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I am submitting herewith a dissertation written by Sean Michael Sullivan entitled "Heat Sensitive Immunoliposomes." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Leaf Huang

Leaf Huang, Major Professor

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# HEAT SENSITIVE IMMUNOLIPOSOMES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Sean Michael Sullivan

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## ABSTRACT

Heat sensitive immunoliposomes were prepared with derivatized antibody. These liposomes are able to bind specifically to target cells and to release their encapsulated contents upon brief heating. Monoclonal anti-$H2K^k$ was covalently derivatized with palmitoyl-N-hydroxysuccinimide by the method of Huang,A., Kennel,S.J. and Huang,L. J. Biol. Chem. 255, 8015 (1980). The palmitoyl antibody was injected at a controlled rate into a suspension of fused unilamellar dipalmitoylphosphati-dylcholine liposomes maintained at a constant temperature. The final protein-to-lipid ratio of the resultant liposomes with incorporated antibody (immunoliposomes) was dependent upon the injection rate of antibody and the lipid concentration. Injection of palmitoyl antibody into a liposome suspension containing 50 mM carboxyfluorescein at $41^{o}C$ resulted in simultaneous antibody incorporation and entrapment of dye. The immunoliposomes were able to release entrapped dye upon heating. Furthermore, this ability was retained when the immunoliposomes were bound to the target cells. $^{3}H$-Uridine was entrapped in the heat sensitive immunoliposomes to examine the cellular uptake properties of entrapped contents upon release. The release of uridine from bound heat sensitive immunoliposomes exhibited very

similar properties to those obtained for carboxyfluorescein release. The rate of uridine uptake for immunoliposome released uridine was 5 fold greater than bare liposome released uridine and 10 fold greater than that obtained for free uridine. Nucleoside uptake inhibitors were able to inhibit uptake of free uridine and uridine released from immunoliposomes showing the release to be extracellular and uridine uptake was mediated by the nucleoside transporter. These results show that a high local concentration of nucleosides released from immunoliposomes bound to their respective target cell can enhance cellular uptake thus promoting efficient drug delivery.

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# I. INTRODUCTION

Liposomes were first developed as a model system for studying the physical properties of membranes. These properties included lipid-lipid (1-4) interactions and lipid-protein interactions (5-8). Liposomes have also served to reconstitute the biological activity of membrane proteins (9). Characterization of the model systems used to study these properties has provided a wealth of information leading to other liposome applications, such as the developement of a targeted drug delivery system.

Liposomes posses several properties which make them quite suitable for drug delivery. First, liposome encapsulated drugs have a longer circulation half life than unencapsulated drug thus increasing the therapeutic index of the drug (10). Second, liposome encapsulated drugs are protected from degradative agents existing in the administration route. Third, liposomes are relatively nontoxic and are composed of biodegradable material. Finally, liposomes are able to encapsulate a wide variety of biologically active molecules (11-15).

Recognition molecules attached to the liposome surface mediates the binding of the liposomes to cells expressing a cell surface receptor. This mode of drug delivery was

designed for delivery of chemotherapeutic reagents to cancer cells. Binding of the liposomes to cancer cells would result in delivery of drug solely to the tumor and minimize drug delivery to non-cancerous cells. Monoclonal antibodies raised against cell surface antigens have proven to be excellent recognition molecules. They offer the following advantages: a.) specificity of antigen recognition, b.) monoclonal antibody selection and production are fairly routine, and c.) large quantities can be produced.

Methods for coupling antibody to liposomes fall under two general categories: a.) crosslinking of antibody and liposomal lipid head groups by conventional cross linking reagents (16-18) or by chemical modification of the lipid and/or antibody (19-21), b.) increasing antibody hydrophobicity by covalent modification with hydrophobic reagents thus facillitating incorporation into the liposomal bilayer (22-24). Each of these techniques has been developed to suit a particular class of antibody, or a method for liposome formation or a combination of the two. Liposomes with antibody attached to the liposomal surface have been termed immunoliposomes and will be referred to as such.

