P -NMR, indicative of the PE conformational changes, and differential scanning calorimetry (DSC) which reveals broad lipid interactions, are applied to the liposome system. Using both the fluorometric and biophysical methods, the mechanism and results of acid induced fusion can be studied.

pH-Sensitive Liposome Fusion Systems

Using freeze-fracture techniques, Hope (13) demonstrated the fusion of SUV composed of egg transesterified PE: phosphatidic acid (8:2 mole/mole) into large unilamellar and oligomellar vesicles. Included in this work is electron micrographs of intermediate vesicles which display lipidic particles localized to the regions of fusion. Lipidic particles are indicative of PE in a reverse micellar conformation within adjacent membranes; these intermembrane particles have been proposed as being sites of vesicle fusion (24). Bondeson (25) used the

resonance-energy transfer method to show fusion of egg transesterified PE: phosphatidylcholine (8:2 mole/mole) at slightly acid pHs (6.5-7.0). In this work, though, lectin was incorporated into the liposomes and exogenous agglutinin was added to facilitate liposome to liposome binding. Since fusion is dependent on vesicle collision, the inclusion of the agglutinating factor enhanced the fusion rate.

A well studied system using resonance energy transfer to study acid induced liposome fusion, independent of exogenous agglutinin, is the PE:PHC (8:2 mole/mole) pH-sensitive liposomes (10 and Chapter II). These liposomes begin to display fusion activity at a pH < 7.0 with maximum fusion occurring at pH = 4.8. The midpoint of fusion occurs at pH = 6.2. Negative stain electron micrographs of the liposomes before and after the acid addition indicate that approximately six rounds of fusion of the pH-sensitive SUV occur to give the final large vesicles. The PE:PHC liposomes appeared to be very leaky, as determined by calcein dequenching, during the fusion reaction. Inclusion of 40 mole percent of cholesterol, though decreasing the total amount of fusion, allows for the retention of entrapped calcein during the fusion event (Chapter II).

In order to fully characterize the relationship between membrane mixing i.e. resonance energy transfer

assay, and aqueous mixing, by means of the ANTS/DPX assay, Duzgunes et al. (12) established a PE:OA (7:3 mole/mole) liposome fusion system. Aqueous mixing was seen between fusing vesicles as the pH was lowered to 6.3, concurrent to aqueous content mixing, though, was rapid leakage of the entrapped content (12). The acid induced membrane fusion between PE:OA liposomes was confirmed by resonance-energy transfer and irreversible changes in turbidity (12). Similar to the PE:PHC system the liposomes are destabilized upon protonation and become leaky, possibly from the membrane-membrane contact point, upon fusion. ^{31}P -NMR scans of PE:OA liposome at slightly acidic pH indicate the presence of PE in the hexagonal II conformation (21). This destabilizing configuration of PE in the bilayer, though probably responsible for the fusion event, is also a source of rapid aqueous content leakage. Though both PE:PHC and PE:OA liposomes are leaky at fusion pHs, both vesicle types have been successfully demonstrated to be useful vehicles for cellular delivery.

A parallel system has been developed to study acid-induced fusion, which employs cholesteryl hemisuccinate (CHEMS) as the stabilizing factor for PE bilayers. Similar to the PE:OA liposomes, the PE:CHEMS vesicles are sensitive to mildly acidic pH's (11). Using the ANTS/DPX fluorescence assay it was demonstrated that the acid induced pH leakage of these vesicles occurs subsequent to

aggregation and that the destabilization effect of protonation is dependent on bilayer contact (11). The CHEMS molecules are able to stabilize the PE lipid in a bilayer configuration at neutral pH; the negative charge on the succinate head group is important in maintaining bilayer integrity by causing an electrostatic repulsion between liposomes. Upon protonation the CHEMS acquires a cholesterol-like configuration and is still able to maintain PE in a bilayer form, but the CHEMS induced repulsion between liposomes is removed; this allows contact between adjacent liposomes leading to destabilization and leakage of entrapped contents.

In order to characterize the PE/CHEMS interaction Lai et al. (26) studied the biophysical effects of protonation on PE:CHEMS liposomes using differential scanning calorimetry and ^{31}P -NMR. PE alone displays a bilayer to hexagonal phase transition (T_H) at 56°C . The addition of 25 mole % of CHEMS to egg transesterified PE at neutral pH abolishes the T_H up to a temperature of 72°C as measured by DSC (26). At pH 4.5, PE:CHEMS (75:25 mole/mole) the transition again appears but is shifted to a temperature lower than egg transesterified PE alone. The increase of the T_H of PE by CHEMS at neutral pH and decrease of T_H of PE at acidic pH was confirmed using ^{31}P NMR (26). Thus, CHEMS is able to stabilize PE by preventing a transition to the hexagonal II phase, but upon protonation CHEMS

actually increases the ability of PE to revert to the inverted micelle structure. CHEMS in a deprotonated state is capable of both stabilizing PE in a bilayer and maintaining separation from adjacent liposomes. In the presence of an acidic environment the electrostatic repulsion between vesicles is abolished so membrane-membrane contact is increased; in addition the ability of PE to form the hexagonal II phase is increased for these liposomes. These contact points between membranes are the area of fusion between liposomes but in the PE:CHEMS vesicles these points also cause contact induced destabilization and hexagonal II phase formation (27). It appears that in the PE:CHEMS system a decrease in pH causes leakage and collapse of the liposomes before a fusion event can take place.

In contrast to the finding in the PE:OA fusion system, using the PE:CHEMS liposomes Ellen et al. (28,29) demonstrated that a mildly acidic condition causes a mixing of bilayer lipids, as measured by resonance energy transfer, but no mixing of aqueous contents as determined by the ANTS/DPX assay. Thus the liposome-liposome interaction, at acidic pH, allows intermixing of the membrane lipids; but due to the collapse of the vesicular structure the content of the interacting liposome leaks and aqueous mixing cannot occur between vesicles. Whether this liposome composition will be

applicable to in vitro or in vivo delivery is yet to be determined.

Recently a system has been developed using a succinic derivatized PE molecules as the stabilizing factor to maintain PE in a bilayer (14). DOPE was derivatized with succinic anhydride yielding N-succinyl-PE (COPE); this system is unique in that the stabilizing acidic factor contains identical diacyl chain as the PE lipid to be stabilized into a bilayer. Interestingly, liposomes composed of COPE alone are leaky at neutral pH but stable at pH 4.0; while liposomes composed of COPE:DOPE (3:7 mole/mole) act in the opposite direction. The pH induced leakage effect of COPE:DOPE is independent of acyl chain saturation, and lipid composition (14). This indicates that the leakage effect is not due to liposome aggregation but probably packing defect caused by the protonation of the COPE molecules. The apparent difference in the effect of acidic conditions make the COPE-containing liposomes an excellent system for studying lipid packing in pH-sensitive vesicles. It is also possible that these liposomes have a potential for delivering contents to cells.

Divalent Cation Effect

A second characteristic of the pH-sensitive liposomes of both the PE:fatty acid or PE:CHEMS types is the

divalent cation induced fusion (12,21,26,28). It has been demonstrated that Ca^{+2} in the range of 2-3mM and Mg^{+2} in range of 3-4mM can induce fusion between liposomes composed of PE and a negatively charged stabilizer. Similar to protons the divalent cations neutralizes the negative charge of the stabilizing entity allowing close contact between PE molecules; in addition both Ca^{+2} and Mg^{+2} cause a bridging between adjacent liposomes, increasing the number of bilayer contact points. Divalent cations have also been shown to act synergistically with proton, shifting the pH needed to initiate fusion from 6.5 to closer to neutral (21). This synergistic effect of the divalent cation could have an important contribution in cellular delivery experiments, since both Ca^{+2} and Mg^{+2} are present in the growth medium. The divalent cation could allow fusion of liposomes at pHs higher than those required for destabilization and content leakage increasing the chance of delivery.

The model membrane system presented previously allows for an exact study of the mechanism of interaction between fusing liposomes. The systems, though, deal with liposome-liposome fusion. Two interesting demonstrations of acid induced fusion of liposomes with biological membranes have been demonstrated recently. Huang et al. (20) using the resonance energy transfer assay has demonstrated the fusion of liposome composed of PE:OA (8:2

mole/mole) with mitoplast or isolated inner membranes of rat liver mitochondria, with a pH optimum at 5.0-5.5. This method may be an effective way to introduce impermeable macromolecules into the matrix of mitoplasts.

A second demonstration of fusion is between liposomes composed of PC and high mole percent of cardiolipin (CL) and membrane vesicles derived from Bacillus subtilis and Streptococcus cremoris (30). Using both resonance energy transfer and aqueous delivery of colloidal gold particles, PC:CL (1:1 mole/mole) liposomes show a low pH dependent fusion with the biological membranes. Treatment of the biological membranes with trypsin inhibited the fusion event indicating a protein dependence for the acid induced fusion. This low pH fusion process, though, could be useful in delivering substances to bacterial protoplasts.

Cytoplasmic Delivery by pH-Sensitive Liposomes

Liposomes have been used extensively as a tool to deliver biologically active molecules to cells (2,31,32). This has been achieved by use of a fusogenic agent to fuse liposomes to the cell plasma membrane or by allowing cellular endocytosis of the liposomes followed by a passive leakage of the entrapped molecules from the cellular lysosomes. In addition, to accelerate liposome delivery, numerous liposome targeting systems have been developed (33,34,35,36,37). In these systems monoclonal

antibodies, specific to a cell surface antigen, are attached to the liposomes by a variety of methods (2,38). These antibody targeted liposomes, (immunoliposomes), bind to specific cell antigens are endocytosed via a receptor mediated endocytosis mechanism and are delivered, through the endosomes, to the lysosomal compartment. An obvious drawback to immunoliposome delivery is the inability of most biologically active molecules to escape the endosome/lysosome pathway into the cytoplasm; thus, unless the molecule to be delivered can escape the degradative lysosome vacuole, conventional immunoliposomes are a poor method for cytoplasmic delivery. pH-sensitive immunoliposomes, on the other hand, can solve the problem by exploiting the inherent cellular endocytic pathway. Following receptor-mediated endocytosis the liposomes encounter an acidic pH 5.0-6.5 in the endosome vacuoles (39); therefore, upon endocytosis the pH-sensitive liposomes are confronted with a condition which readily activates the fusion activity of the liposomes. Two examples of pH-sensitive liposomes, one targeted and one non-targeted, have been demonstrated for efficient cytoplasmic delivery of an entrapped molecule by exploiting the endocytic pathway.

Non-targeted Delivery

Calcein and FITC-dextran were entrapped in PE:OA (7:3 mole/mole) extrusion vesicles. Calcein has been

demonstrated to be an impermeable, pH-insensitive aqueous space fluorescent marker (40). Dye-entrapped liposomes were incubated with CV-1 African Green Monkey Kidney cells and delivery was monitored by fluorescence microscopy. Cells incubated with PE:OA liposomes containing either calcein or FITC-dextran displayed diffused cytoplasmic fluorescence indicating cytoplasmic delivery. Control liposomes composed of either PS:PE (3:7 mole/mole) or OA:PC (3:7 mole/mole) containing the same fluorescent marker displayed only punctate fluorescence when incubated with the CV-1 cell indicating delivery only to the endosome/lysosome system. In addition pretreatment of CV-1 cell with chloroquine, which is a permeable weak base which raises the internal pH of the endosome/lysosome, inhibited the cytoplasmic delivery of calcein by the pH-sensitive liposomes. Therefore, the cytoplasmic delivery depended upon the acidification of the endosomes.

Targeted delivery

This was done in our laboratory and is described in detail in chapter III. Calcein was entrapped in PE:PHC liposomes prepared by the reverse-phase evaporation method and bearing an attached anti H-2K^k antibody. Anti H2K^k antibody is a monoclonal antibody against the murine histocompatibility H-2K^k antigen. These immunoliposomes were incubated with both L-929, H-2K^k antigen expressing target cells and A-31, H-2K^d antigen expressing control

cells and delivery was monitored by fluorescence microscopy (41). pH-sensitive immunoliposomes incubated with L-929 cells displayed diffused cytoplasmic fluorescence, while A-31 cells displayed no fluorescence at all, since the vesicles do not bind with the control cells. Control immunoliposomes composed of PC showed only punctate fluorescence when incubated with L-929 cells indicative of lysosomal delivery only. Similar to the non-targeted liposome experiments, chloroquine was able to block the cytoplasmic delivery of calcein by pH-sensitive immunoliposomes (41).

To further clarify the ability of pH-sensitive immunoliposomes to deliver their contents to the cytoplasm, a cytotoxic drug was used to monitor delivery. In these experiments (described in Chapter V) Ara-C was chosen as the cytotoxic agent since it has previously been demonstrated that Ara-C delivered with pH-insensitive immunoliposomes was ineffective in killing cells due to its delivery to and subsequent degradation in the lysosomes (33,42). Ara-C was encapsulated in PE:OA pH-sensitive and PC (pH-insensitive) immunoliposomes and incubated with L-929 cells. Cell killing was monitored by inhibition of ^3H -Thymidine incorporation into DNA.

Ara-C entrapped in pH-sensitive immunoliposomes was five times more effective in killing L-929 cells than free Ara-C. Both untargeted pH-sensitive liposomes and

pH-insensitive immunoliposomes were ineffective in killing the L-929 cells. As in the previous work pretreatment of cells with chloroquine or NH_4Cl , another lysosomotropic agent, inhibited the cytoplasmic delivery (43).

The ability to deliver protein via pH-sensitive immunoliposomes has also been demonstrated in this model system. Diphtheria toxin A fragment (DTA) is unable to cross lipid membranes and is therefore non-toxic to the L-929 cells. When DTA is encapsulated in PE:OA pH-sensitive immunoliposomes it is effectively delivered to the cytoplasm resulting in killing the L-929 cells. Delivery and killing was monitored by inhibition of protein synthesis (44). DTA entrapped in PC immunoliposomes or administered alone is ineffective in killing the target cells. Once again both chloroquine and NH_4Cl effectively inhibit the delivery of DTA by the pH sensitive immunoliposomes. An additional example of pH-sensitive liposome mediated delivery has been demonstrated with plant protoplasts. Liposomes composed of DOPE:cholesterol:OA (4:4:2 mole/mole) with entrapped calcein demonstrated enhanced cytoplasmic delivery to carrot protoplast over several conventional pH-insensitive liposomes (45).

This thesis will examine the development and characterization of an acid pH inducible liposome fusion system and the application of these pH-sensitive liposomes to cellular delivery of the liposome contents.

CHAPTER II

pH-SENSITIVE LIPOSOMES COMPOSED OF DIOLEOYL PHOSPHATIDYL-
ETHANOLAMINE AND PALMITOYLHOMOCYSTEINE:
ACID-INDUCED LIPOSOME FUSION

Membrane fusion under moderately acidic conditions is responsible for the infection of a number of the enveloped viruses, including Semliki Forest virus (46,47), vesicular stomatitis virus, and influenza virus (47). It has been proposed that the viral membrane fuses with the endosome membrane once the virion is endocytosed and exposed to the acidic pH in the endosomes (47). While the precise mechanism of the acid-induced membrane fusion is not known, this phenomenon is of considerable interest for cell biologists. Studies with the liposome model membranes have revealed that liposomes made of asolectin (mixed phospholipids) can fuse with mitochondrial inner membranes at pH 6.5 (48). Serum albumin (4) and its proteolytic fragments (4) can induce liposome fusion at a somewhat lower pH (below 4). Recently Blumenthal et al. have shown that clathrin induces the fusion of neutral liposomes at pH below 6.5 (5). In all these cases, liposome fusion requires the presence of protein. In the present study using liposomes made pH-sensitive as described by Yatvin et al. (49), we report an acid-induced

liposome fusion that does not involve either proteins or other macromolecules.

Materials and Methods

Materials

Palmitoylhomocysteine (PHC) was synthesized and purified as described (49). Dioleoyl phosphatidylethanolamine (PE), dioleoyl phosphatidylcholine (PC), and bovine brain phosphatidylserine (PS) were used in this study. All phospholipids, including N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE (N-NBD-PE) and N-(lissamine rhodamine B-sulfonyl)-PE (N-Rh-PE), were purchased from Avanti Biochemicals. Cholesterol and calcein were obtained from Sigma.

Liposome Preparation

Solvent-free lipid films were suspended in phosphate-buffered saline (pH 7.4) at 10 mol/ml and sonicated at room temperature for 15 min with a bath sonicator (Laboratory Supplies, Hicksville, NY). Various lipid compositions were used as indicated in the text. Fluorescence-labeled liposomes containing 1 mol % each of N-NBD-PE and N-Rh-PE were prepared identically as the unlabeled liposomes.

Liposome Fusion

Ten microliters of labeled liposomes and various amounts of unlabeled liposomes were added to 2 ml of phosphate-buffered saline. After the relative fluorescence was measured, 5-20 μ l of HCl at various concentrations was added to achieve the desired pH while the sample was vigorously mixed. After about 2 min at room temperature, appropriate amounts of NaOH were added to return the pH to 7.4. The relative fluorescence of the sample was again measured.

Fluorescence Measurements

A Perkin-Elmer LS 5 fluorescence spectrophotometer was used. The emission spectrum was taken for each sample and samples were excited at 468 nm. The excitation and emission slit widths were 5 nm and 3 nm, respectively. Light scattering was about 5-6% of the total fluorescence signal. The ratio, R , of N-NBD-PE emission at 530 nm to the N-Rh-PE emission at 580 nm is a sensitive measure of the efficiency of the resonance energy transfer between N-NBD-PE and N-Rh-PE (22). The value of R was 0.20 for the unfused liposomes, due to the high efficiency of energy transfer. As the labeled liposomes fused with the unlabeled liposomes, dilution of the fluorescent lipids occurred, resulting in a decrease in the efficiency of energy transfer and an increase in R values (5,22). Hence

the increase in R value is a quantitative measure for the degree of liposome fusion. A total mixing of lipids upon complete fusion would result in a maximal R value that is determined by the ratio of unlabeled to labeled liposomes in the fusion mixture. The percent of liposome fusion used in this communication is defined as:

$$\% \text{ fusion} = \frac{R_f/R_i - 1}{n} \times 100 \quad [1]$$

in which R_i and R_f are the R values before and after the fusion reaction, respectively, and n is the concentration ratio of unlabeled to labeled liposomes in the mixture. To check the validity of Eq. 1, labeled and unlabeled liposomes were mixed at different ratios and the R_i values were obtained. The mixtures were then sonicated thoroughly to mix all the lipids and the R_f values were obtained. The plot of $R_f/R_i - 1$ vs. n was a straight line with a slope of 1 up to $n = 3$, indicating that Eq. 1 was valid. For $n > 3$ the fluorescence enhancement was lower than theoretical, therefore, most experiments were performed with $n = 3$. The R values were reproducible to within $\pm 5\%$ in the range used.

Liposome Leakage

To determine liposome leakage during fusion, calcein, a water-soluble self-quenching fluorescent dye was used as

an internal aqueous space marker (50). Liposomes were prepared from lipid mixtures devoid of fluorescent phospholipids by sonication in phosphate buffered saline containing 140 mM calcein. The untrapped calcein was removed by passing liposomes through a Sepharose-4B column (Pharmacia) equilibrated in phosphate buffered saline. The calcein-containing liposomes were treated with acid and base as described above. The calcein fluorescence before and after fusion, and after addition of 0.2% Triton X-100 to disrupt the liposomes, was measured at excitation and emission wavelengths of 490 and 518 nm, respectively. The latency of calcein fluorescence is defined as:

$$\% \text{ latency} = (1 - F/F_t) \times 100 \quad [2]$$

in which F and F_t are the calcein fluorescence intensity in the absence and presence of Triton X-100, respectively.

Electron Microscopy

Liposomes were negatively stained with 0.5% uranyl acetate and viewed in a Hitachi 600 electron microscope operating at 75 kV. Pictures were taken at x35,000 and further enlarged photographically. To determine the size distribution of the liposomes a histogram was drawn by measuring >200 liposomes from micrographs taken of three different liposome preparations.

Results

Sonicated liposomes containing DOPE and PHC fuse readily when the medium pH is lowered below 7. This was indicated by an increase in liposome size as seen with negative stain electron microscopy. Fig. 1 shows typical pictures of liposomes composed of PE/PHC (8:2, mol/mol) before and after the acid treatment. The diameter of the original sonicated liposomes was 490 ± 230 Å. After the acid treatment (pH 4.8), about one-third of the liposomes appeared unchanged in size--i.e., in the range of 300-700 Å in diameter. The rest of the liposomes became much larger, with a diameter of 2900 ± 100 Å. The fused liposomes did not appear aggregated. Since the increase in liposome diameter is approximately 6-fold, several rounds of fusion must have occurred.