Binding specificity of immunoliposomes has been demonstrated for several different systems (18,25-27). Once bound to the target cell, the liposome can remain on the cell surface or be internalized. This is governed by the nature of the cell surface antigen and cell (27-29) type, etc. For internalization, binding of the antibody to the cell surface receptor results in receptor mediated endocytosis. This leads to the delivery of the immunoliposomes to the lysosomes where the liposomes and entrapped their contents are degraded. For effective drug delivery, the drug must be able to exit the lysosome and still retain its therapeutic activity. Several drugs possess this capability (30-32); however, this mode of delivery places a restriction on the type of drugs which can be delivered. To avoid delivery to the lysosome, pH sensitive immunoliposomes have been developed (33-35). These immunoliposomes have been engineered to fuse with other membranes upon exposure to an acidic enviorment. The purpose is to have immunoliposomes fuse with the endosomal membrane thereby releasing its contents into the cytosol before they are delivered to the lysosome . This mode of delivery has proved to be very effective (36), but it relies on the ability of the target cell to actively endocytose. We have developed an alternative approach for

those cell types which do not actively endocytose. It utilizes the controlled release of immunoliposome entrapped contents at the target cell surface. This would produce a transient, high, localized concentration of drug at the target cell surface resulting in an enhanced rate of drug uptake over that of drugs released from liposomes not bound to the target cell surface.

Controlled release of liposome entrapped contents has been achieved using " heat sensitive " liposomes. Heating of these liposomes to a temperature at which the liposomal lipid is phase separated, phase transition temperature ($T_M$), results in the release of entrapped contents (37,72). These liposomes have been used in vivo to deliver chemotherapeutic agents, such as methotrexate, and cis-dichlorodiamineplatinum (39,40,73) to tumors.

In the case of methotrexate (MTX), L1210 murine tumors were implanted subcutaneously in the hind feet of mice and the drug laden liposomes were administered intravenously. Uptake of liposome encapsulate MTX by tumors heated to $42^{\circ}C$ was six fold greater than that obtained for free MTX. Furthermore, liposome encapsulated MTX delayed tumor growth two days longer than free MTX. The liposomes exhibited an increase in the therapeutic index of the encapsulated MTX, but the effect was limited.

This may have been due to several factors. Localized heating of the tumor area was not completely restricted to the tumor resulting in uptake by other tissues in the vicinity of the tumor. This limited effect may also have been reflective of the inability of the MTX transport rate to compete with the rate of MTX clearance from the heated area. Release of the drug directly at the tumor cell surface should enhance the rate of uptake and reduce uptake of the drug by tissues other than the tumor. Confering binding specificity of these heat sensitive liposomes will enhance the delivery efficiency if high local concentrations of drug at the membrane surface are to significantly enhance target cell uptake.

To demonstrate this principle we have constructed heat sensitive immunoliposomes using a monoclonal antibody to the mouse major histocompatability antigen $H\text{-}2K^{k}$. Ideally, the drug-laden, heat-sensitive, target-specific liposomes bind to the tumor a few degrees below the release temperature, Once bound, the cells are heated to the release temperature creating a local ized concentration of drug at the site of the tumor. This local ized concentration, though transient, serves as a driving force to increase the rate of drug transport into the target cell. The results shown in Section 2 summarize the

preparation and characterization of the heat sensitive liposomes. Emphasis is placed on a.) optimization of antibody incorporation, b.) binding specificity, and c.) release properties of immunoliposomes free in suspension and bound to target cells. In Section 3, nucleoside uptake was used as a model system to study the delivery efficiency of the heat sensitive immunoliposomes. Nucleoside transport was chosen for the following reasons: a.) the transport rate is extremely rapid, b.) nucleoside transport and metabolism have been well characterized c.) cytotoxic nucleosides have been used in cancer chemotherapy and the kinetic parameters for uptake are similar to those of naturally occuring nucleosides (41).

## II. MATERIALS AND METHODS

### Reagents

Anti-H2K$^{k}$ was isolated from ascites fluid generated by the mouse hybridoma 11-4.1 (24). The ascites fluid was diluted 1:4 with 0.5 M potassium phosphate buffered saline pH 7.2 making the final buffer concentration 0.1 M potassium phoshate and passed through a protein A-Sepharose (Pharmacia,N.J.) column. After washing the column with 0.1M phosphate buffered saline pH 7.4, the antibody was eluted with 0.05 M glycine pH 4.0. The antibody was dialyzed overnight against 4L PBS, 1mM EGTA, and 0.2% NaN pH 8.0. The P3 IgG was isolated from ascites fluid generated by P3 lymphoma using DEAE chromatography (24). Antibody iodination was carried out using chloramine T (24). The specific activity ranged from 5 x $10^{3}$CPM/ug for the uridine uptake studies to $10^{5}$ CPM/ug for binding studies. Free iodine was separated from the protein by centrifugation through a 1 ml sephadex G-25 column. Sephadex G-25 slurry was placed in a 1 ml syringe plugged with glass wool. The syringe was centrifuged at 1500 rpm in a desk top centrifuge for 3 min to remove the void volume. The antibody solution was applied (0.2 ml containing 1 to 5 mg of antibody) and centrigued under the