The increase in liposome size was also demonstrated by gel filtration (Fig. 2). In this experiment, labeled liposomes and 3-fold excess of unlabeled liposomes were mixed. An aliquot was chromatographed on a Bio-Gel A-50 m column at pH 7.4. A heterogeneous liposome population produced the broad peak in the elution profile. Another aliquot was treated with acid (pH 4.8) and then chromatographed on the same column after the pH was returned to 7.4. The eluted liposomes were detected by the emission of N-NBD-PE as measured at 530 nm. The fluorescence of each fraction was also measured in the presence of 0.2%

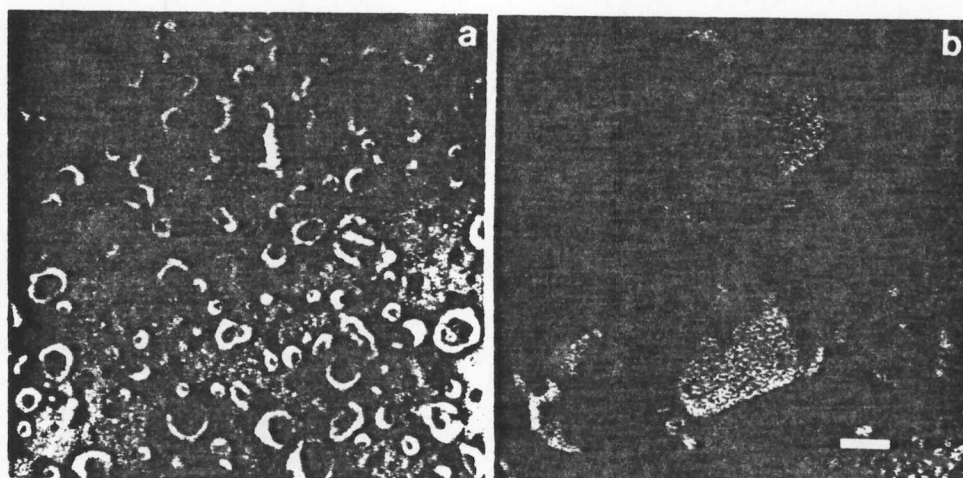


Figure 1. Negative stain electron micrograph of DOPE:PHC (8:2) liposomes before (a) and after (b) the acid treatment. Fusion pH= 4.8; lipid concentration= 0.2mM. (Bar=0.1 μ m)

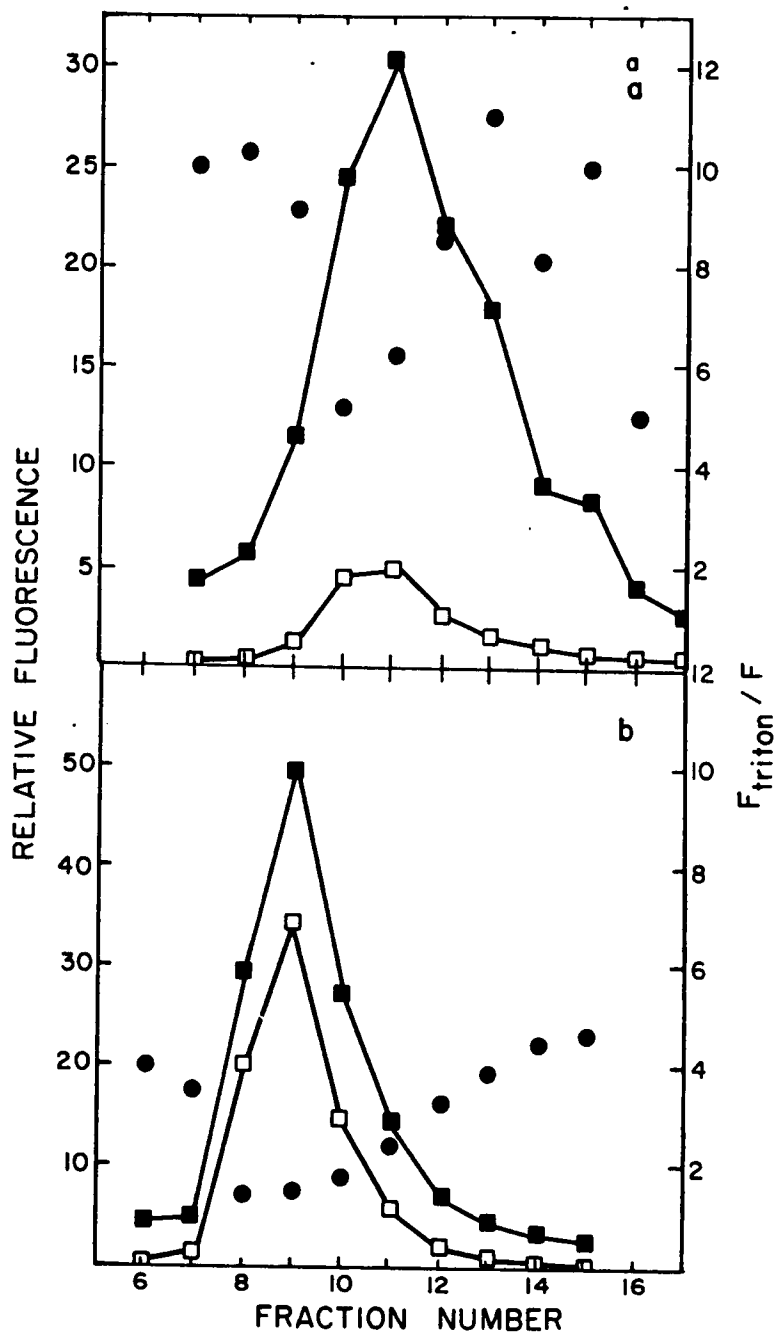


Figure 2. Gel filtration of DOPE:PHC (8:2) liposomes through Bio-Gel A-50m (BioRad) before (a) and after (b) the acid treatment. Fractions were measured for fluorescence of NBD-PE in the absence (□) and presence (■) of 0.2% Triton X-100 as described in Methods, chapter II. The ratio (●) of fluorescence in the absence and presence of Triton is also plotted.

Triton X-100 to completely dissolve the liposomes. As can be seen, the sonicated liposomes, originally eluted in the included volume fractions of the column, had shifted to the void volume fractions (fraction 9) after the acid treatment, indicating an increase in liposome size. Furthermore, the ratio of N-NBD-PE fluorescence in the presence and absence of Triton X-100 was very different in these liposomes. Before fusion, the ratios were generally greater than 5, indicating a high degree of quenching of the N-NBD-PE fluorescence due to efficient energy transfer. After the acid treatment, these ratios were significantly reduced to below 5, indicating less efficient energy transfer. This result is expected if the acid treatment induced a fusion between the labeled and the unlabeled liposomes. This would result in a dilution of the fluorescent probes in the fused membranes.

The acid-induced liposome fusion could be quantitated by a resonance energy transfer method described by Struck, et al. (22). In this assay, the extent of fusion is measured by the dilution of the fluorescent probes embedded in the membranes. As can be seen in Fig. 3, the percent fusion, as calculated by using Eq. 1, for PE:PHC (8:2) liposomes was dependent on the pH of the medium. Fusion occurred when pH was below 7.0 and reached maximum at pH approximately 4.8 with a midpoint pH 6.2.

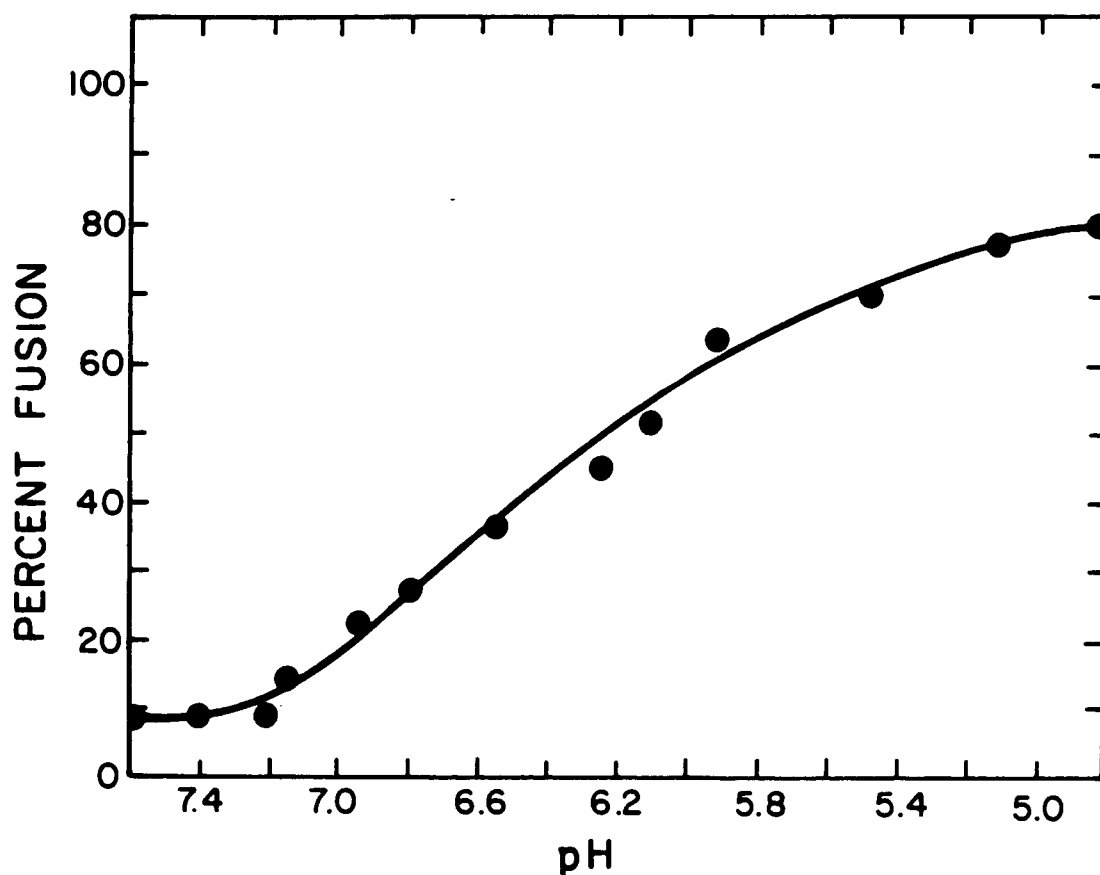


Figure 3. pH dependence of fusion. DOPE:PHC (8:2) liposomes were used at 0.2mM lipid concentration and $n=3$. Percent fusion is calculated according to equation 1 of chapter II.

The lipid composition of the liposome strongly determined the efficiency of the acid-induced fusion. Table 1 shows the percent fusion at pH 4.8 for liposomes made of various lipid compositions. PHC was required for fusion. The combination of PE and PHC (8:2) was very effective for fusion, whereas the addition of PC to such liposomes diminished fusion. Fusion was reduced from 89% to 36% when 25% of the PE (PE/PC/PHC = 6:2:2) was replaced by PC. The maximal fusion was observed with liposomes containing large amounts of PE and no PC.

Although the PE/PHC (8:2) liposomes fused best, the fusion process was very leaky as indicated by experiments using calcein. At high concentrations calcein fluorescence is efficiently self quenched; however, fluorescence is markedly enhanced when the dye is diluted as it leaks from the liposomes (50). Table 2 shows that nearly all the entrapped calcein in the DOPE:PHC (8:2) liposomes was rapidly released during fusion. However, when 40% cholesterol was included in these liposomes, less leakage of calcein was observed (55% latency). The cholesterol-containing liposomes could still be efficiently fused as examined by the resonance energy transfer method in a separate experiment.

Table 1. Acid-induced fusion of liposomes of various compositions.

Lipid composition	mol ratio	Fusion,* %
DOPE/PHC	8:2	89.0 $\pm$ 2.14
DOPE/PHC	6:4	53.0 $\pm$ 1.58
DOPE/PHC	3:7	62.0 $\pm$ 1.87
DOPE/DOPC/PHC	6:2:2	36.0 $\pm$ 1.29
DOPE/DOPC/PHC	4:4:2	21.0 $\pm$ 2.01
DOPC/PHC	8:2	8.0 $\pm$ 1.00
PS/PHC	8:2	16.0 $\pm$ 1.29
DOPE/DOPC	7:3	6.0 $\pm$ 1.87
DOPE/PS	7:3	13.0 $\pm$ 1.29
PS/DOPC	7:3	5.3 $\pm$ 1.58

Fusion pH = 4.8; lipid = 200 μ M; n = 3.

*Calculated according to Eq. 1 and expressed as mean $\pm$ SD.

Table 2. Liposome leakage during fusion

Liposome composition	mol ratio	Fusion, [*]	Latency of calcein fluorescence, % [†]	
			Before fusion	After fusion
DOPE/PHC	8:2	89 ± 2.14	76 ± 2.88	5 ± 0.75
DOPE/Chol/PHC	4:4:2	52 ± 2.37	71 ± 2.31	55 ± 1.21

Fusion pH = 4.8, lipid = 200 μ M, n = 3, Chol, cholesterol.

^{*}Calculated according to Eq. 1 and expressed as mean ± SD.

[†]Calculated according to Eq. 2 and expressed as mean ± SD

Discussion

To our knowledge, an acid-induced liposome fusion that is not mediated by proteins or other macromolecules has only been demonstrated in a few cases. In the case of liposome fusion mediated by proteins--e.g., serum albumin (4), clathrin (5), and viral glycoproteins (47,51,52)--it is clear that the protein conformation change is the primary driving force for fusion. In the present study, the driving force comes from the lipid itself, most likely PHC. Homocysteine forms a thiolactone ring at acidic pH (53). This mechanism was thought to be responsible for the pH-sensitive leakage in liposomes composed of disaturated PC and PHC (49). However, recent evidence argues against this mechanism (unpublished observation). Rather the pH effect may be explained by a change in "acid-base" equilibrium--i.e., the ratio of charged to uncharged N-acylamino acid with subsequent changes in the electrostatic interactions among the lipid headgroups. Alternatively, the bilayer solubility of the protonated PHC might be so low that domains of PHC are formed at acidic pH. Such lateral phase separation of the bilayer lipids could be a major cause of fusion (54,56). The latter mechanism appears particularly attractive because we have found that the presence of PE greatly enhances the acid-induced liposome fusion. Pure unsaturated PE, such as dioleoyl DOPE used in this study, is prone to form

hexagonal phase or inverted micelles (16,56) and this tendency has been proposed as a primary mechanism for membrane fusion in other systems (56,58). In either case, it would generate a profound structural alteration of the lipid bilayer. Furthermore, these mechanisms suggest that other long-chain amphiphiles containing carboxylic groups, such as fatty acids, can also be used for acid-induced liposome fusion.

The interactions of liposomes with animal cells have been extensively studied. Although liposome-cell fusion was initially suggested as a primary mechanism, it is clear from the recent studies in this (33) and other (59,60) laboratories that the endocytosis of liposomes is primarily responsible for liposome uptake. Furthermore, Straubinger, et al. have shown that the endocytosed liposomes encounter an acidic environment once they enter the endosomes (39). The pH of endosomes has been determined to be about 5 (61). Therefore, it is conceivable that the liposomes used in this study could provide an effective cytoplasmic delivery system by fusing with the endosome membranes. The less leaky liposomes containing cholesterol may be particularly useful in discharging their contents into the cytoplasm. This is shown in the next chapter.

CHAPTER III

EFFICIENT CYTOPLASMIC DELIVERY OF A FLUORESCENT DYE BY pH-SENSITIVE IMMUNOLIPOSOMES COMPOSED OF DIOLEOYL PHOSPHATIDYLETHANOLAMINE AND PALMITOYLHOMOCYSTEINE

The development of an effective, nontoxic method for the delivery of macromolecules to the cytoplasm of living cells is important for studies of control mechanisms of cellular processes. The versatility of liposomes in their composition, size and ability to encapsulate many different macromolecules, makes them attractive carriers for cellular delivery. Work described in the preceeding chapter, involving a model system for studying membrane fusion, led to the development of a liposome composition of dioleoylphosphatidylethanolamine/palmitoyl homocysteine (DOPE/PHC) (8:2) that undergoes fusion at acidic pH's (10). Concurrent with this model study, our laboratory established a reproducible cell targeting system, which involves the incorporation of a fatty acid derivatized monoclonal antibody into liposomes (62). This allows for the specific binding of the immunoliposomes to target cells (63). Upon binding the immunoliposome is endocytosed and eventually delivered to the lysosomes (33). The processing pathway includes an intermediate step at which the immunoliposomes encounter an acidic environment in the endosomes with a pH that varies from 4 to 6 (39).

In the work presented in this chapter we have combined the established monoclonal antibody targeting system with the pH sensitive liposomes to generate pH-sensitive immunoliposomes that become fusion competent at acidic pH's. Thus, if the liposomes were endocytosed and proceeded into the endosome vesicles they would encounter an acidic environment and become fusion competent. If a fusion reaction occurred between the liposome and the endosome membranes the contents of the liposome would be released into the cell cytoplasm. The experiments in this paper were designed to test this hypothesis.

Materials and Methods

Materials

PHC was synthesized and purified as described (49). DOPE and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Calcein and chloroquine were obtained from Sigma Chemical Co. (St. Louis, MO).

Antibody Preparation

Anti-H2K^k antibody from a mouse hybridoma cell line 11-4.1 was purified, labeled with ¹²⁵I, and derivatized with N-hydroxysuccinimide ester of palmitic acid, as described by Huang et al. (62).

Liposome Preparation

Reverse-phase evaporation vesicles were prepared as follows. Solvent-free lipid films containing DOPE/PHC (8:2) or DOPC were suspended in phosphate-buffered saline (PBS) containing 60 mM calcein. A trace amount of hexadecyl [^3H] cholestanyl ether was included in the lipid mixture to facilitate the monitoring of the lipid. A critical ratio of 65 μl buffer per 5 μmol lipid must be maintained in order to form a stable emulsion with the organic phase. The lipid suspension was sonicated at room temperature for 10 min with a bath sonicator (Laboratory Supplies Inc., Hicksville, NY) and the pH was adjusted to 7.6. The sonicated liposomes were transferred to a 25-ml round-bottom flask, and 4 ml of a 3:1 (vol/vol) mixture of chloroform/ethyl ether was added. The mixture was briefly sonicated (~30 s) to form a stable emulsion. The emulsion mixture was rotovaped at 30°C with a water aspirator using a Buchi Rotavapor-R (Buchi Laboratoriums Technik AG, Switzerland) until all of the organic solvent was removed. The resulting reverse-phase evaporation vesicles were incubated in a fume hood for 1 h to remove any residual organic solvent. PBS was then added to the lipid suspension to bring the final concentration to 10 mM.

Antibody Incorporation

Immunoliposomes were prepared by a modification of

the methods developed by Shen et al. (64). Palmitoyl antibody, in PBS containing 0.15% deoxycholate at an antibody concentration of 1 mg/ml, was mixed with reverse-phase evaporation vesicles at a lipid-to-antibody weight ratio of 10. 5% (vol/vol) of ethyl ether was added to the vortexing antibody-liposome mixture; this final solution was dialyzed against three changes of 4 L PBS to remove ethyl ether, deoxycholate, and untrapped calcein. The resulting immunoliposomes were run on a 5-ml 5-20% linear sucrose gradient spun at 46,000 rpm for 5 h to evaluate the efficiency of incorporation. Sizing of immunoliposomes was done by measurements obtained from electron micrographs. The immunoliposomes were negatively stained with 0.5% aqueous uranyl acetate and viewed in a Hitachi 600 electron microscope (Hitachi Ltd., Tokyo) at 75 kV. Size histograms were produced from micrographs taken from various preparations of liposomes.

Cell Incubations

Mouse L929 cells (k haplotype) and A31 cells (d haplotype) were grown on glass coverslips which had been pretreated with a 1% solution of gelatin. The medium used for the incubation experiments consisted of PBS containing 1 mM Ca^{++} , 1 mM Mg^{++} , and 16 mM D-glucose. Immunoliposomes (DOPE/PHC or DOPC) at a lipid concentration of 50 $\mu\text{g/ml}$ were incubated with both types of cell at 4°C for

1.5 h. The cells were washed three times with medium and incubated in fresh medium for 30 min more at 4°C to reduce the nonspecific binding.

After the second 4°C incubation, the cells were again washed three times with buffer and then observed under a Leitz Orthoplan epiluminescence microscope equipped with an Orthomat-W camera. Both phase-contrast and fluorescent pictures were taken; all of the fluorescent pictures were taken with the same exposure time (1.5 min).

In parallel experiments cells that had been treated with liposomes at 4°C as above were then incubated at either 20 or 37°C for 2 h, washed, and then photographed under the fluorescence microscope. In another series of experiments the cells were incubated with 50 μ M chloroquine before the 4°C binding step, and after all washing steps the fresh incubation buffer was also supplemented with 50 μ M chloroquine. After incubation with immunoliposomes at 4°C these cells were then incubated at 37°C for 2 h in the presence of chloroquine, washed, and then photographed under the fluorescence microscope.

Quantitation of Cytoplasmic Fluorescence Intensity

Calcein in concentrations from 1 μ M to 1 mM was entrapped in large (>20 μ m) multilamellar liposomes composed of DOPC (65). A calibration curve was established using a Leitz Wetzlar MPV-2 Microscope

Photometer, by measuring the fluorescence intensity from an area of $4 \mu\text{m}^2$ of the flattened liposomes that contained varying concentrations of calcein. At least two different areas of 25 liposomes were measured for each calcein concentration. The results showed a linear calibration curve over the concentration range of $1 \mu\text{M}$ to 1 mM (data not shown). The fluorescence intensity from a spot of same surface area in the cytoplasm of the cells incubated at 37°C with pH-sensitive immunoliposomes was measured for two different areas of 15 cells. The cytoplasmic dye concentration was calculated from the calibration curve under the assumption that the thickness of the cytoplasm is about the same as that of the flattened liposomes. In reality the cytoplasm was probably thinner than the liposomes. Therefore, the estimated dye concentration in cytoplasm represents a lower limit of the actual value. Fluorescence intensity determination of untreated cells showed no measurable autofluorescence. The cytoplasmic dye concentrations of the control experiments were not measured, since the calcein fluorescence appeared in granules and was not evenly distributed in the cytoplasm. The fluorescence intensity of the punctate granules did not fall along the established calibration curve.

Results

Immunoliposomes

The average size of the immunoliposomes composed of DOPE/PHC (8:2), as determined by negative stain electron microscopy, was $1,400 \pm 400$ Å. The results of the sucrose gradient centrifugation showed a co-migration of the liposomes and the monoclonal antibody, which appeared at the top of the gradient. Radioactive marker counting indicated an 80% incorporation of the derivatized antibody into the liposomes.

^4C Binding

Previous work of this laboratory (33) demonstrated that there was specific targeting of immunoliposome with the anti H2K^k antibody to k-haplotype target cells whereas the d haplotype cells showed no specific binding. Fig. 4 shows the binding of calcein-entrapped DOPE/PHC (8:2) and DOPC immunoliposomes to L929 cells. The immunoliposomes bound specifically to the target cells that show an external ring of fluorescence. Incubation of immunoliposome with A-31 cells displayed no detectable fluorescence of the cells. Cells incubated with liposomes without antibody showed no binding of liposomes to either cell type as determined by no visible fluorescence

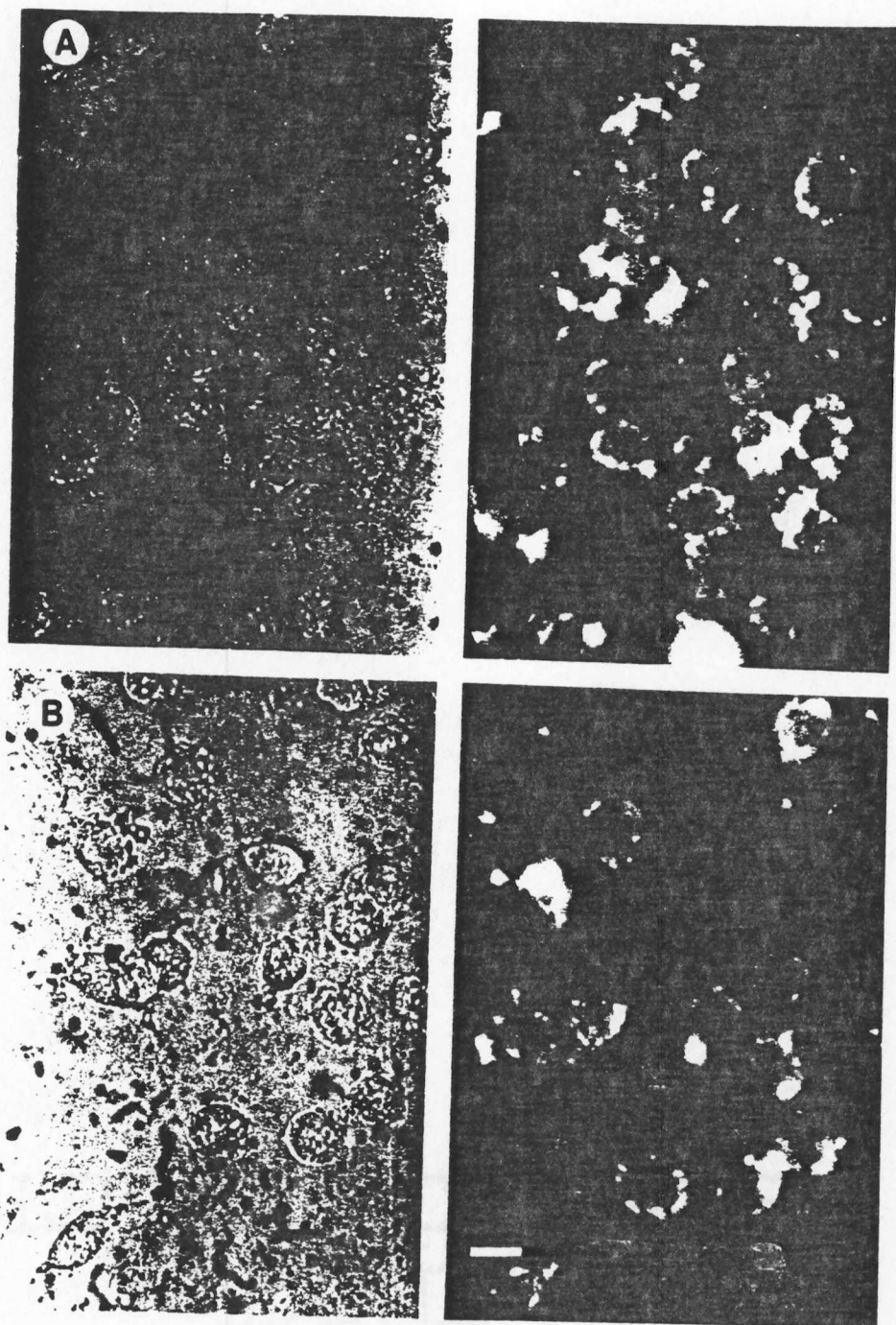


Figure 4. L-929 cells incubated with calcein entrapped immunoliposomes at 4°C as described in Methods, chapter III. A- DOPE:PHC (8:2) immunoliposomes; B-DOPC immunoliposomes. Bar = 0.01mm. Magnification = x1000.

(photographs not shown). Cells incubated with free calcein (0.3 mM) showed no uptake of dye.

20°C Binding

Fig. 5, A and B are photographs of L929 cells treated with immunoliposomes at 4°C, washed, and then incubated at 20°C. Punctate or granular fluorescence appeared inside the cells. Little or no diffused fluorescence in the cytoplasm was observed. It is clear that the liposomes were internalized by the cell, but no cytoplasmic release of dye was observed. The internalized immunoliposomes and their contents remained in the endosome/lysosomes. No significant difference was found between the DOPE/PHC and DOPC immunoliposomes.

37°C Incubation

In these experiments both DOPE/PHC and DOPC immunoliposomes were bound to target cells at 4°C and then incubated at 37°C to allow endocytosis of the bound immunoliposomes. Figure 6A shows photographs of cells treated with the pH-sensitive DOPE/PHC liposomes. The diffused fluorescence observed in these cells clearly indicates a cytoplasmic delivery of calcein. Cells also showed a dark nucleus shadow, which indicates that calcein did not penetrate the nuclear membrane. The calcein released into the cytoplasm would still maintain its

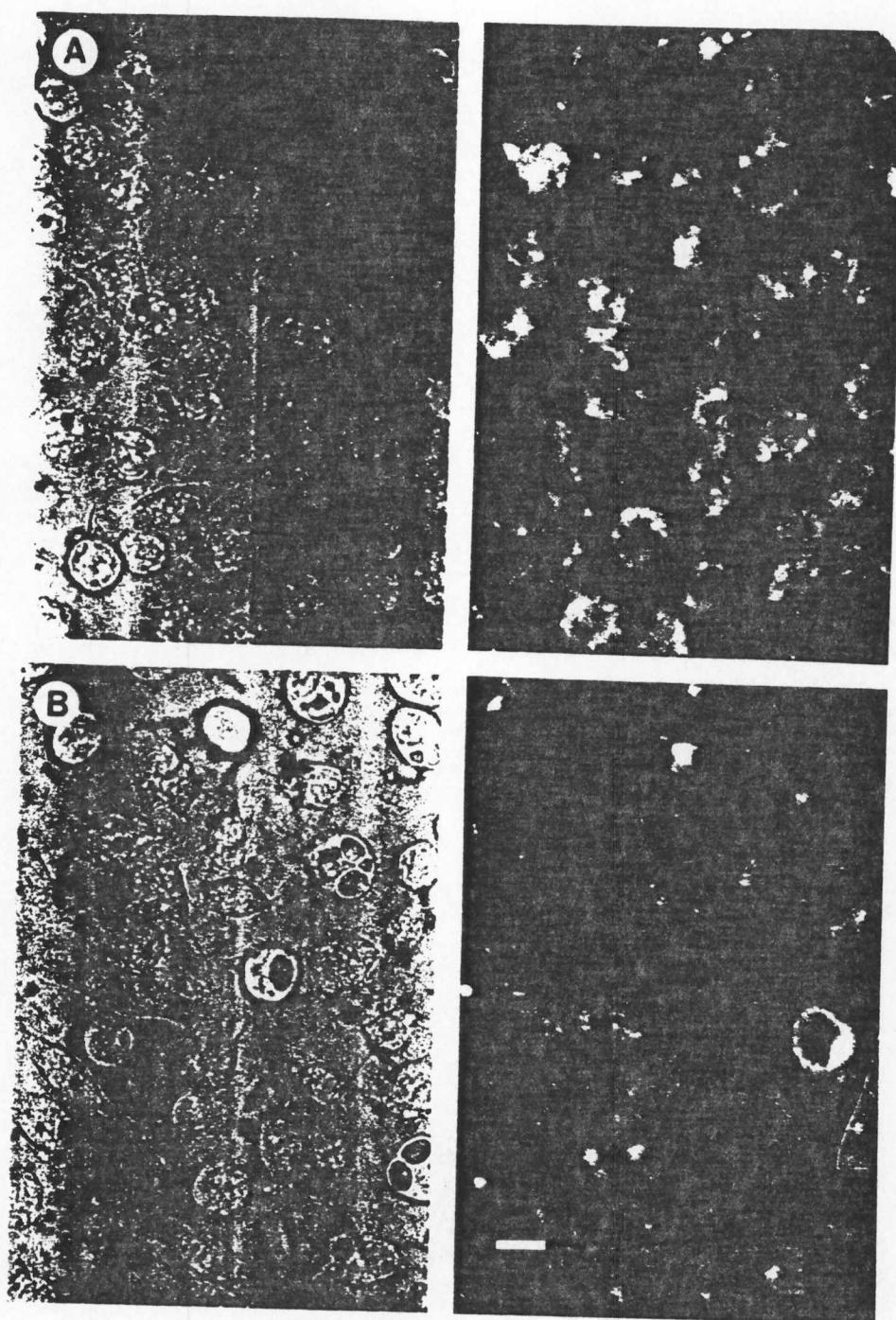


Figure 5. L-929 cells incubated with immunoliposomes at 4°C for 1.5 hr., washed, and then incubated at 20°C for 2.0 hr. as described in Methods, chapter III. A- DOPE:PHC (8:2) immunoliposomes; B- DOPC immunoliposomes. Bar = 0.01 μm . Magnification = $\times 1000$.

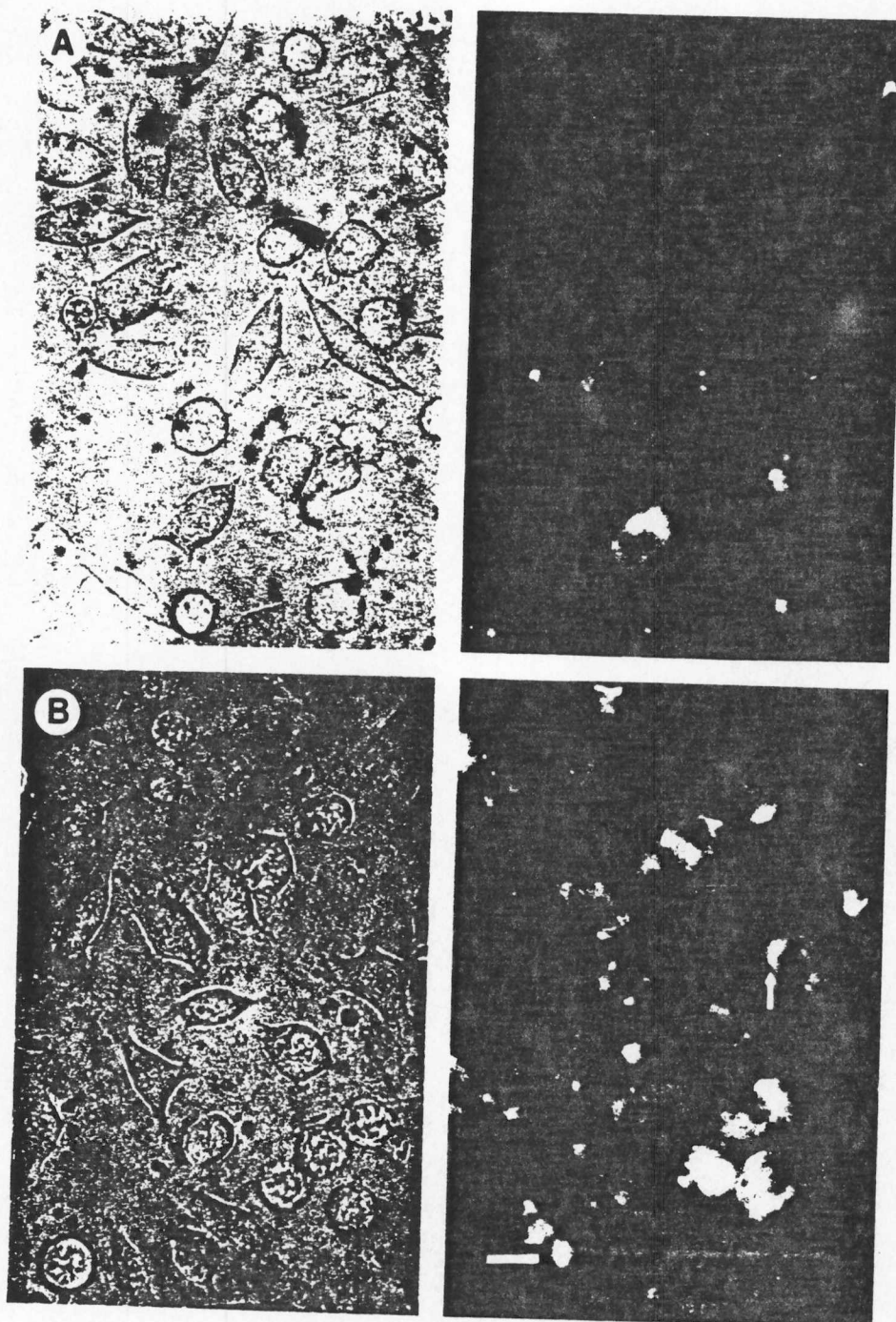


Figure 6. L929 cells incubated with immunoliposomes at 4°C for 1.5 hr., washed, and then incubated at 37°C for 2.0 hr. as described in Methods, chapter III. A- DOPE:PHC (8:2) immunoliposomes; B- DOPC immunoliposomes. Paranuclear fluorescence can be seen in B (↑). Bar = 0.01 mm. Magnification = x1000.

charged nature and should therefore be unable to permeate the nuclear membrane. The calcein may also associate with cytoplasmic macromolecules, which could block its entrance into the nucleus. Using the microscope photometer, we measured the relative fluorescent emission of the cytoplasmic calcein for a number of target cells. Based on the fluorescence intensity calibration curve the concentration of the calcein in the cellular cytoplasm was $50 \pm 20 \mu\text{M}$ ($n = 30$). The average volume of the cell cytoplasm was $\sim 10^3 \mu\text{m}^3$, and that of the immunoliposomes was $\sim 1 \times 10^{-3} \mu\text{m}^3$. The calcein concentration in the immunoliposomes was 60 mM, which is about 1,000-fold higher than the concentration found in the cytoplasm. This result indicates that about one thousand liposomes had released their entrapped calcein into the cytoplasm of a cell.

Cells treated with the pH-insensitive DOPC liposomes did not show any release of dye (Fig. 6B). Only punctate fluorescence was observed with these cells, indicating that the calcein was retained in the endosome/lysosome system. Cells treated with DOPC immunoliposomes did display capping of fluorescence, which has been observed in early work involving targeting of these liposomes (63). Paranuclear fluorescence was also seen in some of the cells treated with DOPC immunoliposomes, indicating delivery of the liposome and its contents to the lysosomes had occurred. Cells incubated with free calcein (0.3 mM)

at 37°C showed some punctate but no diffused fluorescence, probably as a result of pinocytosis.

Incubation with Chloroquine

Chloroquine is a weak base that is readily taken up by cells, and is partitioned into acidic organelles and raises their pH (66-68). It has been demonstrated that chloroquine does not interfere with the receptor-mediated internalization of ligands (66,68,69). Because incubation of target cells with chloroquine blocks the acidification of the endosomes and lysosomes, upon endocytosis the immunoliposomes should encounter a less acidic pH.

Immunoliposomes were bound at 4°C to chloroquine-treated cells, and the incubation temperature was then raised to 37°C to activate endocytosis of the liposomes. Fig. 7, A and B, shows cells that received such treatment. Similarly to the 20°C incubation, the fluorescence appeared punctate both on the periphery of the plasma membrane and inside the cells, which indicates that there was little or no release of the liposome-entrapped calcein. This is expected if the calcein delivery is dependent upon an acid-induced fusion of the immunoliposomes with the endosome membrane. The loss of cytoplasmic delivery with the neutralization of the endosome clearly supports this hypothesis. The cells treated with DOPC

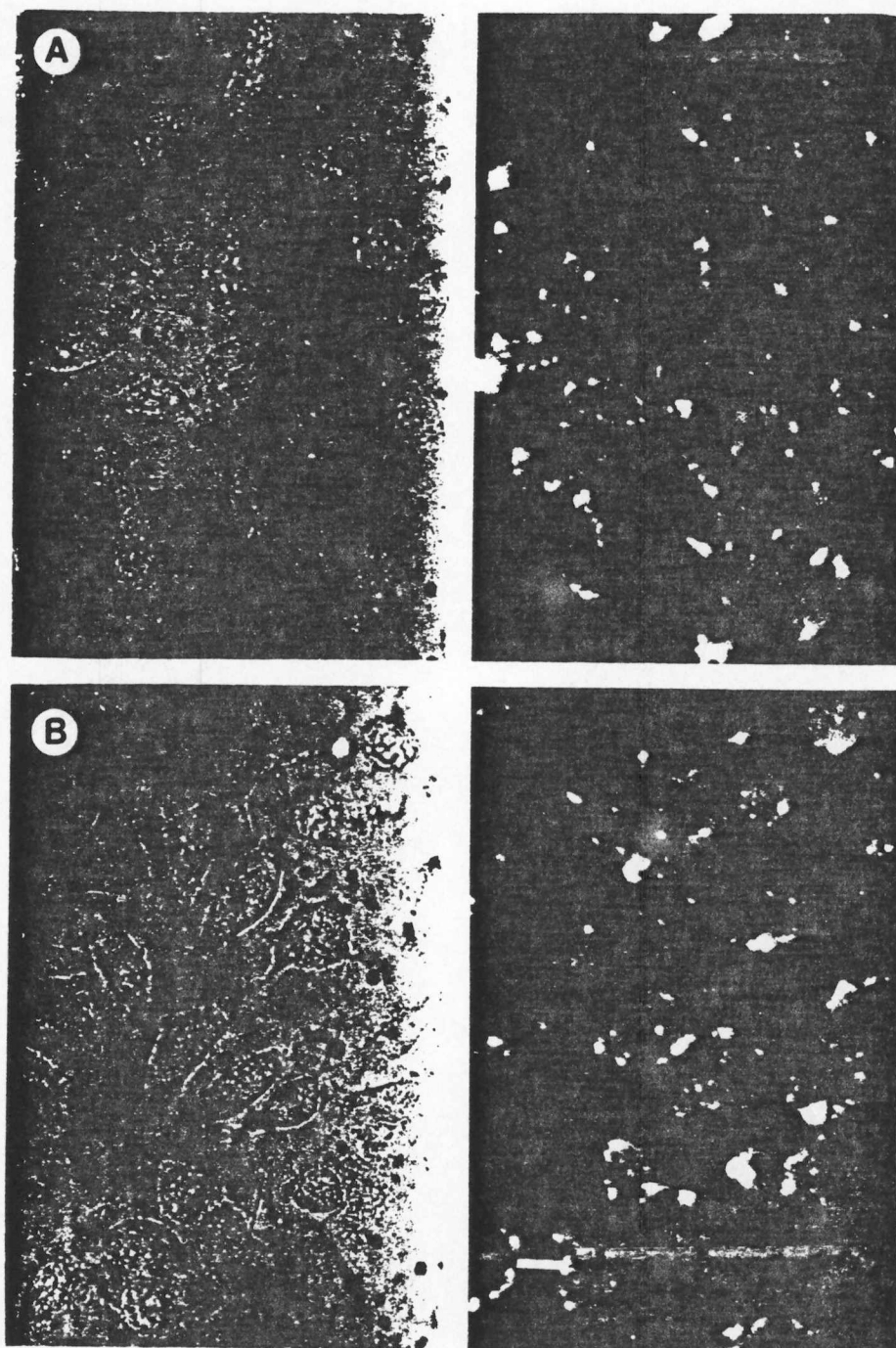


Figure 7. Immunoliposomes were incubated with L-929 cells that had been constantly exposed to 50 μ M chloroquine. Immunoliposomes were incubated at 4°C for 1.5 hr., washed and then incubated at 37°C for 2.0 hr. as described in Methods, chapter III. A-DOPE:PHC (8:2) immunoliposomes; B-DOPC immunoliposomes. Bar = 0.01mm. Magnification = x1000.

immunoliposomes also displayed internal punctate fluorescence similar to that seen with the 20°C incubation.