exact same conditions. Antibodies were acylated using N-hydroxysuccinimide ester of palmitic acid (NHSP) (24). Antibody and NHSP were co-dissolved in deoxycholate. The final concentration of antibody was 10 mg/ml,the NHSP to protein molar ratio was 10:1, the final deoxycholate concentration was 2 % in phophate buffered saline, pH 8.0. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Birmingham,Ala.) and stored in $CHCl_3$ at $-20^{o}C$. Carboxyfluorescein (CF) was isolated according to the method of Ralston et al (42) with the following modifications. Recrystallized CF was dissolved in 95% EtOH and applied to a Sephadex LH-20 column (1.5 x 90 cm) equilibrated and eluted with 50 % EtOH. Fractions were analyzed by TLC using $CHCl_3$/MeOH/$H_2O$ (65:24:4). Those fractions lacking contaminants with an $R_f$ value greater than 0.6 were pooled and used. The extinction coefficient was determined and found to be $5.4 \times 10^4$ $M^{-1}$ $cm^{-1}$. Deoxycholate was purchased from Calbiochem (San Diego,CA) and recrystalized. Hexadecyl cholesteryl ether was synthesized according to Paltauf (43). It was then radiolabelled with catalytic hydrogenation using tritium gas (ICN Radiochemicals). A trace amount was used as a nontransferable/non-degradeable lipid marker (44). Uridine was purchased from Sigma ( St. Louis,MO). $^3$H-uridine was

purchased from ICN (Irvine, CA). Nitrothiobenzylinosine (S-(4-Nitrobenzyl)-6-thioinosine) was purchased from Aldrich Chem. Co. (Milwaukee, WI). Dipyridamole (2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-(5,4-d)pyrimidine) was obtained from Boeringer Mannheim Biochemicals (Indianapolis,IN). Lipid purity was assessed by TLC using $CHCl_3$ : methanol : water (65:35:4) as a developing solvent. Inorganic phosphate was determined by the method of Bartlett(45).

Liposome Preparation

Fused unilamellar DPPC liposome were prepared according to Wong et al (46) with the following modifications. Small unilamellar DPPC liposomes (SUV) were prepared in phophate-buffered saline containing 1 mM EGTA and 0.02% $NaN_3$ pH 7.4. Routinely, 200 mg of DPPC was evaporated to dryness under a stream of nitrogen followed by vacuum descication. A trace amount of $^3H$-cholesteryl ether was added to DPPC to be used as a non-trasferable lipid marker. The specific activity ranged from $10^4$ CPM/mg for uridine uptake studies to $10^5$ CPM/ug for binding studies. The lipid was hydrated in 2 ml of phosphate buffered saline at $43^{\circ}C$ for 5 min. All the lipid was vortexed into suspension and sonicated in a

strong bath sonicator (Laboratory Supplies Co.,Inc., Hicksville, N.Y.) for 10 min at 25$^{o}$C. The lipid suspension was transfered to a 15 ml corex centrifuge tube and sonicated with a small probe (Heat Systems-Ultrasonics,Inc.) at 43$^{o}$C for at least 45 min or until the suspension was opalescent (a minimal amount of turbidity). The SUV suspension was centrifuged at 2 x $10^{5}$ x G for 60 min in a SW 50.1 rotor (Beckman). The supernatant was fractionated into 0.25 ml aliquots starting from the top and incubated at 4$^{o}$C for 4-30 days. After a 4 day incubation, the top fraction was used for the uridine uptake studies. Stable liposomes were separated from unstable liposomes by a 30 ml 5-20 % continuous sucrose gradient with a 3.5 ml 65 % sucrose cushion. The suspension was heated at 43$^{o}$C for 20 min prior to loading on the gradient. The maximum loading capacity was 35 mg in 2 ml of buffer. The preparative sucrose gradient was centrifuged in a Beckman SW 27 rotor at 8.5 x $10^{4}$ x G for 16 hrs. The liposomes were collected from the buffer-5 % sucrose interface and dialyzed against phosphate-buffered saline at pH 8.0 for 8 hrs.