Discussion

We have previously showed that liposomes composed of DOPE/PHC (8:2) become highly fusion active at acidic pH's (10). The pH for half-maximal fusion lies at ~6.4, whereas almost 100% fusion occurs at pH 4.8 or lower. Although the mechanism of liposomes fusion is not known, it probably involves the formation of the hexagonal phase of DOPE when PHC is protonated at the low pH. Since the pH range at which the liposome becomes fusion competent falls in the same range as the endosome and lysosome pH, the liposomes may become fusion active if they are delivered to these organelles. We previously showed that palmitoyl antibody can be incorporated into liposome membranes and the immunoliposomes are rapidly endocytosed by the target cells by a process that resembles the receptor-mediated endocytosis (33). If fusion of the liposome with the endosome membrane occurs, one would expect to see the release of the liposome content into the cytoplasm of cells. We have used a fluorescent dye, calcein, for this purpose. It is a water-soluble and self-quenching dye. It does not permeate cell membrane due to the high charge content (39).

Immunoliposomes were bound to target cells at 4°C,

washed, and then incubated at 37°C so that normal endocytosis and cellular processing, including endosome acidification, would occur. Cells incubated with liposomes composed of DOPE/PHC, which become fusion competent at acidic pH's, display diffused fluorescence throughout the cytoplasm, indicating a release of the entrapped calcein (Fig. 6A). Since the calcein cannot penetrate through the endosome membrane the dye must have been released directly into the cytoplasm probably by a fusion reaction between the liposome and the endosome membranes. The inability of calcein to cross membranes can also be seen by the lack of fluorescence in the nucleus. The pH-insensitive DOPC liposomes showed little or no diffused fluorescence after the 37°C incubation. Some cells showed high degree of paranuclear fluorescence, indicating this type of liposomes are delivered to the lysosomes.

The cytoplasmic delivery of calcein by the pH-sensitive liposomes could be blocked by chloroquine (Fig. 7A). Since chloroquine effectively raises the pH of the endosomes/lysosomes (66-68), this result strongly indicates the importance of the acidic environment for calcein release. Incubation at 20°C also prevents the dye release by the pH-sensitive liposomes, but the liposomes appear to be intracellular (Fig. 5A). Studies with mutants of Semliki Forest Virus have shown that at 20°C cells actively endocytose but that the fusion of endosomes

with lysosomes is blocked (70,71); concurrent with this blocking effect is a large degree of heterogeneity of the endosome pH (72). The lack of dye release from the pH-sensitive liposomes may indicate that the liposomes are located in those endosomes whose pH's are not sufficiently acidic. Alternatively, the dye release may take place in the lysosomes, which would be blocked if the endosome-lysosome fusion did not occur at 20°C.

It is not clear from the present study if the liposomes actually fused with endosome or lysosome membranes. We also do not know the fate of the liposomes after the dye release. In any case, the cells appeared to be morphologically normal and maintained normal doubling time after the treatment with the pH-sensitive immunoliposomes. If the liposomes fuse with the endosome or lysosome membrane as we suspect, the situation is very similar to the infection pathway of the enveloped virus such as the Semliki Forest, influenza, and vesicular stomatitis viruses. The viral membrane fuse with the endosome membrane in response to the acidic pH (references 73-76). The difference here is that the driving force for fusion in liposomes is a weakly acidic lipid such as PHC, whereas the driving force for the viruses is the viral glycoproteins.

The efficiency of this pH-sensitive immunoliposome delivery system is apparent from the high number of

liposomes ($\sim 10^3$ liposomes/cell) that released their contents to the cytoplasm of the cell. This high level of delivery should prove very useful in future studies. For example, to know that immunoliposomes encapsulated with a monoclonal antibody at 10 mg/ml could deliver to each cell $\sim 10^4$ antibody molecules would obviously be very helpful in studying various cellular functions. There are a great many potential uses for this pH-sensitive immunoliposome system, depending only upon the ability to trap the desired molecule.

In conclusion, we have designed a liposome system that efficiently delivers the contents to the cytoplasm of the cells in a target-specific manner. Since water-soluble molecules can be easily encapsulated in liposomes, this system should be very useful for the cytoplasmic delivery of drugs, enzymes, antibody, nucleic acids, and other biologically active molecules into the living cells. The work of this type is described in Chapter V.

CHAPTER IV

EFFECTS OF PROTONS AND DIVALENT CATIONS ON pH-SENSITIVE
LIPOSOMES COMPOSED OF DIOLEOYL PHOSPHATIDYLETHANOLAMINE
AND OLEIC ACIDIntroduction

Previously we have demonstrated the ability of liposomes composed of DOPE:PHC (8:2) to become fusogenic at a mildly acidic pH (10). Furthermore, these pH-sensitive liposomes have been shown to successfully deliver their entrapped contents to the cytoplasm of target cells using an established monoclonal antibody targeting system (33). The cytoplasmic delivery occurs presumably by an acid triggered event of the liposome which encounters a low pH in the cellular endosomes (39). This acid induced cytoplasmic delivery event is a common pathway for viral infection of cells by such viruses as Semliki Forest Virus (46), Influenza Virus (47) and Vesicular Stomatitis Virus (47). The route of virus entry is an acidic pH induced glycoprotein conformation change which initiates release of the viral genome into the cytoplasm (77,78). Since the pH-sensitive liposomes contain no protein, the proton mediated membrane fusion and cytoplasmic delivery must involve a different mechanism. In order to elucidate the mechanism of the pH-sensitive liposome fusion event this chapter describes a

series of biophysical studies of the DOPE:oleic acid (OA) liposomes under various acidic conditions in order to monitor the molecular events which led to fusion of these liposomes.

Materials and Methods

Materials

Dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE and N-(lissamine rhodamine B-sulfonyl)-PE were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol, oleic acid and calcein were purchased from Sigma Chemical Co.

Liposome Preparation

Dried lipid film, of the appropriate lipid composition was hydrated in phosphate buffered saline (PBS) at pH = 7.8. The lipid suspension was sonicated using a bath sonicator (Laboratory Supplies Inc.) for 15 min, forming small unilamellar vesicles (SUV). The pH was monitored during sonication in order to maintain a constant pH of 7.8. The final liposome concentration was 10 mM.

Membrane Mixing Assay

DOPE:OA (8:2 mole/mole) SUV containing 1.0 mole %

each of NBD-PE and Rh-PE, along with an unlabeled population of DOPE:OA (8:2 mole/mole) were prepared as described under liposome preparation. The liposomes in a 3:1 ratio of unlabeled to labeled were added to 2 ml of PBS (pH = 7.8) at room temperature (final concentration = 0.2mM). The relative fluorescence intensity of both NBD-PE and Rh-PE were measured using a Perkin-Elmer LS-5 fluorescence spectrophotometer. The samples were excited at 468 nm using a slit width of 5 nm and emission was measured at 530 nm for NBD-PE and 580 nm for Rh-PE using a 3 nm slit width. Following fluorescent measurements, the pH of the solutions was lowered by addition of varying amounts of 0.1N HCl. After a 5 min. incubation the pH was returned to pH = 7.8 by addition of 0.1N NaOH and the fluorescence of both fluorophores was measured again. The percent lipid mixing was determined as described by equation 1 of Chapter II and plotted as percent fusion versus pH. A lipid mixing versus pH curve was also established with DOPE:Cholesterol:OA (6:2:2) following the same protocol. In order to determine the effect of divalent cations on the pH lipid mixing curve, varying amounts of Ca^{+2} or Mg^{+2} from 1-4mM was added to the PBS prior to liposome addition. Lipid mixing was determined following the same protocol described above.

Concentration Effect of Lipid Mixing

DOPE:OA SUV at a 3:1 unlabeled to labeled ratio were added to PBS at a final lipid concentration ranging from 0.05 mM to 3.2 mM. A membrane lipid mixing versus pH curve was established using the same procedure outlined above. In order to compensate for light scattering effects at high concentrations, all of the samples were adjusted to a 0.2 mM concentration following the acid-base cycle. The pH at which 50% of maximal lipid mixing took place, determined from the lipid mixing vs. pH curve, is termed pH_{50} . pH_{50} is a measure of the acid sensitivity of the liposome system; the higher pH_{50} is, the greater the system is sensitive to acid.

^{31}P -Nuclear Magnetic Resonance (NMR)

Dried lipid of the appropriate composition was hydrated with 50 mM Tris - 100 mM NaCl buffer (pH = 7.8) containing 30% D_2O at a lipid concentration of 50 mM. The lipid solution was sonicated using a bath sonicator with maintenance of pH at 7.8. The liposomes were incubated at the experimental condition; i.e. acidic pH, presence of divalent cation or a combination of both, as indicated in the results section, for 12 hours. ^{31}P -NMR was measured with a JEOL FX90Q (90 MHz) Fourier Transfer NMR. A proton decoupled signal was measured from 6000 scans with the following parameters: Frequency = 50,000 Hz , pulse width

2.5 μ s, pulse delay time = 2 ms, delay = 0.5 μ s, acquisition time = 81.9 ms observed frequency 36.19 MHz and offset frequency = 82.5 KHz. H_3PO_4 in 30% D_2O was employed as an external standard.

Liposome Light Scattering

When lipids are in a stable bilayer configuration as in SUV, they scatter little light. As the temperature increases and the lipids transition into the hexagonal phase, light scattering increases significantly. We have measured the tendency of the liposome system to go into the hexagonal phase by measuring light scattering at different temperatures. 90° light scattering of DOPE:OA (8:2) or DOPC liposomes, at 0.2 mM in PBS, were measured using a Perkin Elmer LS-5 fluorescence spectrophotometer at 600 nm. The liposomes, at varying pH from 6.6 to 7.8, in the presence of the divalent cations Ca^{+2} and Mg^{+2} at concentrations from 0.0 to 4.0 mM, were measured as a function of temperature. The temperature was scanned at $0.2^\circ C/min$ starting at $12^\circ C$, ending at $65^\circ C$. T_{50} is defined as the temperature at which 50% maximal increase of light scattering takes place (see Fig. 15). T_{50} is a quantitative measure of the liposome's tendency to transform into hexagonal phase; lower T_{50} means higher tendency for the hexagonal phase and vice versa.

Results

Figure 8 displays the effect of lowering the pH from 7.8 to 6.2 on the ability of SUV to undergo membrane mixing as monitored by resonance energy transfer. DOPE:OA (8:2 mole/mole) liposomes began to undergo lipid mixing at pH = 7.1 and continued to display a pH dependent membrane mixing effect until pH = 6.3 where the percent lipid mixing leveled at 80%. The pH_{50} for these liposomes was 6.9. Vesicles composed of DOPE:Cholesterol:OA (6:2:2 mole/mole) had an identical pH dependent lipid mixing curve as the DOPE:OA vesicles, showing the inclusion of a relatively small amount of cholesterol, which may inhibit leakage, does not affect lipid mixing. The addition of 1 mM Ca^{+2} increased the pH_{50} to 7.1, while an addition of 2 mM Ca^{+2} displayed a $pH_{50} = 7.5$. A Ca^{+2} concentration of 3 mM or higher caused complete mixing of the liposomes at pH 7.8 equal to the lipid mixing of divalent cation free liposomes at pH = 6.3. Mg^{+2} addition also caused a shifting of the pH_{50} to more alkaline pH's but to a lesser extent than Ca^{+2} . 1 mM Mg^{+2} gave a pH_{50} just below 7.0, while 2mM Mg^{+2} had a $pH_{50} = 7.1$. Increasing the Mg^{+2} concentration to 3 mM shifted the pH_{50} to 7.4; 4 mM Mg^{+2} caused a greater than 50% lipid mixing at pH 7.8 but did not cause complete mixing until pH = 7.2.

In order to demonstrate the effects of increased liposome-liposome interactions on lipid mixing, pH

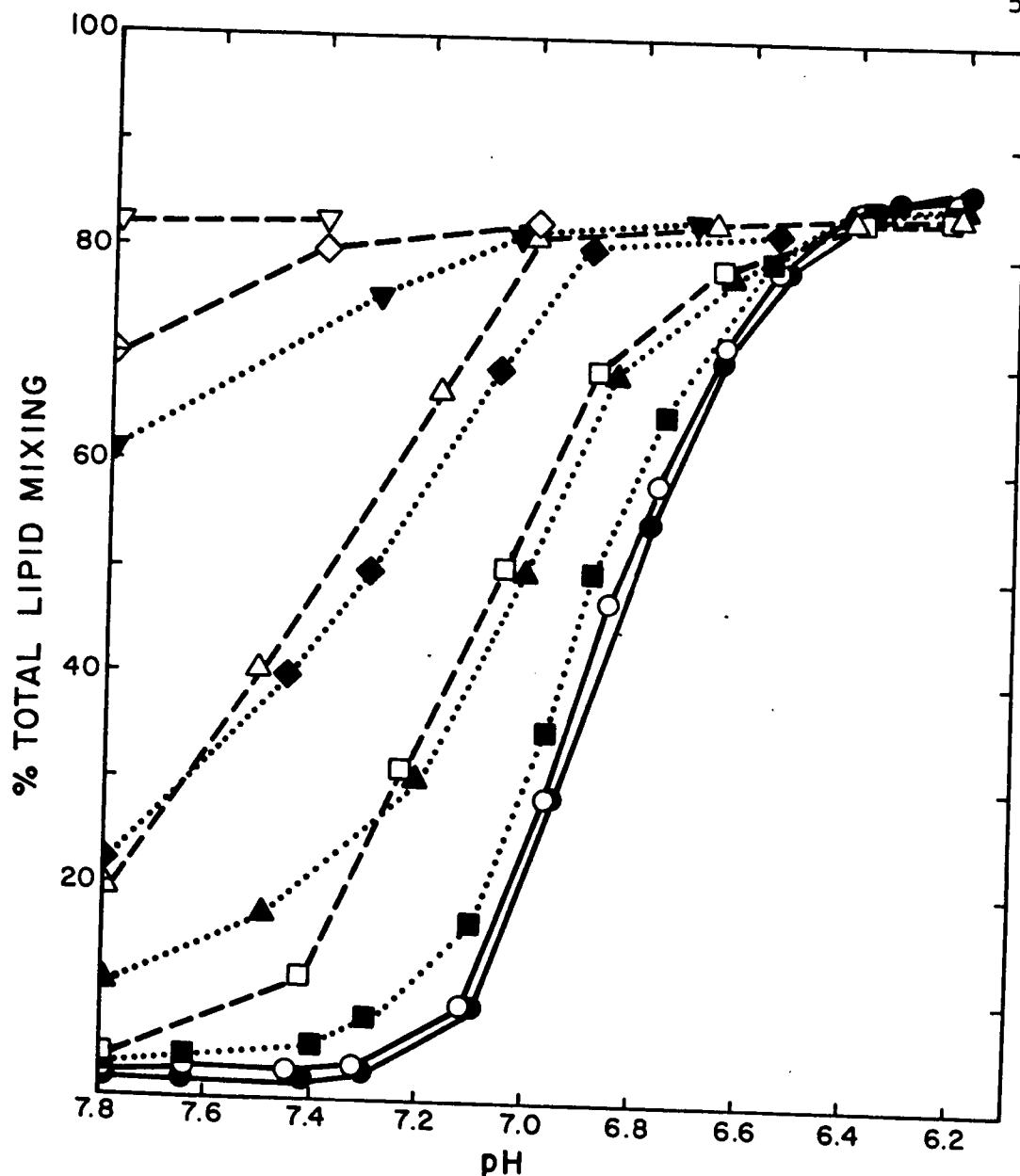


Figure 8. pH and divalent cation effect of lipid mixing. DOPE:OA (8:2) liposomes were measured for lipid mixing using resonance energy transfer. Percent lipid mixing was determined as described in Methods, chapter IV. pH curves were established in the presence of varying amounts of Ca^{++} and Mg^{++} . — (○) DOPE:OA (8:2); — (●) DOPE:Cholesterol:OA (6:2:2); --- (○) Ca^{++} (open symbols); (○) Mg^{++} (closed symbols), (□) - 1 mM cation; (△) - 2mM cation; (◇) - 3mM cation; (▽) - 4mM cation.

dependent curves of lipid mixing for DOPE:OA (8:2 mole/mole) liposomes were determined at different lipid concentrations. The lipid concentration dependence of membrane mixing is shown in Figure 9. As the total liposome concentration is increased the values of pH_{50} also increase. The higher the lipid concentration the easier it is for protons to induce lipid mixing.

^{31}P -NMR is an effective tool for distinguishing between the bilayer and hexagonal II phases of PE molecules. PE lipid in a bilayer configuration displays a characteristic peak at -5.0 ppm with a slight left handed shoulder. When the PE exists in a hexagonal II phase the main peak shifts to 5.0 ppm with a right handed shoulder. The ^{31}P -NMR spectra of PE in small unilamellar liposomes displays an isotropic symmetrical peak at -5.0 ppm. The symmetry is a product of the fast tumbling rate of small liposomes (16). Figure 10 shows ^{31}P -NMR spectra of DOPE:OA (8:2 mole/mole) liposome at $pH = 7.8, 7.2$ and 6.2 . At $pH = 7.8$ the vesicles display a typical isotropic bilayer profile, upon reduction of the pH to 7.2 the lipid begins to show characteristic hexagonal II pattern along with the bilayer pattern. If the DOPE:OA vesicles are exposed to $pH = 6.2$ a complete hexagonal II configuration is displayed. The effects of the divalent cations Mg^{+2} and Ca^{+2} at $pH 7.8$ on the phase behavior of DOPE is shown in Figure 11 and 12,

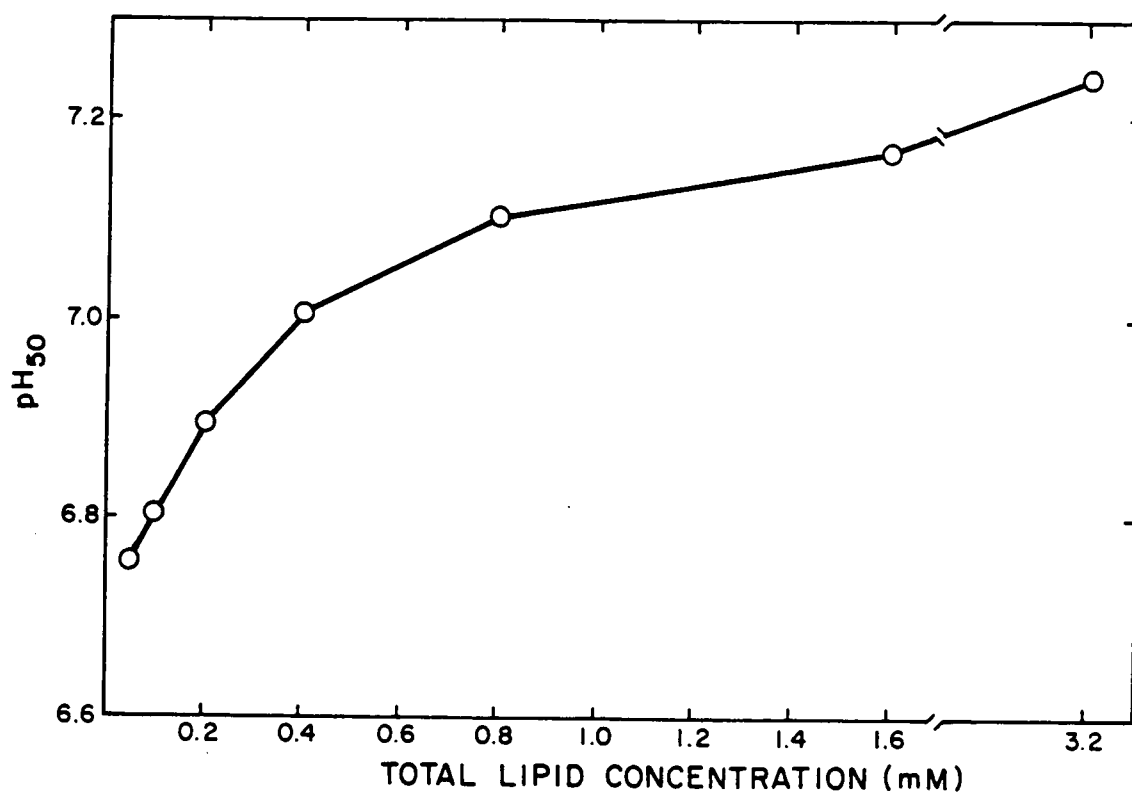


Figure 9. Concentration dependence of liposome fusion. The pH dependence of fusion of DOPE:OA (8:2) liposomes was determined at various lipid concentrations. The pH₅₀ for each concentration was determined as described in Methods, chapter IV.

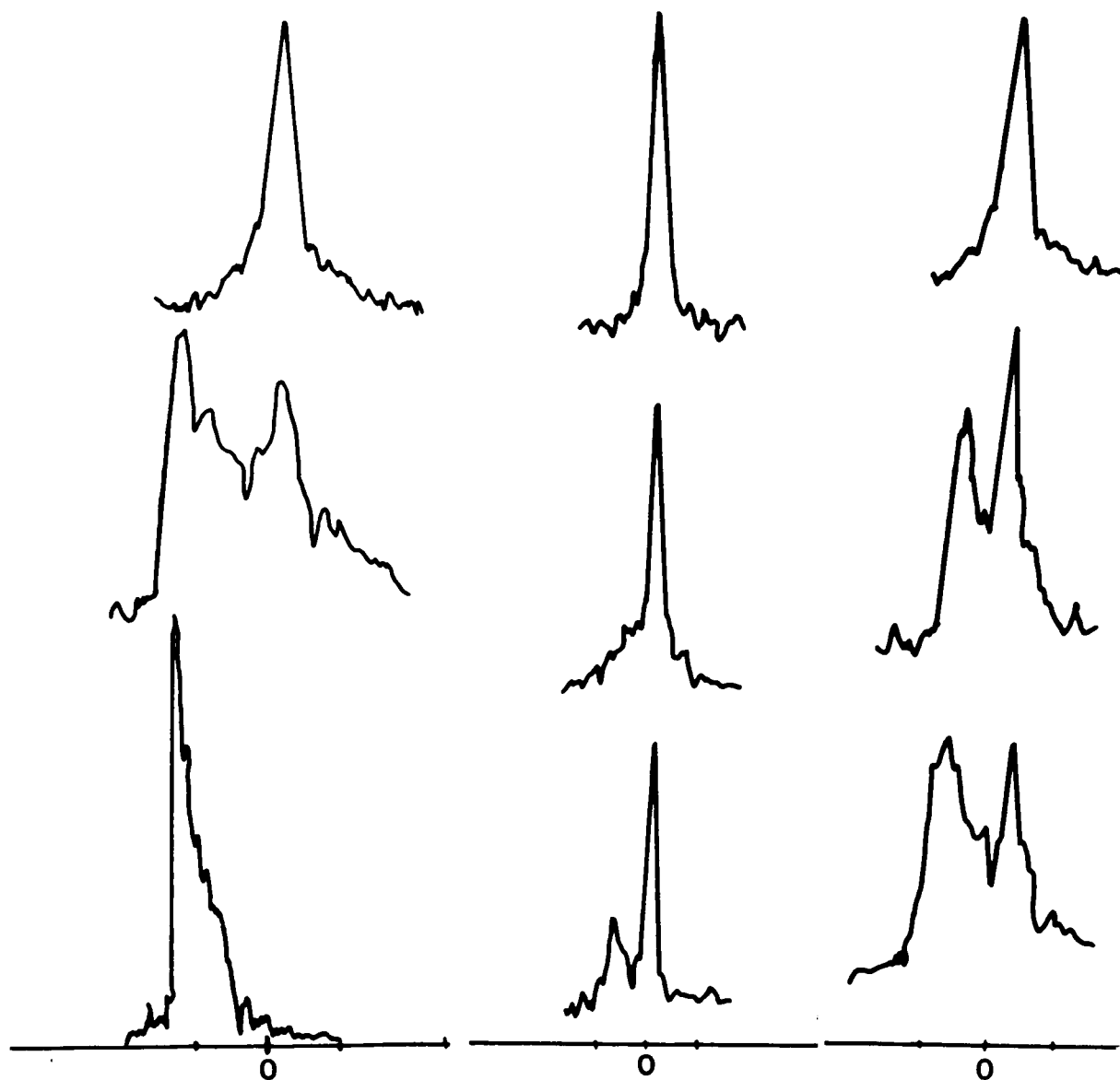


FIGURE 10

FIGURE 11

FIGURE 12

Figure 10. ^{31}P -NMR spectra of DOPE:OA (8:2) liposomes at a) pH=7.8; b) pH=7.2; c) pH=6.2

Figure 11. ^{31}P -NMR spectra of DOPE:OA (8:2) liposomes at pH=7.8 in the presence of a) 2mM Mg^{++} ; b) 4mM Mg^{++} ; or c) 6mM Mg^{++} .

Figure 12. ^{31}P -NMR spectra of DOPE:OA (8:2) liposomes at pH=7.8 (a) in the presence of b) 2mM Ca^{++} ; or c) 4mM Ca^{++} .

respectively. 2 mM Ca^{+2} is able to begin inducing the hexagonal II phase in DOPE:OA liposome; upon increasing the Ca^{+2} concentration to 4 mM an increase in the amount of DOPE in the hexagonal II phase is displayed in the spectrum, though some lipid still remains in the bilayer configuration. Mg^{+2} is less effective in promoting hexagonal II phase formation in DOPE:OA liposome. At a 4 mM Mg^{+2} concentration the DOPE still displays a bilayer NMR pattern. At a concentration of 6 mM, Mg^{+2} begins to promote a small amount of hexagonal II phase in the vesicles but a majority of the lipid still maintains a bilayer structure. The synergistic effect of protons and Ca^{+2} to promote DOPE hexagonal II configuration is seen in Figure 13. The addition of Ca^{+2} at 2 mM and 4 mM increase, respective to concentration, the amounts of DOPE in the hexagonal II formation; though, even at pH = 7.2 in the presence of 4 mM Ca^{+2} , the lipid still displays a small amount of DOPE in the bilayer structure. ^{31}P -NMR scans of DOPC at pH's varying from 7.8 to 6.2, in the presence and absence of Ca^{+2} at a concentration of 2 mM and 4 mM, display a profile indicating complete isotropic bilayer configuration under all conditions (Fig. 14).

To determine the effects of temperature on the lipid phase behavior of DOPE:OA vesicles, changes in light-scattering were measured as a function of increasing temperature. When DOPE exists in a bilayer configuration



FIGURE 13

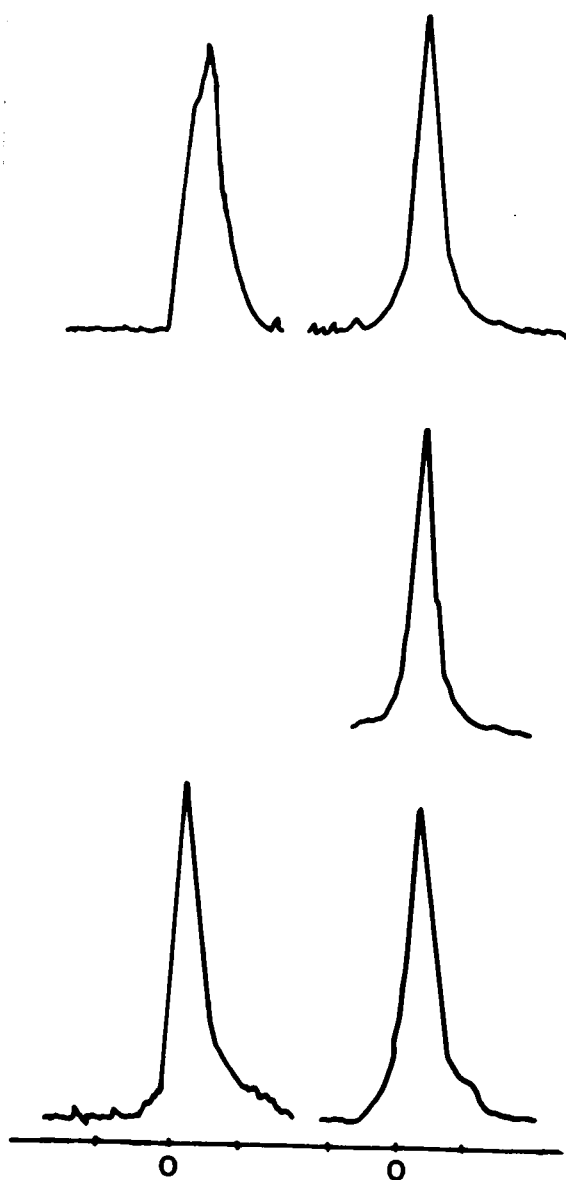


FIGURE 14

Figure 13. ^{31}P -NMR spectra of DOPE:OA (8:2) liposomes at pH=7.2 (a) in the presence of b) 2mM Ca^{++} or c) 4mM Ca^{++} .

Figure 14. ^{31}P -NMR spectra of DOPC liposomes at pH=7.8 (a) in the presence of b) 2mM Ca^{++} or c) 4mM Ca^{++} ; and at pH=6.2 (d) in the presence of e) 4mM Ca^{++} .

light scattering is at a minimum; upon a transition to the hexagonal II phase the DOPE undergoes aggregation and/or change in refractive index which can be measured as an increase in light scattering. Table 3 lists the T_{50} for DOPE:OA (8:2 mole/mole) liposomes at a 0.2 mM concentration under various conditions. As the pH of the buffer was reduced from 7.8 to 6.6 T_{50} progressively decreased, indicating that the system had a higher tendency to transform into the hexagonal phase. Similar to lipid mixing the inclusion of Ca^{+2} increased the temperature-dependent bilayer destabilization. Mg^{+2} was also able to act synergistically with protons but to a lesser extent than Ca^{+2} . Figure 15 is an example of the light scattering profile produced by increasing the temperature of DOPE:OA at various pH's and exposure to increasing amounts of divalent cations. DOPE:OA at physiological pH shows no transition up to 65°C (Figure 15 trace a). DOPE:OA at a pH = 6.9 displays a phase transition with the T_{50} equal to 36°C (Figure 15 trace b). Trace c of figure 15 represents DOPE:OA at pH = 7.4 in the presence of 2 mM Ca^{+2} ($T_{50} = 25^{\circ}\text{C}$). Upon cooling of samples which had undergone a phase transition no reverse of light scattering was observed.

Discussion

Similar to the previously described DOPE:PHC

Table 3

Temperature Dependence of T_{50} of DOPE:OA Liposomes

Divalent Cation Concentration							
0 mM		1 mM		2 mM		3 mM	
pH		Ca ⁺⁺	Mg ⁺⁺	Ca ⁺⁺	Mg ⁺⁺	Ca ⁺⁺	Mg ⁺⁺
7.80	>65	49	>65	32	48	23	39
7.40	>65	38	49	25	38	<15	28
7.10	45	31	39	19	32	<15	24
6.90	36	<15	23	<15	<15	<15	<15
6.60	<15	<15	<15	<15	<15	<15	<15

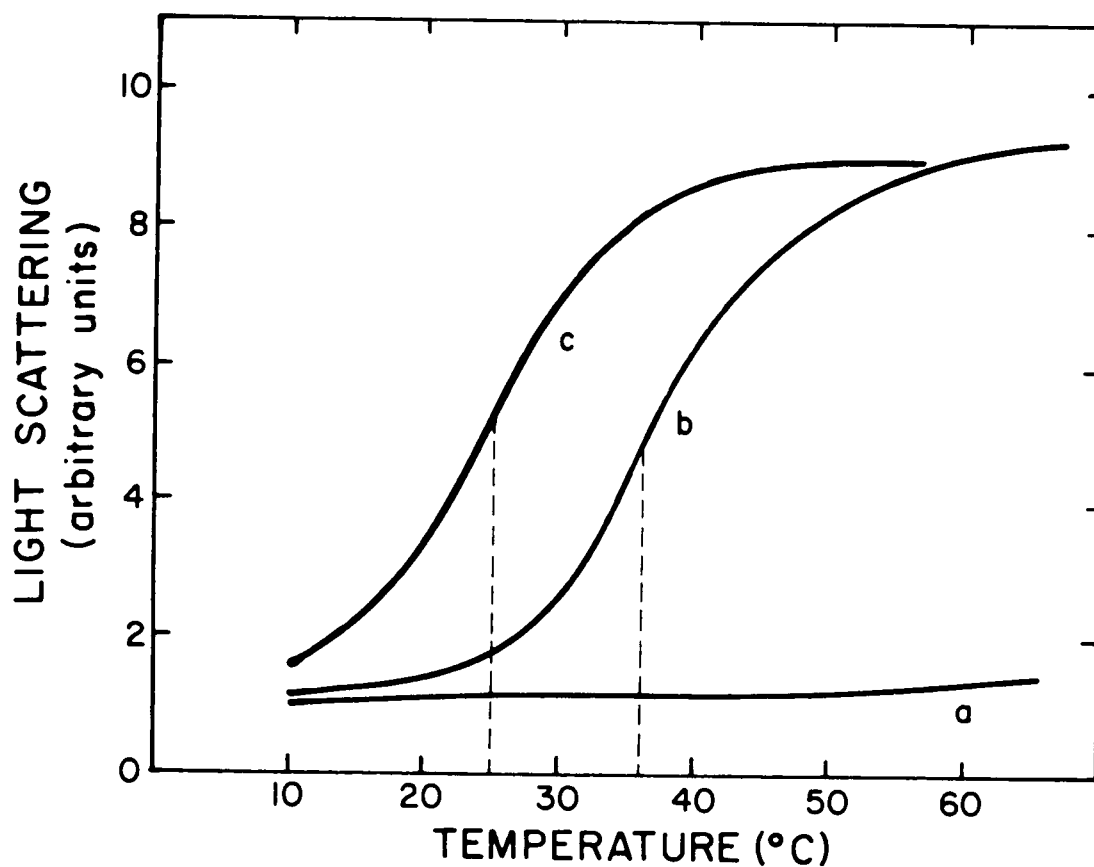


Figure 15. Temperature dependence of light scattering. 90° light scattering as a function of increasing temperature was measured for DOPE:OA liposomes as described in Methods, chapter II. a) pH= 7.8 without divalent cations, b) pH= 6.9 without divalent cation, c) pH= 7.4 in the presence of 2mM Ca^{+2} . Dashed line indicates T_{50} .

liposomes (10), DOPE:OA SUV undergo a fusion event when the pH is decreased below 7.2. The pH of fusion falls within a short range of pH's from 7.1 to 6.6. As can be seen in figure 9, increasing the lipid concentration causes a shifting of the pH_{50} to more alkaline pH's. Since a fusion event is dependent upon the number of liposome-liposome interactions, the increase in concentration will increase the collision frequency between liposomes. Thus the increase in liposome content at higher concentration will increase the probability of a fusion event occurring between vesicles. The curve of lipid mixing versus pH can also be shifted toward higher pH by the addition of the divalent cations Ca^{+2} and Mg^{+2} . Both divalent cations have the ability to cause a bridging effect between negatively charged OA molecules. Bridging between OA molecules within the same membrane would neutralize the stabilizing effect of the OA on the DOPE, similar to protonation; this allows the DOPE to undergo a transition to the hexagonal II phase, initiating a fusion event. Bridging between OA molecules on different membranes would increase the bilayer to bilayer interactions, since fusion is dependent on bilayer collisions this intervesicular bridging would increase the frequency of fusion events. As can be seen in figure 8, Ca^{+2} is more effective than Mg^{+2} in increasing the value of pH. The propensity of Ca^{+2} to induce the hexagonal II

phase of DOPE:OA liposomes at a much lower concentration than Mg^{+2} can also be seen in the ^{31}P -NMR spectra (Fig. 11 and 12). The ability of Ca^{+2} to induce the DOPE:OA liposomes to assume the hexagonal II phase would increase its effect on acting synergistically with protons to promote liposome fusion. This synergistic cooperation between Ca^{+2} and protons can also be seen as an increase in the hexagonal II phase of the pH-sensitive liposomes displayed using ^{31}P -NMR (fig. 13). Thus, both divalent cations increase vesicle to vesicle interaction which promotes fusion at higher pH's; but in addition Ca^{+2} is able to induce hexagonal II phase transition in the pH-sensitive liposomes therefore increasing its ability to promote fusion.

The appearance of hexagonal II phase in the DOPE:OA liposomes at higher pH's than the corresponding pH needed for the lipid mixing assay is due to the higher concentration of lipid used in the NMR preparations. As discussed earlier, increasing the lipid concentration increases the number of liposome collisions; this produces lipid mixing and fusion at higher pH's. This concentration effect has also been demonstrated previously with Ca^{+2} induced fusion of PS containing liposomes (79). Also, using kinetic analyses of membrane fusion Ellens et al. (11) demonstrated a positive correlation between concentration and lipid mixing using pH-sensitive

liposomes composed of PE:cholesterylhemisuccinate.

In order to elucidate the thermostability of the DOPE:OA liposome, light scattering was employed to analyse temperature induced hexagonal II phase formation. Light scattering represents an indirect method of observing changes in the DOPE lipid conformation. The physical transition from the bilayer to hexagonal II phase causes an increase in light scattering due to the aggregated state of the hexagonal II phase. In addition the hexagonal II form of DOPE may have a different index of refraction. DOPE alone has a bilayer to hexagonal II phase transition temperature (T_H) at about 11-13°C (16). The inclusion of 20 mole percent of OA allows bilayers to be formed which are stable to a temperature greater than 65°C (Table 3). As the OA is protonated by a decrease in pH, the T_{50} decreases approaching the original T_H of DOPE alone; this confirms that the protonation of OA disrupts its stabilizing ability forcing the DOPE to revert to its thermodynamically stable hexagonal II configuration. The addition of divalent cations, similar to the lipid mixing, acts synergistically with protons to promote hexagonal II formation by disrupting the stabilizing effect of OA. Again, Ca^{+2} is more effective than Mg^{+2} in inducing the hexagonal phase formation.

The results of these studies led to the following conclusion of the mechanism of fusion. OA is able to

stabilize DOPE into a bilayer configuration by increasing the hydration layer of the lipid head groups, by decreasing intramembrane interaction between adjacent DOPE head groups and by decreasing intermembrane interactions (16). Upon protonation of OA the stabilizing effect is neutralized. Once neutralized, the OA no longer reduces the inter- and intra-membrane interactions of DOPE; upon liposome-liposome contact (i.e. liposome collisions) the DOPE begins to revert to the hexagonal II phase and lipid mixing and fusion result. Divalent cations accelerate the fusion event both by neutralizing the negative charge of OA and by increasing the liposome-liposome interactions.

These pH-sensitive liposomes appear, though, to be an efficient method for delivering entrapped macromolecules to the cytoplasm of cells by exploiting the acidic pH of endosome (41), which are an inherent intermediate in the endocytic pathway. Cytoplasmic delivery of calcein and FITC-dextran by untargeted DOPE:OA pH-sensitive liposomes has been demonstrated with CV-1 cells (40). In a subsequent chapter evidence will be presented for cytoplasm delivery of cytotoxic drugs using antibody targeted DOPE:OA liposomes.

CHAPTER V

pH-SENSITIVE IMMUNOLIPOSOMES COMPOSED OF DIOLEOYL
PHOSPHATIDYLETHANOLAMINE AND OLEIC ACID AS AN
EFFICIENT AND TARGET-SPECIFIC CARRIER FOR
ANTITUMOR DRUGS

Introduction

Liposomes have been extensively studied as a system for the delivery of drugs, proteins, and biological agents to cells (1,32). A major step in the establishing of an effective mode of delivery has been previously developed with the use of monoclonal antibody covalently attached to liposomes, known as immunoliposomes (62). Ours and other laboratories have successfully developed model target systems which specifically deliver liposomes to target cells (33-37). These systems deliver the liposomal contents to the cellular endosomes/lysosomes where, unless the entrapped molecule can effectively escape, it is degraded by the lysosomal enzymes and rendered inactive (33). Thus, although a target-specific delivery system has been well established, no method has been developed to deliver the liposome contents to the cellular cytoplasm.

Previously it has been demonstrated that liposomes composed of DOPE and acylated amino acids such as palmitoylhomocysteine (PHC) (10) or fatty acids such as,

arachidonic acid (Kanda and Huang, unpublished data), oleic acid (OA) (12,20), palmitic acid (20), or a cholesterol derivative cholesterylhemisuccinate (11) are fusion competent when exposed to an acidic environment. Exposure of liposomes of these compositions to a pH of approximately 5.0-6.5, which is the inherent pH of endosomes, will cause them to fuse with adjacent membranes. Combining our anti-H2K^k antibody targeting system with pH-sensitive liposomes composed of DOPE:PHC we have developed a pH-sensitive immunoliposome system which is both specifically targeted and fusogenic at acidic pH (41). Using this system, we have previously demonstrated the ability of these pH-sensitive immunoliposomes to deliver an entrapped dye, calcein, to the cytoplasm of target L-929 cells; while untargeted liposomes and pH-insensitive immunoliposomes were unable to promote cytoplasmic delivery, but only endosomal/lysosomal delivery (41). In addition, Straubinger et al. have demonstrated the ability of untargeted liposomes composed of egg PE and oleic acid (7:3) to promote cytoplasmic delivery of both calcein and FITC-dextran to CV-1 cells (40).