Immunoliposome Preparation

Palmitoyl antibody was concentrated to 15 mg/ml by ultrafiltration (Amicon) and dialyzed against 2 L 0.15% deoxycholate in phosphate-buffered saline, pH 8.0 for 16 hrs. A liposome suspension (2.5 mg/ml) was placed in a 4 ml, plastic vial (omnivial, Wheaton) and then fitted into a water-jacketed chamber. The temperature was controlled by a circulating water bath (Neslab,N.H.). Concentrated palmitoyl antibody was injected into the liposome suspension with a 50 ul Hamilton syringe attached to an infusion pump. The syringe needle pierced a water jacket septum and the wall of the plastic vial. The injection rate of palmitoyl antibody into the liposome suspension was routinely 0.2 ul/min. and the injection temperature was $41^{\circ}C$. The amount of palmitoyl antibody injected was such that the lipid-to-deoxycholate molar ratio was greater than 60. For simultaneous antibody and dye or uridine entrapment, 50 mM carboxyfluorescein in 10 mM $KH_2PO_4$ or 50 mM $^{3}H$-uridine (sp.act. = $10^{5}$ CPM/nmole) in phophate buffered saline (136.9 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 1.1 mM $Na_2HPO_4$) was added to the liposome suspension prior to injection of palmitoyl antibody. After injection, the suspension was incubated at $44^{\circ}C$ for 20 min followed by a slow cooling (30 min) to room temperature. Dialysis

against 4 L phosphate-buffered saline containing 1 mM EGTA and 0.02% $NaN_3$, pH 8.0 for 16 hrs. was used to remove residual detergent and untrapped dye. Immunoliposomes prepared on a large scale were separated from unincorporated palmitoyl antibody by the preparative sucrose gradient procedure described above. Fractions containing palmitoyl antibody, lipid, and dye were pooled and dialyzed against 4 L phosphate-buffered saline, pH 7.4 for 12 hrs. The degree of antibody incorporation was determined using a 5 ml analytical 5 to 20 % continuous sucrose density gradient centrifugation. A 4.5 ml 5 to 20 % continuous sucrose gradient was layered over 0.5 ml 65 % sucrose cushion. A 0.2 ml sample containing 150 ug of lipid was applied to the gradient and centrifuged at 2 x $10^5$ x G for 5 hrs. Gradients were fractionated into approximately 20 fractions and the amount of lipid, palmitoyl antibody, and carboxyfluorescein was determined for each fraction.

Release of Carboxyfluorescein by Heating

Carboxyfluorescein fluorescence was measured by exciting at 490 nm and the emission eas monitored at 520 nm using a Perkin-Elmer LS5 spectrofluorimeter equipped with a water-jacketed cell holder. The temperature scanning rate was $0.3^{\circ}$ C/min using a programmable tempera-

ture controller (Neslabs,N.H.). For a rapid increase in temperature, the cuvette holder was equilibrated at 48°C using a water jacketed cuvette holder attached to a circulating water bath. The cuvette and contents were equilibrated at 4°C. Placement of the cuvette into the cuvette holder resulted in an increase in temperature to 42°C within 3 min. A thermocouple was used to monitor the cuvette temperature directly. 100 % Carboxyfluorescein was achieved by addition of deoxycholate to the liposome suspension above the DPPC phase transition temperature. The final deoxycholate concentration was 0.2 %.

Cell Binding

The attached cell lines L-929 and A-31 were grown in McCoy's 5A medium (Flow,MA) containing 10 % donor calf serum (Gibco,NY). The RDM4 and P3 cells were grown in RPMI 1640 medium containing 10 mM pyruvate and 10 % fetal calf serum. The liposomes were added in a volume of 1 ml of media to $2 \times 10^6$ cells in a 35 mm petri dish and incubated for 2 hrs. at 4°C, washed three times with phosphate-buffered saline and solubilized with 1 ml 1 % Triton X-100. For RDM4 and P3 cells, the sample volume was 0.5 ml and cell number was $2 \times 10^6$ cells. Unbound material was separated from the cells by centrifugation

through 6% ficoll(Pharmacia,NJ) in phosphate buffered saline, pH 7.4 using an Eppendorf microfuge. The cell pellet was solubilized with 0.1 ml 1% Triton X-100 and analyzed for $^{125}I$ and $^{3}H$.