In this chapter we demonstrate the ability of pH-sensitive immunoliposomes composed of DOPE:OA (8:2) to enhance the cytotoxic effect of the drug Ara-C to target cells as compared to free drug, and drug entrapped in untargeted liposomes, or in the pH-insensitive

immunoliposomes. Furthermore, the mechanism of action for the pH-sensitive immunoliposomes was elucidated by studying the effects of competitive blockers and lysosomotropic amines on the immunoliposome's ability to achieve cytoplasmic delivery. The results of this work support the hypothesis that the pH-sensitive immunoliposomes deliver their entrapped contents to the cellular cytoplasm by an acid induced fusion of the liposomes with the endosome membrane following a receptor-mediated endocytosis.

Materials and Methods

Antibody Preparation

Anti H-2K^k antibody from the mouse hybridoma cell line 11-4.1 was purified, labeled with ¹²⁵I, and derivatized with the N-hydroxysuccinimide ester of palmitic acid, as described by Huang et al. (62).

Liposome Preparation

Reverse-phase evaporation vesicles were prepared as follows. Solvent free lipid films of DOPE:OA (8:2) or DOPC were suspended in phosphate buffered saline (PBS) containing the cytotoxic drug to be entrapped. [³H] Ara-C, at 1×10^{-4} cpm/ μ g, or [³H] MTX, at 0.5×10^{-4} cpm/ μ g, were used to monitor the concentration of the respective drug encapsulated. A ratio of 25 μ l of buffer per μ mole

of lipid was maintained in order to form a stable emulsion with the organic phase in the subsequent step. The lipid suspension was sonicated at room temperature for 5 min with a bath sonicator (Laboratory Supplies, Inc.). The pH of the DOPE:OA suspension was adjusted to pH 9.5, while the pH of the DOPC was maintained at 8.0. The sonicated vesicles were transferred to a 25 ml round bottom flask and 3 ml of distilled diethyl ether was added. The mixture was vigorously vortexed and briefly sonicated (30s) to form a stable emulsion. The mixture was rotovaped at room temperature using a water aspirator until all of the organic solvent was removed. The liposome suspension was then incubated for 30 min in a fume hood to remove residual organic solvent. PBS at the respective pH's was then added to the reverse phase evaporation vesicles to bring the final lipid concentration to 10 mM.

Immunoliposome Preparation

Immunoliposomes were prepared by a modification of the method developed by Shen et al. (64) as modified by Connor and Huang (41). Palmitoyl antibody, in PBS containing 0.15% deoxycholate and at an antibody concentration of 3 mg/ml, was mixed, with constant stirring, with the liposome suspension at a lipid to antibody weight ratio of 10. Diethyl ether was added to

the vortexing solution to a final concentration of 2% (v/v). This antibody-liposome mixture was dialyzed against 3 changes of 4 liters PBS (pH 7.9) over 24 hr to remove diethyl ether, deoxycholate, and untrapped drug. The final entrapped drug concentrations for the dialysed immunoliposome preparations were 4.0 μ g Ara-C/ μ mole lipid and 3.0 μ g MTX/ μ mole lipid, respectively. Drug-free or "empty" immunoliposomes were prepared identically except no drug was added.

Ara-C Cytotoxicity Assay

L-929 cells and A-31 cells, grown in McCoy's medium, were seeded onto 24-well tissue culture plates and grown to 50% confluency. Various concentrations of DOPE:OA and DOPC immunoliposomes containing Ara-C, ranging from 4.0 $\times 10^{-7}$ M to 8.0 $\times 10^{-6}$ M were added to both the L-929 and the A-31 cells and incubated for 3 hr at 37°C. Parallel to the drug entrapped immunoliposomes, antibody free liposomes containing Ara-C, empty immunoliposomes, and free Ara-C were added to the cells for comparison. Following the incubation, the cells were washed 3 times with fresh McCoy's medium. [3 H]-Thy, at 2 μ Ci/well, was added to the cells, which were then incubated at 37°C for 24 hr. Cells which received no liposome treatment were also incubated with [3 H]-Thy in order to obtain the control uptake level. Subsequent to the [3 H]-Thy

incubation the media was removed and 0.5 ml of 0.25% trypsin was added to the cells to facilitate removal from the tissue culture plates. The cells were harvested using a 12-well Millipore filter manifold onto microfiber glass filter papers which were subsequently incubated in 5% trichloroacetic acid at 4°C for 30 min, followed by washing in ice cold 95% ethanol and acetone. The filters were dried and counted for ^3H -cpm. Measurements were done in quadruplicate.

Cytotoxicity Blocking

Underivatized anti H-2K^k antibody or immunoliposomes containing no Ara-C was incubated with the L-929 cells at 37°C for 1 hr prior to the Ara-C containing immunoliposome addition. The free antibody concentration was 12 fold excess of the antibody concentration on the immunoliposomes. The blocking immunoliposomes were composed of DOPE:OA (8:2). The lipid concentration of the blocking immunoliposomes was a 5 fold excess of the lipid concentration of the drug encapsulated immunoliposome. In some experiments, chloroquine or NH_4Cl , both at 50 μM , were added to the L-929 cells 1 hr prior to the immunoliposome addition. Following the preincubation with blocking reagents the pH-sensitive immunoliposome addition, ^3H -Thy incubation, and the cell harvesting were done as described above.

MTX Cytotoxicity Assay

MTX containing DOPE:OA and DOPC liposomes with and without incorporated antibody were added, at varying concentrations of MTX from $1.0 \times 10^{-7} \text{M}$ to $2.0 \times 10^{-6} \text{M}$, to L-929 cells, which had been grown as described above, at 37°C for 1.5 hr. A parallel set of cells were incubated with free drug at the same concentrations as offered in the immunoliposomes. Following the incubation, the cells are washed 3 times with fresh media and [^3H] Leu at 2 $\mu\text{Ci}/\text{well}$ was added. After 24 hr incubation the cells were harvested and counted for ^3H -cpm using the same procedures as outlined above.

Materials

DOPE and DOPC were purchased from Avanti Polar Lipids, Inc. Chloroquine, oleic acid, NH_4Cl and MTX were purchased from Sigma Chemical Co. Ara-C was purchased from Calbiochem. [^3H] Leu and [^3H] Ara-C were purchased from Amersham, [^3H] MTX was purchased from New England Nuclear, and [^3H] Thy was purchased from ICN Biochemicals.

Results

It has previously been shown that liposomes composed of DOPE:OA become unstable (40) and fusion competent (20,21) at weakly acidic pH of 5-6.5. In contrast, DOPC

liposomes are insensitive, both in terms of membrane fusion and content leakage, at acidic pH's (10,20,21). Incorporation of derivatized anti-H-2K^k antibody into the pH-sensitive liposomes had no effect on the pH-sensitivity of the liposomes (data not shown). The target L-929 cells are mouse fibroblasts of the k halotype and express H-2K^k antigen which is recognized by the monoclonal antibody 11-4.1 (33). A-31 cells were chosen as a control cell since they belong to the d haplotype and do not express the H2K^k antigen. The use of these cells as a model system for liposome targeting has been well established (33,41,62).

Ara-C Cytotoxicity

Figure 16 displays the cytotoxic effect of Ara-C on L-929 cells as determined by the inhibition of [³H] Thy incorporation as compared to untreated cells. DOPE:OA (pH-sensitive) immunoliposomes showed a distinct increase in cell killing over free Ara-C. The concentration of Ara-C which gives 50% uptake of [³H] Thy for the pH-sensitive immunoliposomes was approximately $2.0 \times 10^{-6}M$ while the free drug did not cause a drop below 60% [³H] Thy incorporation at a concentration of $8.0 \times 10^{-6}M$. The DOPE:OA pH-sensitive displayed almost a five-fold enhancement of cell killing over the free Ara-C. DOPC (pH-insensitive) immunoliposomes were less effective at delivering Ara-C to the cell than free drug, with only a

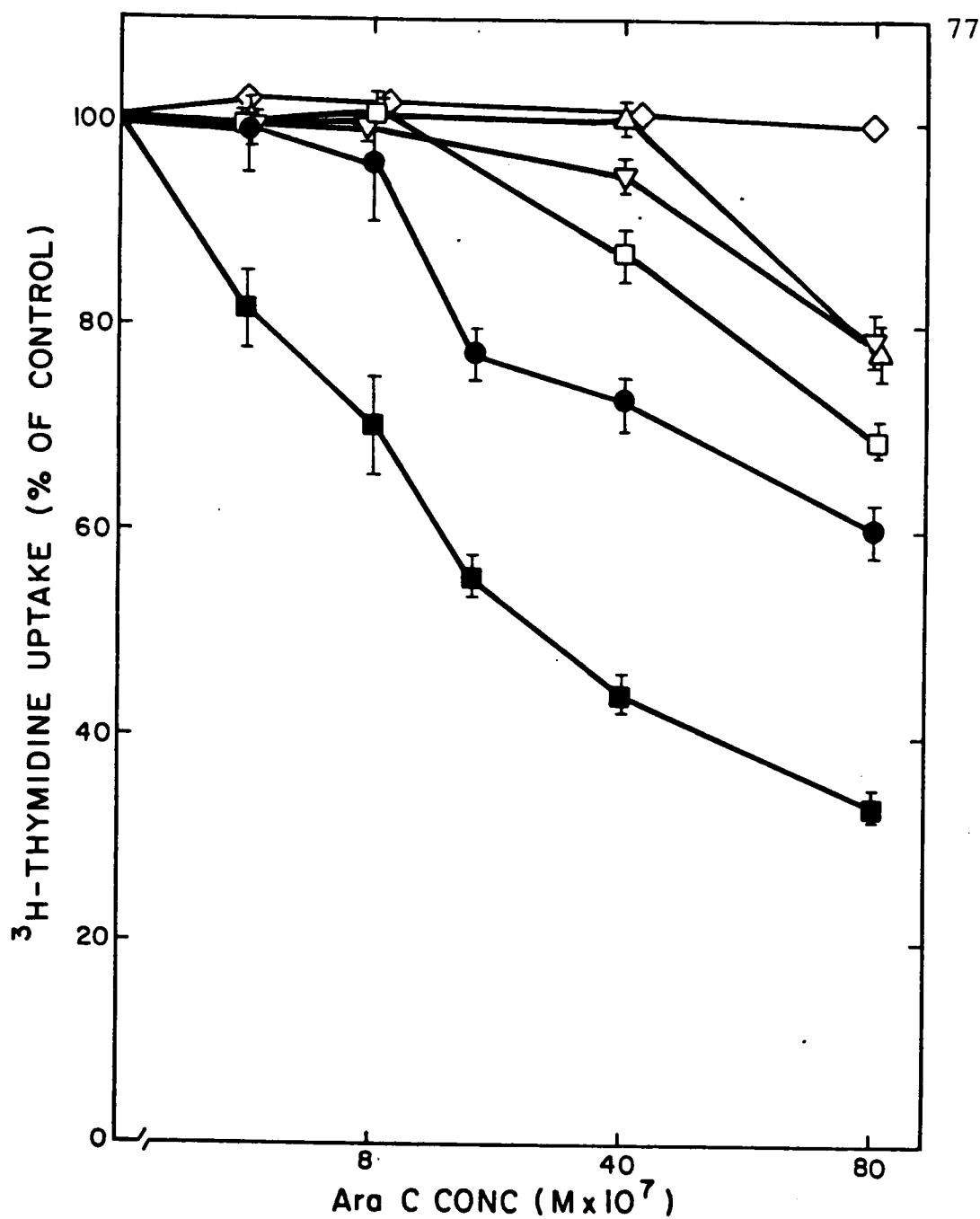


Figure 16. Ara-C cytotoxicity assay with target cells. L-929 cells were incubated with DOPE:OA (8:2) immunoliposomes (■); DOPC immunoliposomes (▽); DOPE:OA bare liposomes (□); DOPC immunoliposomes (Δ) all containing Ara-C. Ara-C administered as free drug (●) and empty DOPE:OA (8:2) immunoliposomes (◇) were also incubated with L-929 cells. Cytotoxicity was assayed as inhibition of [^3H] Thy incorporation.

20% loss of [^3H] Thy incorporation, at the highest Ara-C concentration tested. Both DOPE:OA and DOPC liposomes without antibody showed little ability to kill cells by delivering Ara-C; these untargeted liposomes showed about 80% [^3H] Thy incorporation even at the highest Ara-C concentration tested. DOPE:OA immunoliposomes without entrapped Ara-C added at an equivalent dose as in the Ara-C containing immunoliposomes were completely nontoxic to the L-929 cells.

Figure 17 displays the result of the same experiment as the one shown in Fig. 16 to a non-target cell line, A-31 cells. Free Ara-C was capable of killing the A-31 cells with 50% [^3H] Thy incorporation at a concentration of $8.0 \times 10^{-6}\text{M}$. None of the liposome incubations were able to deliver their encapsulated drug to the A-31 nontarget cells.

Figure 18 shows the effect of blocking agents on the ability of the pH-sensitive immunoliposomes to deliver their encapsulated Ara-C and kill L-929 cells. Underivatized free antibody, at 12-fold excess, was able to completely block the cytotoxic effect of Ara-C entrapped in the DOPE:OA immunoliposomes. Empty immunoliposomes, at a 5-fold excess, were also able to partially block the cytotoxic effect; the blocked immunoliposomes were about 4 times less effective in killing the L-929 cells than the DOPE:OA immunoliposomes.

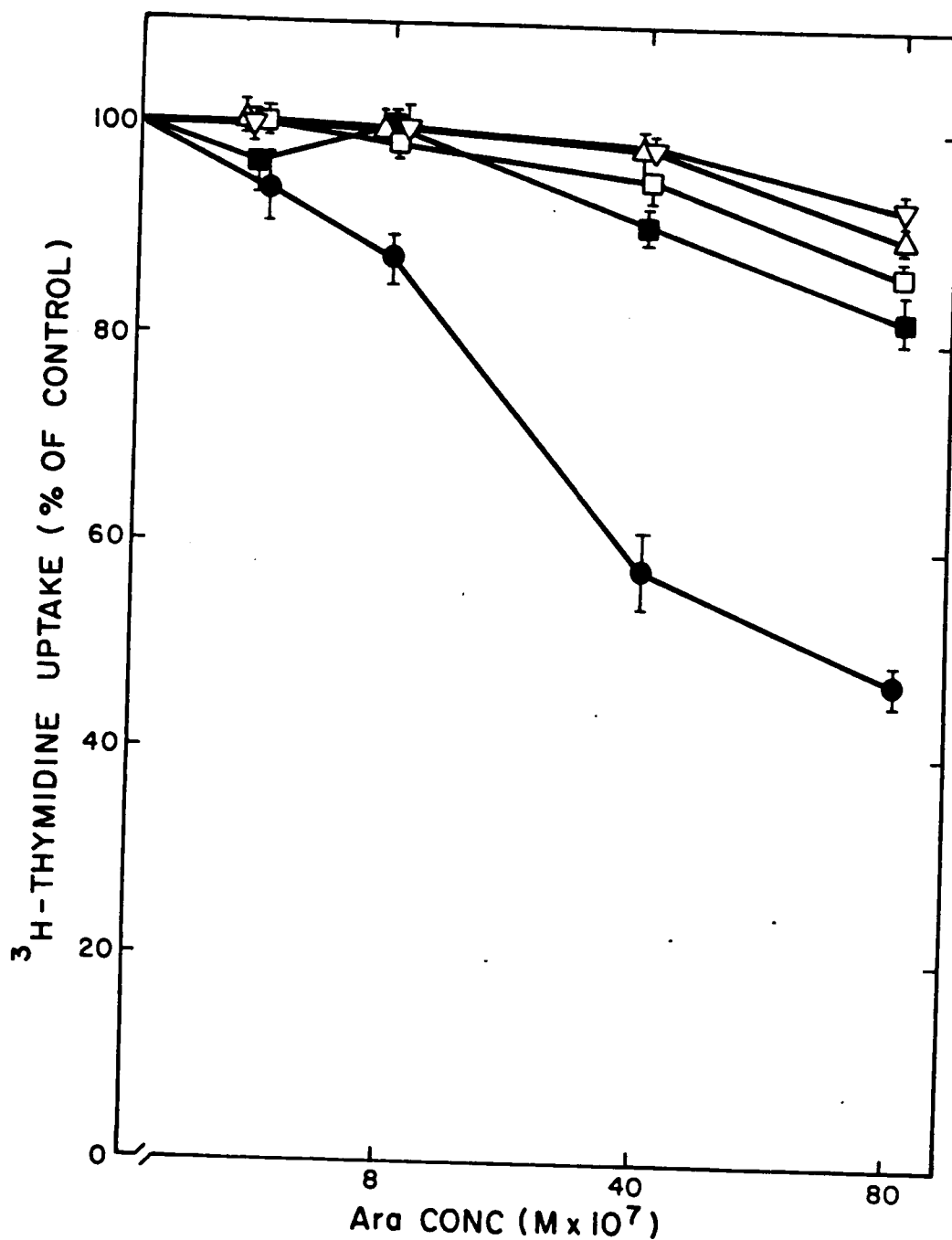


Figure 17. Ara-C cytotoxicity assay with nontarget cells. A-31 cells were incubated with DOPE:OA (8:2) immunoliposomes (■); DOPE:OA (8:2) bare liposomes (□); DOPC immunoliposomes (▽); DOPC bare liposomes (△), all containing Ara-C. Free Ara-C (●) was also incubated with A-31 cells. Cytotoxicity was assayed as inhibition of [3 H] Thy incorporation.

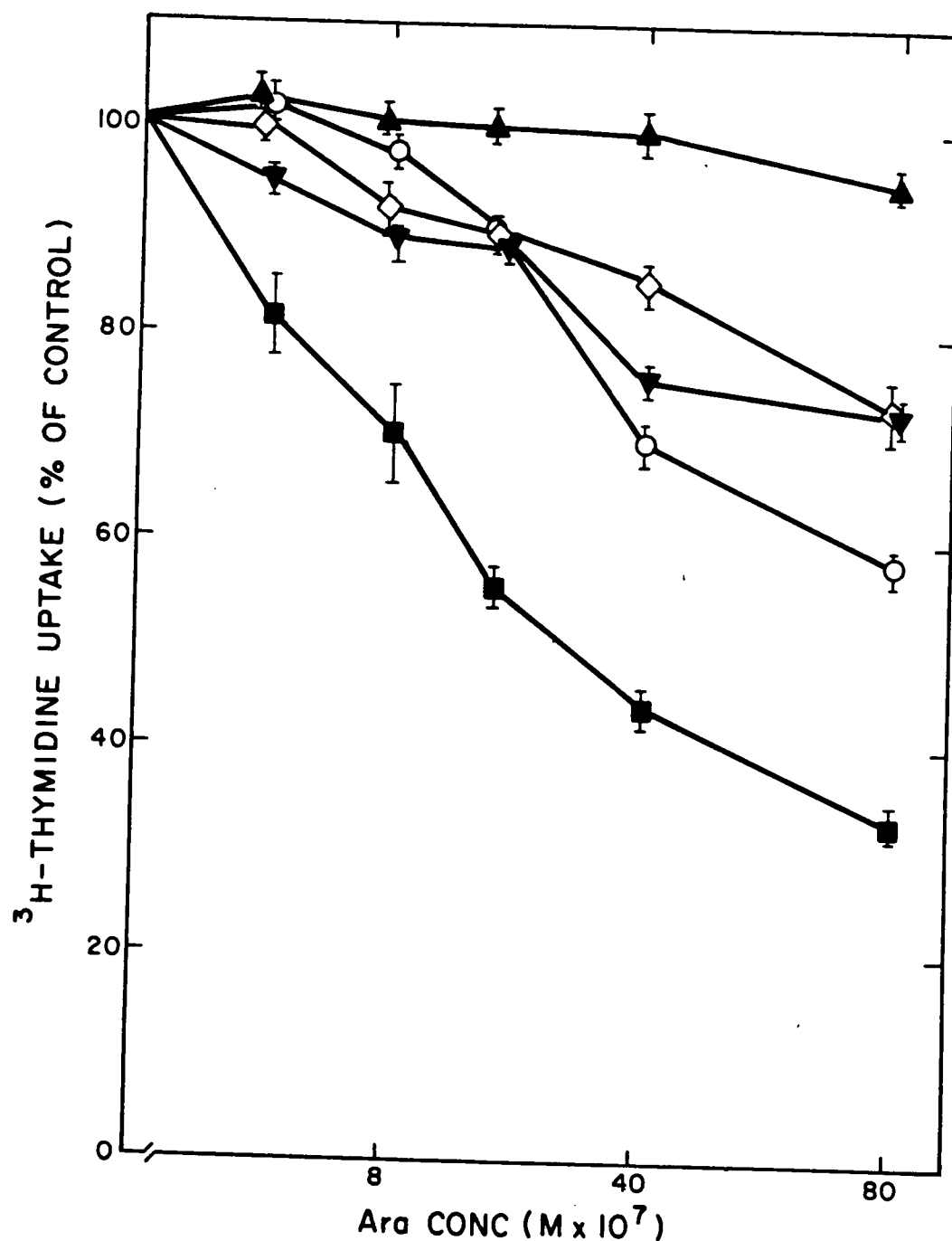


Figure 18. Blocking of Ara-C cytotoxicity. L-929 cells were pretreated with excess free antibody (▲); excess empty DOPE:OA (8:2) immunoliposomes (○); chloroquine (▼); NH_4Cl (◇) or nothing (■) prior to administering the Ara-C entrapped in pH-sensitive immunoliposomes. Cytotoxicity was assayed as inhibition of [3H] Thy incorporation.