Release of Entrapped Carboxyfluorescein from Immunoliposomes Bound to RDM4 Cells

Immunoliposomes were bound to RDM4 cells according to the cell-binding protocol. The cell pellet was resuspended in 100 ul phosphate-buffered saline and divided into two 50 ul aliquots. Each aliquot was added to 2 ml of phosphate-buffered saline. One sample remained at room temperature while the other suspension was subjected to a rapid increase in temperature as described above with simultaneous monitoring of carboxyfluorescein fluorescence. Both samples were then layered over 1 ml of 6 % Ficoll-phosphate-buffered saline and centrifuged at 3 x $10^3$ rpm in a desk top centrifuge. $^{125}I$ was counted in a cell pellet.

Negative Stain Electron Microscopy

The liposome and immunoliposome suspensions (1 mg/ml) were applied to 400 mesh Formvar-coated copper grids and allowed to settle for 1 min. Samples were stained with 0.5

% phosphotungstic acid. An Hitachi 600 electron microscope was used to visualize the samples.

Determination of Uridine Uptake

RDM4 cells were grown in RPMI 1640, 1mM sodium pyruvate, 10% fetal bovine serum. For incubation with liposomes, cells were collected by centrifugation at 500 rpm for 5 min using a desk top centrifuge. Cells were resuspended in RPMI, 1 mM sodium pyruvate, 10 % dialyzed donor bovine serum to a concentration of $2 \times 10^7$ cells per ml. 0.5 ml of cell suspension was added to a 6 ml culture tube and allowed to equilibrate to $4^{\circ}C$ for 10 min. This was followed by addition 0.5 ml of the immunoliposome suspension (1 ug palmitoyl antibody, 35 ug of DPPC) to the RDM4 cells followed by an 1 hr. incubation. The cell suspension was then incubated at $18^{\circ}C$ for 10 min., diluted to a final volume of 4 ml with culture media, and placed in a $42.5^{\circ}C$ water bath. A 1 min lag was observed for the cell suspension to reach $41^{\circ}C$. Hence, the zero time for uptake was taken at 1 min after cell suspension was placed in water bath. The incubation dose for bare liposomes was measured in terms of encapsulated uridine. The same amount of bare liposome encapsulated uridine was added to the RDM4 cells as that of the immunoliposomes. Addition of free uridine occured after the cell suspension was placed

in the water bath for 1 min. and the amount of free uridine added was equal to that released from the immunoliposomes. After heating for the designated time, the samples were placed on ice, centrifuged at 1 x $10^3$ x G for 10 min., The supernatant was measured for released CPM, pellets were frozen in and ethanol/dry ice bath, solubilized with 1 % Triton-X 100 containing 10 mM uridine and applied to DE-81 filters. The filters were washed with 4 x 500 ml 10 mM ammonium formate/ 30 filters and a 2 X 500 ml water wash. Filters were incubated over night with 1 ml protosol tissue solubilizer and counted with 10 ml toluene based scintillation fluoror (2.8 gm 2,5-diphenyl-oxazole, 0.035 gm 1,4-bis(2-(5-phenyloxazolyl)) benzene/ L of toluene). For aqueous samples, 2 parts toluene based scintilla tion fluoror was mixed with 1 part Triton X-100. The ratio of aqueous fluoror to aqueous sample was 3.4 ml of fluoror to 0.5 ml of sample.

Determination of Temperature Dependent Uridine Release and Uptake

Immunoliposomes were incubated with RDM4 cells for 1 hr. at 4 $^{o}$C followed by a ten minute incubation at 18$^{o}$C. The suspension was diluted to a final volume of 4 ml with cell culture medium, incubated for 5 minutes at the designated temperature, and placed on ice. Percent release was monitored by centrifugation of liposomes in an air-

driven ultracentrifuge (Beckman). 0.2 ml of suspension was centrifuged at 2 x $10^5$ x G for 20 min. The tube was fractionated into two 0.1 ml aliquots along with a .1 ml 2 % triton tube wash and counted. The percent release was determined using the following equation:

% Uridine Release = ( CPM Released )/ ( Total CPM) x 100

The remainder of the suspension was processed using the uridine uptake protocol.

Inhibition of Uridine Uptake by Nucleoside Transport Inhibitors

The incubation conditions were the same as used for uptake measurements with the following modifications. After the 10 min., 18$^{o}$C incubation, the cell suspension was diluted to 4ml with cell culture media containing the inhibitor and the incubation 18$^{o}$C was continued for another 10 min. A 20 uM nitrobenzylthioinosine stock solution was prepared in phosphate buffered saline pH 7.0 and allowed to stir at least 3 hrs prior to use. A 100 uM dipyridamole stock solution was prepared in cell culture media pH 6.8.

## III. PREPARATION AND CHARACTARIZATION OF HEAT SENSITIVE IMMUNOLIPOSOMES

### Incorporation of Palmitoyl Antibody into Liposomes

Palmitoyl anti-$H2K^k$ was injected into a temperature-regulated liposome suspension at a controlled rate of 4 ug per min. The amount of palmitoyl antibody associated with the liposomes was determined by analytical sucrose density gradient centrifugation. The gradient profiles for the immunoliposomes is shown in panel A of figure 1. The incorporated palmitoyl antibody comigrated with the lipid peak, whereas the unincorporated palmitoyl antibody aggregated with itself and sedimented to the bottom of the gradient. This was also observed for sedimentation of palmitoyl antibody alone as shown in panel B of figure 1. Palmitoyl antibody aggregation was due to dilution of the detergent concentration below the critical micelle concentration necessary to keep the hydrophobic antibody in solution. The high backround between fractions 5-12 in panel A may be attributed to the heterogeneity of the protein aggregate size and composition. Although the palmitoyl antibody was bound to the liposomes, the protein-to-lipid ratios across the peak were not constant and those liposomes enriched with palmitoyl antibody sedimented further into the gradient. This result

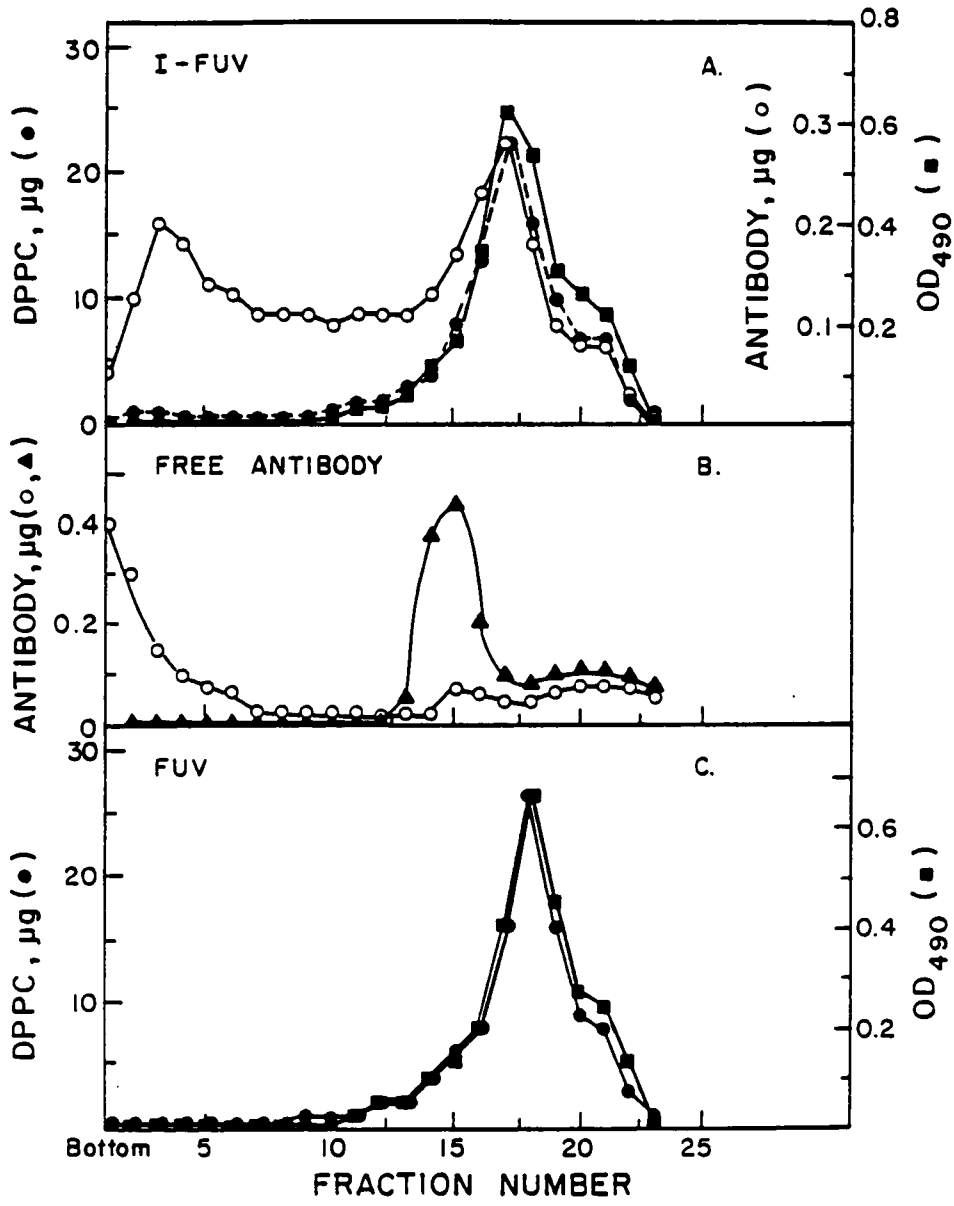