Cells pretreated with the weak bases chloroquine and NH_4Cl showed a resistance to the cytotoxic effect of Ara-C entrapped in the pH-sensitive immunoliposomes (Fig. 18). Chloroquine, which raises the internal pH of cellular acidic organelles, decreased the cytotoxicity of Ara-C entrapped in the pH sensitive immunoliposomes by about 2-fold as measured by [^3H] Thy incorporation. Another lysosomotropic amine, NH_4Cl , also blocked to a similar degree the killing potential of Ara-C entrapped in the DOPE:OA immunoliposomes.

MTX Cytotoxicity

Figure 19 displays the cytotoxic effect of MTX, encapsulated in targeted and untargeted pH-sensitive or insensitive liposomes, on L-929 cells. MTX encapsulated in both DOPE:OA and DOPC immunoliposomes were more effective in killing L-929 cells than free MTX as determined by [^3H] Leu incorporation. The DOPE:OA immunoliposomes were slightly better than the DOPC immunoliposomes in enhancing the cytotoxicity of MTX. Untargeted liposomes of both lipid compositions were ineffective in delivering the drug to target cells.

Discussion

In order to further the potential of immunoliposomes as an effective drug delivery system, a mechanism is

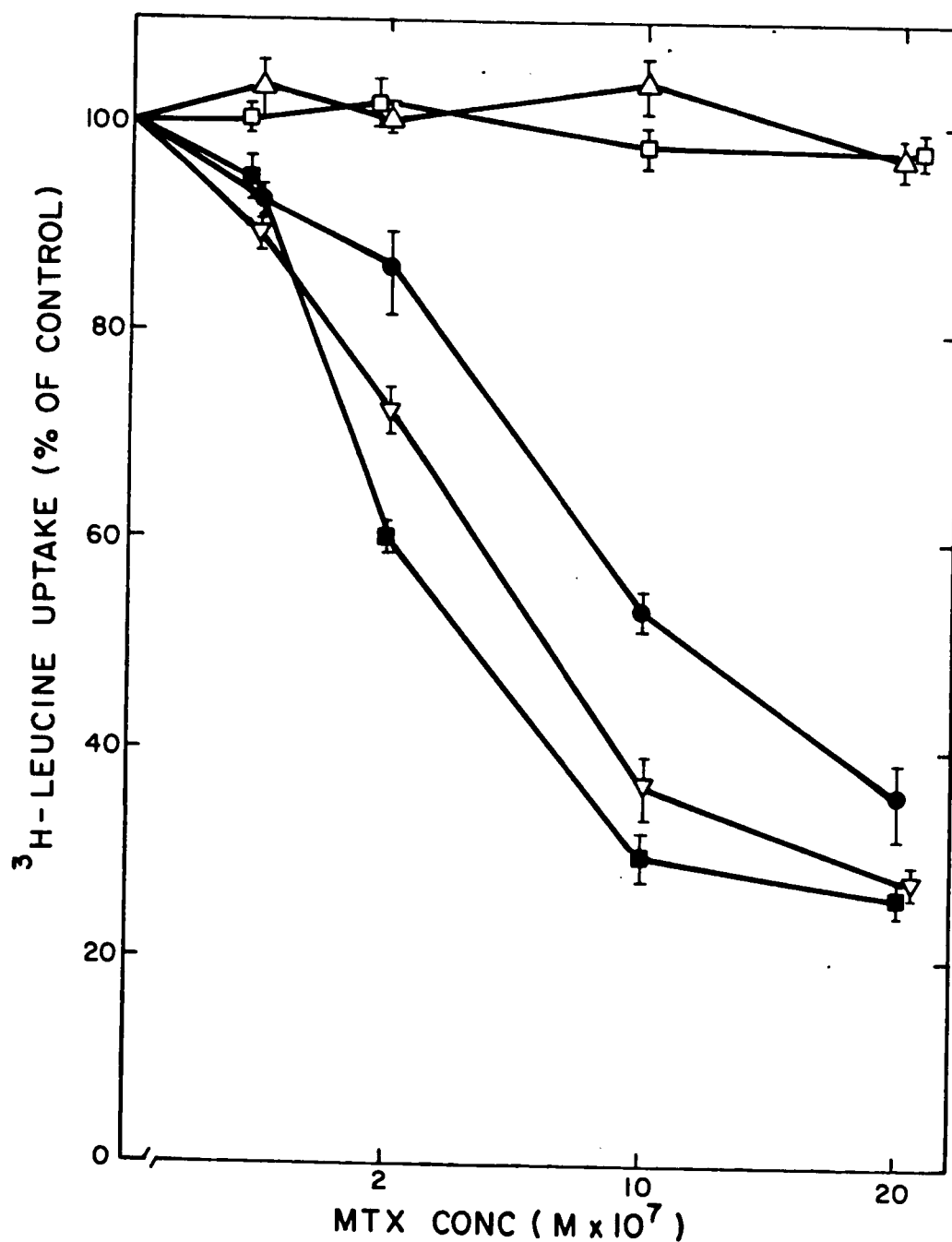


Figure 19. MTX cytotoxicity assay. L-929 cells were incubated with DOPE:OA (8:2) immunoliposomes (■); DOPC immunoliposomes (▼); DOPE:OA (8:2) bare liposomes (△); or DOPC bare liposomes (□), all containing MTX. MTX administered as free drug was also incubated with L-929 cells (●). Cytotoxicity was measured as inhibition of [3 H] Leu incorporation.

needed to insure the delivery of the liposomal content to the cell cytoplasm. This chapter clearly demonstrates the ability of pH-sensitive immunoliposomes composed of DOPE:OA (8:2) to provide a method for the cytoplasmic delivery of its entrapped contents.

The enhanced cytoplasmic delivery of Ara-C by pH-sensitive immunoliposomes is clearly seen in Fig. 16. Targeted DOPC liposomes, though bound and endocytosed normally (41), were unable to deliver their entrapped Ara-C to the cytoplasm, confirming the requirement of a pH-sensitive lipid composition for cytoplasmic delivery. Furthermore, the lack of cell killing by immunoliposomes incubated with non-target cells (Fig. 17) or target cells preincubated with competitive blockers (Fig. 18) indicate the target specificity of this delivery system. By using the weak bases, chloroquine and NH_4Cl , we have shown the absolute dependence on an acidic environment for cytoplasmic delivery (Fig. 18). If the internal vacuole pH of the cell is raised, by preincubation with a weak base, the amount of cytoplasmic delivery of the entrapped Ara-C by the pH-sensitive immunoliposomes is reduced to less than that of the free drug. Thus the acidification of the endosomes/lysosomes is required if the pH-sensitive immunoliposomes are to deliver their contents to the cytoplasm.

The drug Ara-C was chosen in these experiments

because of its susceptibility to degradation by lysosomal enzymes (33,42). Thus in order to be an effective cytotoxic drug, the Ara-C must escape to the cytoplasm from a compartment prior to the lysosome delivery. Previous work using pH-insensitive immunoliposomes demonstrated that the entrapped Ara-C was unable to escape the endosomes/lysosomes and was ineffective as a cytotoxic drug (33). Clearly the enhanced cytotoxicity of Ara-C entrapped in pH-sensitive immunoliposomes demonstrated that the cytoplasmic delivery of the drug occurs prior to accumulation in the lysosomes, i.e. in the endosomes. In contrast, MTX can escape acidic compartments, and thus is an effective cytotoxic drug when delivered in either pH-sensitive or insensitive immunoliposomes (33). As demonstrated in Figure 19, MTX entrapped in DOPE:OA immunoliposomes is slightly more toxic than MTX entrapped in DOPC immunoliposomes; this could be due to pH-sensitive vesicles delivering MTX to the cytoplasm from both the endosomes and the lysosomes, while the pH-insensitive immunoliposomes only deliver MTX from the lysosomes.

While it is highly probable that cytoplasmic delivery of Ara-C from the pH-sensitive immunoliposomes takes place in the endosomes, the exact mechanism of delivery is not clear. This mode of escaping the endosome into the cytoplasm by an acid-triggered mechanism is also seen in certain virus systems, such as Semlike Forest Virus (51),

Influenza Virus (75), and Vesicular Stomatitis Virus (73). It has been extensively shown that pH-sensitive liposomes become fusion active when exposed to an acidic environment ($\text{pH} = 5.0-6.5$) (9-11). It is therefore possible that the endocytosed immunoliposome undergoes an acid induced fusion with the adjacent endosome membrane and releases the encapsulated Ara-C to the cytoplasm. In other words the mechanism of delivery is similar to that of the virus infection. pH-sensitive liposomes are also leaky at low pH (12). If a large amount of Ara-C were released in the interior of the endosomes, escape of free Ara-C from the endosomes into the cytoplasm is a possible mode of entry. Alternatively, DOPE:OA liposomes are prone to undergo a bilayer-to-hexagonal phase transition at low pH (21). The non-bilayer structure of the liposomal lipids could cause a rupturing of the endosomes releasing its contents into the cytoplasm.

While the precise delivery mechanism is unclear, our studies clearly demonstrate the superiority of the pH-sensitive immunoliposomes as a target-specific carrier for antitumor drugs. Besides the obvious potential in cancer chemotherapy, this liposome system may also be important for delivering macromolecules, such as enzymes, nucleic acids, and immunomodulators, which cannot by themselves escape the endosomes/lysosomes. These possibilities are currently under investigation.

CHAPTER VI

BIODISTRIBUTION OF pH-SENSITIVE IMMUNOLIPOSOMES

Introduction

A primary goal in the development of liposome targeting systems is to establish an effective method for delivering chemotherapeutic drugs to cells in vivo. Though there is little information concerning the pharmacokinetics, there has been some investigation into the blood clearance and organ distribution of bare liposomes administered intravenously. The conclusion of these studies indicates that the primary accumulation of liposomes appears in the liver, followed by the spleen and very slightly in the lung (80,81,82). In addition, it appears that the resident macrophages in each organ and circulating macrophage are the main cell type responsible for liposome accumulation (83). Initial studies of intravenous application of immunoliposomes indicate that antibody coated liposomes are cleared faster from the blood into the liver than bare liposomes (84,85).

Previously in our laboratory we have developed a liposome system capable of fusing with adjacent membranes upon exposure to acidic environment (10). Using these vesicles, which are composed of dioleoyl phosphatidylethanolamine (DOPE) and oleic acid (OA) and an established monoclonal antibody target system (33), we synthesized

pH-sensitive immunoliposomes with the ability to specifically deliver entrapped dye (calcein) (41), and cytotoxic drugs (Ara-C and MTX) (43) to the cytoplasm of cells in vitro. The delivery is based on an acid triggered event of the immunoliposomes within an acidic endosome following endocytosis.

In order to determine the feasibility of employing these pH-sensitive immunoliposomes as a therapeutic delivery agent in vivo, we determined the tissue distribution of intravenously administered DOPE:OA with and without targeting antibody.

Materials and Methods

Materials

DOPE and DOPC were obtained from Avanti Polar Lipids Inc. (Birmingham Al). Oleic acid, inulin, tyramine and calcein were obtained from Sigma Chemical Co. (St. Louis, MO).

Synthesis of ^{125}I -tyraminyl-inulin

^{125}I -labeled tyraminyl-inulin was synthesized according to Sommerman et al. (86). Briefly, inulin was dissolved in H_2O at 1.0 g per 100 ml and incubated with 10 ml of 0.1 M periodic acid at 4°C for 15 min. in the absence of light. The reaction was terminated by $\text{Ba}(\text{OH})_2$ addition and the resulting salts were removed. To the

oxidized inulin, 4.25 g NaH_2P_4 and 0.55g tyramine were added and the pH was adjusted to 7.5. Subsequently, 0.25 g of NaBH_4 was added and the solution was stirred for 4 hr. at room temperature. Aliquots of the resulting solution were then applied to a 1.5 X 80 cm Sephadex G-25 column at 4°C and 5 ml fractions were collected. The fractions were monitored for sugar using the anthrone reagent technique (87), and for tyramine by absorbance at 279 nm. The tyraminyl-inulin eluted in the void volume and had a constant tyramine: inulin mole ratio of 0.7. The final product was lyophilized and stored at 4°C . Tyraminyl-inulin is dissolved at 10 mg/ml in phosphate buffer saline (PBS) at pH = 7.4 followed by radioiodination with ^{125}I performed by the Chloramine T method.

Plasma Induced Liposome Leakage

DOPE:OA (8:2) or DOPC:OA (8:2) reverse-phase evaporation vesicles (REV) containing 50mM calcein were prepared as previously described (41 and chapter V). 0.2mM of liposomes of either composition were added to 100% mouse serum at 4°C and 37°C respectively; calcein fluorescence was monitored using 490 nm excitation wavelength and 520 nm emission wavelength. Percent leakage

was determined from the equation:

$$\% \text{ leakage} = \frac{F - F_i}{F_T - F_i} \times 100$$

where F and F_T are the calcein in the absence and presence of Triton X-100, respectively and F_i is the initial quenched fluorescence. Percent leakage of both DOPE:OA (8:2) and DOPC:OA (8:2) liposomes was also determined in PBS following the same protocol.

Light Microscopy of Liposome Aggregation

DOPE:OA (8:2) and DOPC:OA (8:2) in PBS at 1 mM concentration after incubation for 2 min at 22°C were photographed at 400X magnification using an Olympus phase contrast microscope. Liposomes at the same magnification were also photographed in the presence of 100% mouse plasma.

In Vivo Biodistribution

DOPE:OA (8:2) and DOPC:OA (8:2) REV's containing ^{125}I -tyraminyl-inulin were prepared (41 and chapter V). The final liposome preparation contained ^{125}I -tyraminyl-inulin at 5×10^3 cpm/ μl and ^3H -cholesterol-ether as a lipid marker at 5×10^6 cpm/ μmole of lipid.

A second set of identical liposomes were prepared into which the palmitoyl derivatized monoclonal antibody

anti H-2K^k, was incorporated following the procedure established previously (43 and chapter V). 1 μ mole of lipid in 0.1 ml PBS was administered intravenously, by tail vein injection, into C3H and Balb/c mice. After 15 min., 3 hr., 6 hr., 24 hr., or 48 hr., the mice were sacrificed by cervical dislocation and the organs and blood were removed. The individual organs were counted for ¹²⁵I- counts in a gamma counter. The tissue was then solubilized with Protosol and counted for ³H-cpm. An aliquot of blood was counted for ¹²⁵I-cpm and ³H-cpm. The total radioactivity in the blood was determined by assuming the total volume of blood was 7.3% of the total body weight (2). Tissue distribution is recorded as percent of counts recovered from the major organs at each time point. Data represent the average of 3 mice. Standard deviations ranged from 3-7%.

Results

Figure 20 illustrates the leakage properties of the DOPE:OA pH-sensitive (panel B) and the DOPC:OA pH-insensitive (panel A) liposomes in the presence of either 100% mouse plasma or PBS. In PBS at both 4°C and 37°C both types of liposomes displayed no calcein leakage over a one hour time period. The DOPE:OA liposomes were slightly leaky in the presence of the plasma at 4°C; the liposomes released about 20% of the entrapped dye after 10

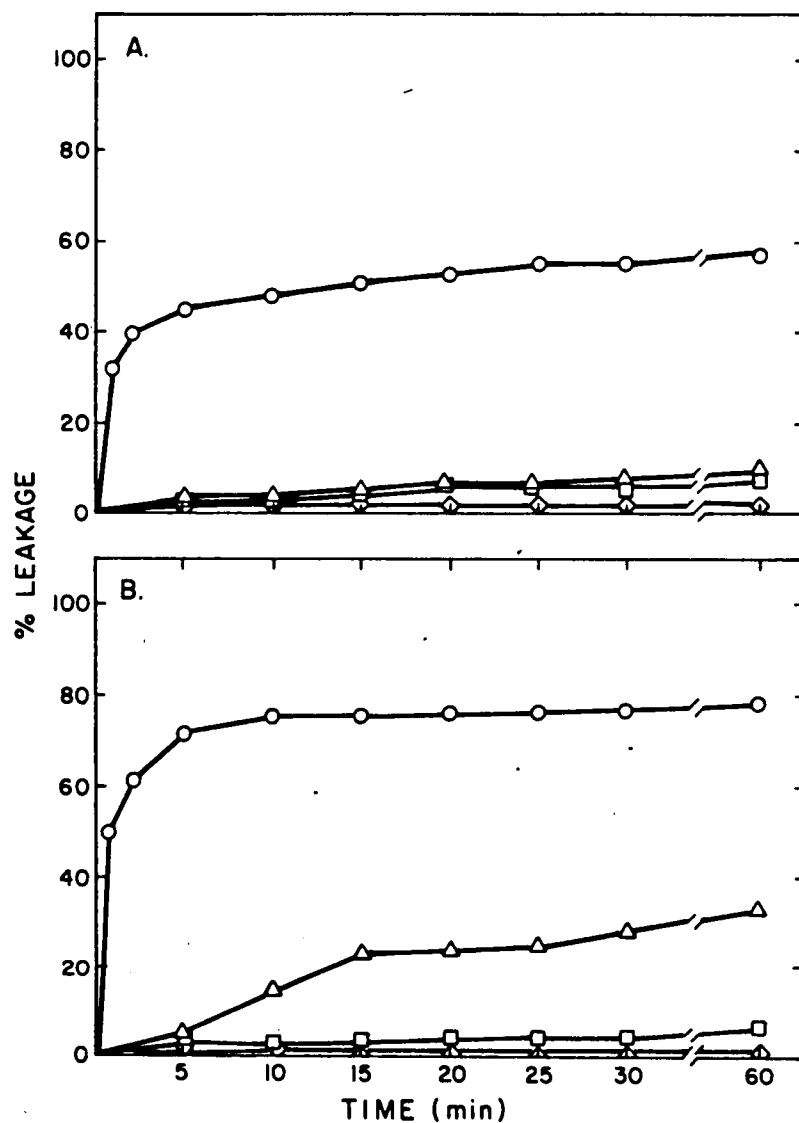


Figure 20. Plasma induced leakage from liposomes. DOPC:OA (8:2) liposomes (panel A) and DOPE:OA (8:2) liposomes (panel B) were measured for calcein leakage at 4°C in PBS (◇) or 100% mouse plasma (□), and at 37°C in PBS (△) or 100% mouse plasma (○). Percent leakage was determined as described in Methods, chapter VI.

min. In contrast, the pH-sensitive liposomes displayed rapid leakage in the mouse plasma at 37°C, releasing 70% of the encapsulated calcein within 5 min of incubation. Following the initial release the amount of leakage remained at 70% for the entire 60 min incubation. The DOPC:OA pH-insensitive liposomes showed no leakage in plasma at 4°C. There was, though, a rapid release of the entrapped calcein from the DOPC:OA liposomes in plasma at 37°C, with a maximum of 50% leakage. Parallel experiments with pH-sensitive and insensitive immunoliposomes gave identical leakage profiles as their corresponding antibody-free liposomes (data not shown).

The light micrographs of the DOPE:OA and DOPC:OA liposomes in the presence and absence of 100% mouse plasma indicate the possible source of the content leakage (Figure 21). Figure 21 a and c are the pH-sensitive and insensitive liposomes, respectively, in PBS. The liposomes appeared intact and free of aggregation. In the presence of plasma, the DOPE:OA liposomes displayed a high degree of aggregation and the liposomes were more refractile (figure 21b). In contrast the DOPC:OA liposomes appeared to have little aggregation and the liposomes were largely intact (figure 21d).