Figure 1. Analysis of Liposomes-associated Palmitoyl Antibody by Sucrose Density Centrifugation. The amount of DPPC ( ● ), palmitoyl antibody ( ○ ), and native antibody ( ▲ ) and the $A_{490}$ ( ■ ) were determined. Gradient profiles are shown for immunoliposomes (A), palmitoyl and native anti-$H2K^k$ (B), and bare liposomes (C).

indicated that the palmitoyl antibody was somewhat heterogenously distributed among the liposomes. Incorporation of antibody into liposomes absolutely required acylation by NHSP. Injection of native underivatized antibody into a liposome suspension under the identical conditions as those for palmitoyl antibody resulted in no antibody association with the liposomes.

To show that the vesicular structure of the liposomes was maintained after antibody incorporation, 50 mM carboxyfluorescein was entrapped simultaneously with the incorporation of palmitoyl antibody. Coincident with the lipid and protein peaks was the absorbance of carboxyfluorescein at 490 nm. The carboxyfluorescein fluorescence of the peak fractions was self-quenched 80 %. Also, the carboxyfluorescein-to-DPPC ratio for immunoliposomes was very similar to that obtained for liposomes without antibody (bare liposomes) shown in panel C. Determination of the trap volume per mole of phospholipid for immunoliposomes was 11.0 L/ mole of lipid and for bare liposomes it was 10.3 L/mole of lipid. These trap volumes are characterisitc of those obtained for large unilameelar liposomes (LUV) (47).

Liposome integrity was further demonstrated by electron microscopy. Fig.2 shows negative stain electron