DOPC:OA liposomes injected into C3H mice displayed a rapid clearance from the blood (15 min), followed by accumulation in the liver and the spleen (Table 4). The

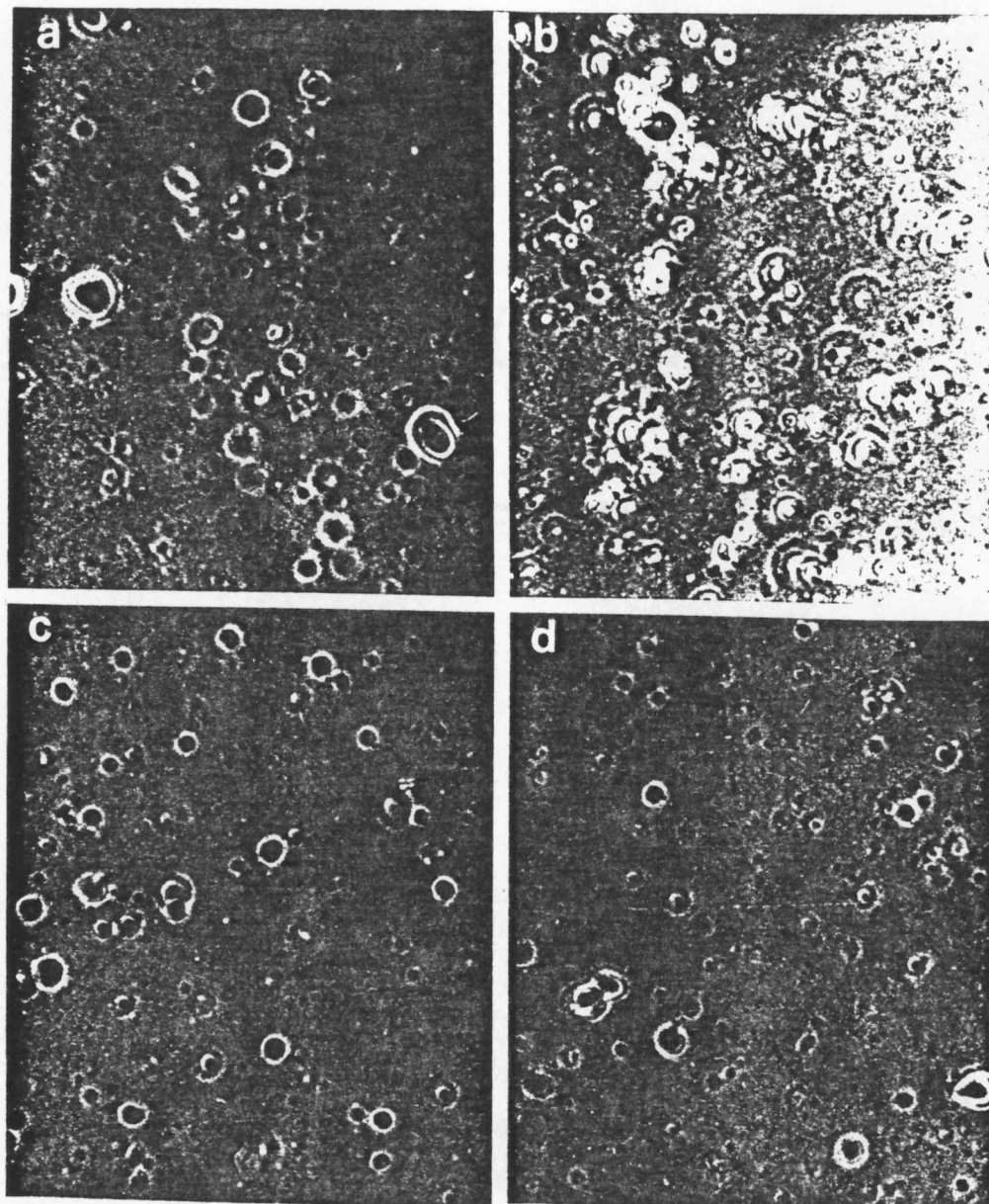


Figure 21. Light micrographs of liposomes. Liposomes at 1.0mM concentration were photographed. a) DOPE:OA (8:2) liposomes in PBS; b) DOPE:OA (8:2) liposomes in 100% mouse serum; c) DOPC:OA (8:2) liposomes in PBS; d) DOPC:OA (8:2) liposomes in 100% mouse plasma. Bar is -- μ m. Magnification = $\times 4000$.

Table 4

Distribution of PC:OA liposomes in C3H Mice^a

Organ	Liposome With Ab					Liposome Without Ab				
	15 min	3 hr	6 hr	24 hr	48 hr	15 min	3 hr	6 hr	24 hr	48 hr
	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I
Organ										
Stomach	0	3	0	9	0	3	0	0	0	0
Spleen	21	8	13	10	24	12	21	14	23	16
Kidneys	0	11	0	3	0	3	0	3	0	3
Liver	61	38	81	66	69	74	74	74	74	74
Heart	0	0	0	0	0	0	0	0	0	0
Lung	3	5	0	2	0	0	0	0	0	0
Blood	9	30	1	4	2	2	0	4	0	3

^a Percent cpm recovered in organs are shown: hexadecyl ³H-cholestanyl ether and ¹²⁵I-tyraminyl-inulin.

majority of the lipid was found in the liver (~75%), while the spleen accumulated about 20% of the recovered radioactivity. The DOPC:OA immunoliposomes followed an identical pattern as the free liposomes. When injected into the Balb/C mice the DOPC:OA liposomes with and without targeting antibody displayed the same profile (Table 5). Similar to the C3H mice, there was a rapid clearance from the blood followed by a majority of accumulation in the liver (~85%). A small amount of accumulation was seen in the spleen (10%) and the kidneys (2%).

DOPE:OA pH-sensitive liposomes gave a different profile than the pH-insensitive liposomes. After injection into C3H mice (Table 6), DOPE:OA immunoliposomes showed a rapid clearance from the blood, with a separation of the ^{125}I -tyraminyl-inulin aqueous marker and the ^3H -cholesteryl-ester lipid marker. The labeled inulin appears in the stomach (20%), the heart (15%), the kidneys (20%) and the liver (26%) at the 3 hr. time point; subsequently the aqueous marker accumulated in the liver (70%). The lipid appears at earlier stages in the lungs (50%) and the liver (35%). The lipid remained in the lung tissue up to 6 hr. where it then redistributed to the liver (60%) and the spleen (12%). The DOPE:OA bare liposomes displayed a similar profile, with the exception of a higher accumulation of lipid in the lungs at 15 min.

Table 5

Distribution of PC:OA Liposomes in Balb/C Mice^a

	Liposome With Ab					Liposome Without Ab				
	15 min	3 hr	6 hr	24 hr	48 hr	15 min	3 hr	6 hr	24 hr	48 hr
Organ	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I
Stomach	0	2	1	3	0	1	0	0	0	0
Spleen	15	6	8	6	5	6	9	7	9	5
Kidneys	1	6	1	3	1	4	1	3	1	3
Liver	71	52	83	78	85	82	84	84	84	88
Heart	0	1	0	0	0	0	0	0	0	0
Lung	2	3	1	0	0	0	0	0	0	0
Blood	5	20	1	4	0	2	0	1	0	0

^aPercent cpm. ³H-cholesteryl ether; ¹²⁵I-inulin.

Table 6
Distribution of PE:OA liposomes in C3H Mice^a

Organ	Liposome With Ab						Liposome Without Ab					
	15 min	3 hr	6 hr	24 hr	48 hr		15 min	3 hr	6 hr	24 hr	48 hr	
	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I
Stomach	0	6	0	26	0	19	0	3	0	2	0	7
Spleen	3	1	4	2	5	3	10	4	13	5	1	1
Kidneys	1	22	0	17	0	21	0	15	1	25	1	31
Liver	41	24	35	26	35	33	63	37	60	59	20	16
Heart	0	13	0	19	0	9	0	15	0	4	1	12
Lung	51	1	55	1	55	1	20	3	21	1	72	2
Blood	1	29	0	6	0	14	1	4	0	3	1	28

^aPercent cpm. ³H-cholesteryl ether; ¹²⁵I-inulin.

(72%), followed by redistribution beginning after 3 hr.

In Balb/C mice the DOPE:OA immunoliposomes displayed the characteristic rapid clearance from the blood, followed by redistribution into other tissue (Table 7). The ^{125}I -tyraminyl-inulin appeared first in the lung (10%), kidneys (24%), stomach (40%), spleen (4%) and liver (8%) followed by a redistribution to the liver (80%) within 6 hr. The lipid initially accumulated in the lungs (24%), but at a lower amount than in the C3H mice, the liver (70%), and spleen (9%). After 6 hr. the lipid began redistributing from the lungs to the liver (80%). In the Balb/C mice the liposomes and immunoliposomes followed basically the same tissue distribution profile.

Discussion

Previously we established an in vitro model system for specific targeting and cytoplasmic delivery of cytotoxic drugs, using pH-sensitive immunoliposomes. In order to determine the applicability of these immunoliposomes as an effective tool for in vivo therapeutic applications, we determined their tissue distribution following intravenous injections. To follow both the lipid component and the internal aqueous compartment two radioisotopic markers were employed, ^3H -cholesteryl ether for the lipid and ^{125}I -tyraminyl-inulin for the entrapped aqueous space.

Table 7

Distribution of PE:OA liposomes in Balb/C Mice^a

Organ	Liposome With Ab						Liposome Without Ab					
	15 min	3 hr	6 hr	24 hr	48 hr		15 min	3 hr	6 hr	24 hr	48 hr	
	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I	³ H 125 _I
Stomach	1 5	4 4	2 3	0 0	0 0		2 9	0 11	0 2	0 3	0 1	
Spleen	4 4	10 5	7 4	10 5	13 6		9 3	4 2	10 5	14 6	10 7	
Kidneys	3 24	3 9	2 16	2 11	2 10		3 19	2 14	2 16	2 12	2 11	
Liver	69 8	57 67	73 70	78 79	79 79		50 35	73 62	66 70	72 74	76 78	
Heart	0 2	1 0	0 1	0 0	0 0		1 0	0 0	1 0	0 0	0 0	
Lung	21 12	24 5	15 2	8 1	6 1		28 8	17 4	17 2	9 1	10 1	
Blood	4 45	4 7	3 5	2 2	1 2		5 25	2 5	2 3	1 2	0 0	

^a Percent cpm. ³H-cholesteryl ether; ¹²⁵I-inulin.

^3H -cholestanyl-ether is a non-exchangeable non-metabolizable lipid marker which remains with the lipid components of the system. ^{125}I -tyraminyl-inulin is a convenient water soluble marker which is not metabolized but is excreted from the animals.

Liposomes composed of DOPC:OA followed the expected tissue distribution seen in other laboratories (80,81,82). The liposomes were rapidly cleared from the blood stream and accumulated in both the liver and the spleen, with the liver being the dominant organ. The presence of targeting antibody on the surface of the liposomes had no effect in the Balb/c mice which do not express the H2K^k antigen. While in the C3H mice, which express the antigen, the clearance from the blood into the liver of the immuno-liposomes appeared to be slightly accelerated by the presence of the antibody probably because of the formation of immune complex and subsequent uptake by the reticuloendothelial cells.

The DOPE:OA pH-sensitive liposomes gave a very different distribution profile. Following the rapid clearance from the blood, the DOPE:OA liposomes appeared predominately in the lung tissue. This effect was much more pronounced in the C3H mice than the Balb/C mice; furthermore, in both strains of mouse the bare liposomes showed more accumulation in the lungs than the immuno-liposomes. The presence of the antibody increases the

ability of the liver to accumulate the liposomes. In the C3H mice the lung was the dominant organ of accumulation for up to 6 hr. after which the liposomes redistributed to the liver and the spleen in similar ratios as the pH-insensitive liposomes.

A second phenomenon observed in the DOPE:OA liposome distribution is the separation of the internal aqueous space marker and the lipid marker. As can be seen in figure 20 the DOPE:OA liposomes are very leaky in the presence of plasma due to their interactions with serum components. The DOPC:OA liposomes also release calcein in the presence of plasma but to a lesser extent than the pH-sensitive liposomes. In the mouse, though, there is very little separation of the aqueous and lipid marker indicating there is very little release of entrapped contents from these liposomes in vivo. This effect is probably due to the size difference between calcein and inulin. Inulin is a larger molecule (MW=6000) and thus will be more readily retained in the liposomes. Figure 21 a and b display the aggregation effect of plasma on DOPE:OA liposomes. The aggregation of PE containing liposomes by serum protein has been observed in other systems (88). In addition it has been reported that lung accumulation of liposomes is size dependent; large vesicles are trapped in the capillaries while smaller liposomes are retained in the circulation (89). What

appears to be occurring upon injection of DOPE:OA liposomes into mice is both the release of the entrapped marker and aggregation of the liposomes due to interactions with serum components. What follows is a distribution of the aqueous marker into the stomach and kidney with subsequent excretion. A similar distribution profile is seen with free ^{125}I -tyramnyl-inulin intravenously injected into mice (data not shown). The aggregated lipid continues to circulate and accumulates in the lung due to the increase in size caused by aggregation.

The inability of the pH-sensitive liposome to retain the entrapped contents upon intravenous injection makes them an ineffective tool for delivery of trapped aqueous molecules. It may be possible, though, to alter the lipid composition; such as the inclusion of cholesterol, in order to increase stability without substantial loss of pH-dependent fusion activity.

More interesting is the possibility of using the DOPE:OA liposomes to deliver lipophilic molecules to the lungs. Lipophilic drugs will be maintained in the lipid phase of the liposome and thus should be delivered to the lung during the DOPE:OA accumulation in the tissue. This phenomenon has been seen in the increased ability to deliver the lipid soluble muramyldipeptide to the lung by PE containing liposomes over those lacking PE (89). The

use of DOPE:OA liposomes for delivery to the lung has yet to be explored.

CHAPTER VII

SUMMARY AND CONCLUSIONS

In order to further the use of liposomes as delivery agents of macromolecules to cells, it became necessary to develop a method of releasing the entrapped contents into the cell, once delivered by the liposomes. Conventional liposomes using a monoclonal antibody targeting system could be readily targeted to cell; but, due to their lipid composition, the liposome and its aqueous content were degraded in the lysosomes following endocytosis. In an effort to exploit the inherent acidic pH of the endosomal vacuole of the cellular endocytic pathway we developed liposomes whose unique lipid composition endows them with the ability to fuse under acid conditions.

Using a variety of biochemical and biophysical methods, it has been demonstrated the liposomes composed of a majority of DOPE and a second weakly acidic amphiphile; such as palmitoylhomocysteine or oleic acid, are fusogenic at mildly acidic pH. The second lipid component acts as a stabilizer of the DOPE by inhibiting DOPE head group interactions and by increasing the head group hydration. Upon protonation of the second component at acidic pH, the stabilization is reversed and the DOPE begins to revert to the hexagonal II phase. In order to compensate for the destabilizing effect of the induced

phase transition adjacent liposomes fuse with each other. In addition to protons, divalent cations are also effective in inducing fusion by a similar mechanism. Furthermore, divalent cations act synergistically with protons in promoting the fusion activity, shifting the pH at which the fusion occurs to nearly neutral pH.

To test the potential of using pH-sensitive liposomes as cytoplasmic delivery agents in vitro, immunoliposomes were developed using the established model targeting system. The pH-sensitive immunoliposomes were able to effectively deliver both a dye and cytotoxic drugs to the cytoplasm of target cells. pH-insensitive immunoliposomes were ineffective in cytoplasmic delivery; these liposomes followed the normal endocytic pathway and were degraded in the lysosomes (Fig. 22, Step 8). Clearly pH-sensitive immunoliposomes can effectively deliver a variety of compounds; i.e. dyes, cytotoxic drugs and proteins to the cellular cytoplasm which cannot be delivered by conventional (pH-insensitive) immunoliposomes. The exact mechanism of delivery has not been elucidated, but the delivery occurs prior to lysosomes and is dependent upon acidification of the cellular endosomes. Several possibilities are illustrated in Fig. 22. It is possible that the "acid-triggered" event is a fusion reaction between the liposome and the adjacent endosome membrane (step 5 in Fig. 22). Alternatively, the immunoliposomes

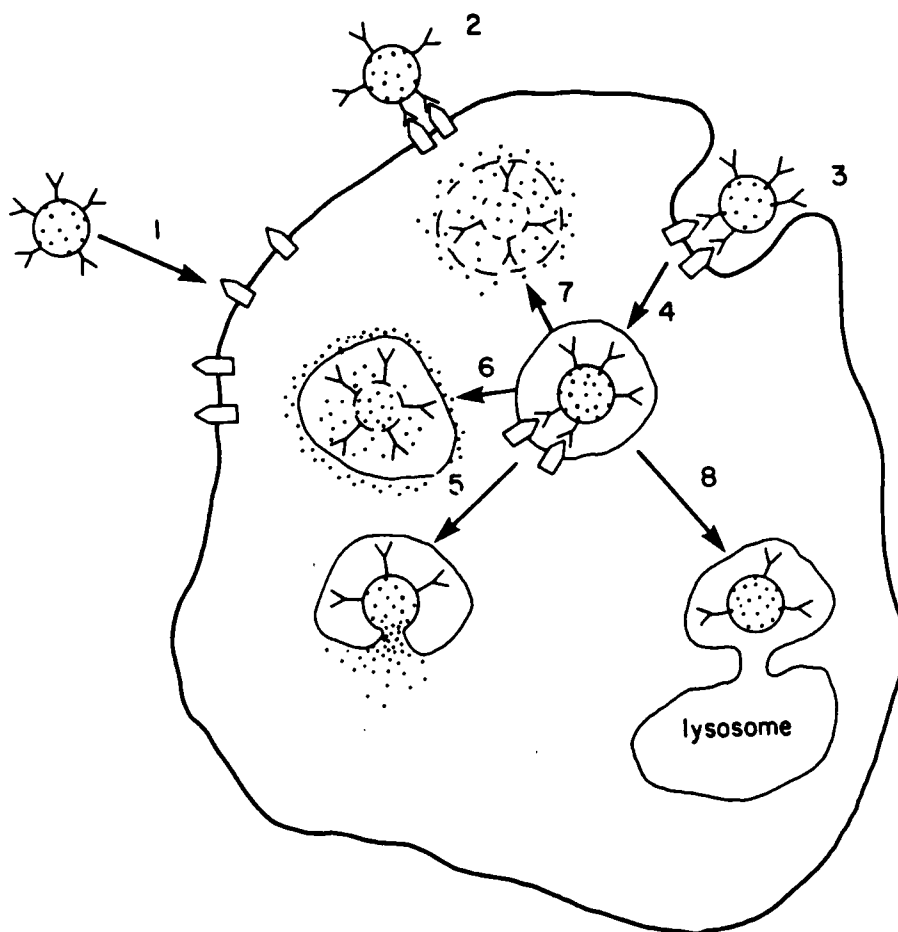


Figure 22. Schematic presentation of the interaction of immunoliposomes with the target L-929 cells. 1. Immunoliposomes bearing antibody (Y) are incubated with target cells expressing antigens (Δ). 2. Binding of the immunoliposomes by the cell surface antigens. 3. Initiation of the receptor-mediated endocytosis at the coated pits. 4. Internalization of the immunoliposomes into the acidic endosomes. 5. Fusion of the pH-sensitive immunoliposomes with the endosome membrane with the liposomal contents released into the cytoplasm. 6. Leakage of the liposomal contents into the endosome with subsequent escape into the cytoplasm. 7. Rupturing of the endosomal vacuole causing release of liposome content into the cytoplasm. 8. Fusion of the endosome with lysosomes and the delivery of the pH-insensitive immunoliposomes into the lysosomes.

could discharge their entrapped contents in the endosome interior where, at high concentrations, the molecules could leak out of the endosomes (step 6). While this is a possibility with calcein and cytotoxic drugs, it is not likely for DTA to move across membranes by itself; therefore, the enhanced delivery of DTA by pH-sensitive immunoliposomes cannot be explained by this mechanism (44). Collins and Huang (44) demonstrated the ability of DTA encapsulated in pH-sensitive liposomes to effectively kill cells, while free DTA and DTA entrapped in pH-insensitive liposomes were unable to deliver DTA to the cytoplasm. Since DTA cannot cross lipid membranes these experiments rule against the possibility of endosome leakage. Another possible mode of cytoplasmic delivery is the rupturing of the endosome vacuole (Step 7). The destabilizing effect of the DOPE:OA vesicles undergoing a bilayer to hexagonal phase transition could rupture the endosome membrane, releasing its contents into the cytoplasm. This possibility cannot be ruled out at this point. The propensity of pH-sensitive liposomes to undergo an acid induced fusion and content mixing with adjacent membrane gives strongest support to the hypothesis of a fusion event between the pH-sensitive immunoliposome and the endosome membrane. Though further work must be done to clarify the mode of delivery, the obvious potential for pH-sensitive liposomes as a delivery

system in vitro for biologically active molecules such as immunomodulators, proteins, drugs and nucleic acids is enormous.

The in vivo application of the DOPE:OA pH-sensitive liposomes as a delivery agent for aqueous macromolecules is, at this point, ineffective due to the loss of entrapped molecules in the presence of serum. It is possible that these liposomes could be rendered more stable by the addition of a third lipid component, such as cholesterol, to increase the stability in serum without compromising its ability to fuse under acid conditions. The DOPE containing liposomes do offer an excellent potential as a mode of delivering lipophilic drugs to the lung tissue. The high accumulation of pH-sensitive liposomes in the lung make them a useful tool for delivering hydrophobic molecules to this organ. The in vivo application of pH-sensitive liposomes, though, is still in the developmental stage.

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