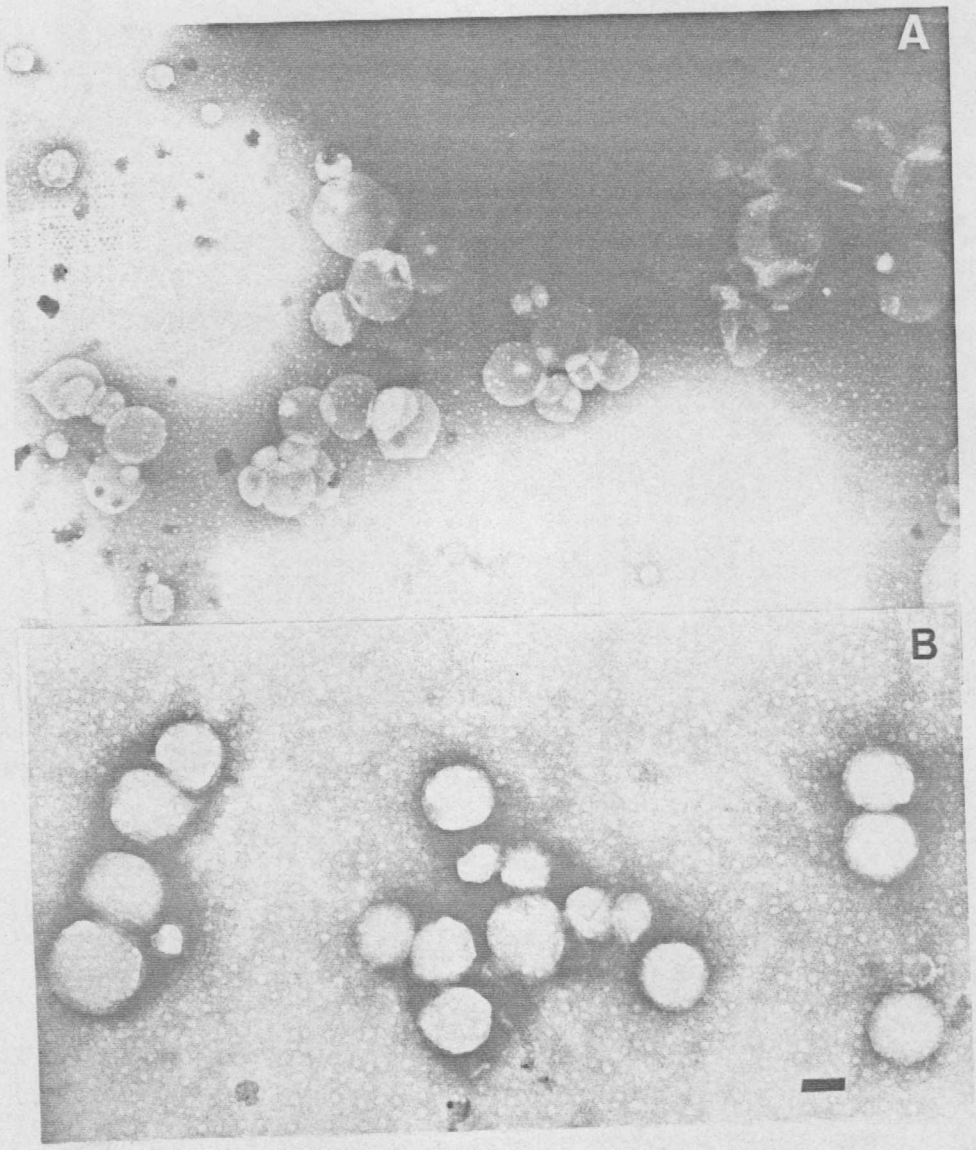


Figure 2. Negative Stain Electron Micrograph of Bare Liposomes (A) and Immunoliposomes (B). The protein to lipid ratio was $1.33 \times 10^{-4}$; the bar is equivalent to 0.1 um.

micrographs of the immunoliposomes (lower panel) and bare liposomes which have been subjected to the same conditions as the immunoliposomes (upper panel). Each preparation displayed heterogeneity in size. The bare liposomes had an average diameter of 180± 80 nm and the immunoliposomes had an average diameter of 190± 70 nm. These data taken together with that of the sucrose density centrifugation show the liposomes to be large and unilamellar.

These results indicate that a substantial amount of palmitoyl antibody can be incorporated into liposomes with simultaneous entrapment of carboxyfluorescein. The entrapment of carboxyfluorescein by immunoliposomes was >90 % for a period of 7 days.

Optimization of Immunoliposome Formation

Several conditions were examined for efficient antibody incorporation. These conditions were liposome concentration, rate of antibody injection, lipid-to-deoxycholate molar ratio, injection temperature, and protein-to-lipid ratio. A summary of the results are shown in Table I. The condition which exerted the largest effect was the liposome concentration, with the optimal concentration being greater than or equal to 10 mg/ml. The percent of antibody incorporation was unchanged for the palmitoyl antibody injection rates tested. However, rapid addition

TABLE I

PARAMETERS REGULATING PALMITOYL ANTIBODY INCORPORATION

| Parameter Varied | pAb x $10^{-5}$ DPPC initial | pAb[e] x $10^{-5}$ DPPC final | % pAb Incorp. |
|---|---|---|---|
| DPPC[a] Concentration (mg/ml) | | | |
| 40.1 | 8.85 | 4.74 | 42 |
| 20.2 | 8.33 | 4.18 | 43 |
| 10.2 | 8.77 | 3.70 | 42 |
| 5.2 | 7.35 | 2.61 | 30 |
| 2.7 | 7.81 | 1.95 | 27 |
| Injection Rate (ug pAb/min) | | | |
| 2.7[b] | 13.89 | 5.88 | 44 |
| 5.4 | 10.64 | 5.35 | 44 |
| 5.4[c] | 11.24 | 6.71 | 52 |
| 10.8 | 12.05 | 6.58 | 52 |
| Injection Temp.[d] ($^{o}$C) | | | |
| 25 | 12.35 | 6.62 | 47 |
| 41 | 14.08 | 7.63 | 47 |
| 45 | 14.93 | 6.85 | 46 |

[a]DPPC/DOC = 100, injection rate = 3ug/min.
[b]DPPC concentration = 20 mg/ml, DPPC/DOC = 200.
[c]DPPC concentration = 20 mg/ml, DDPC/DOC = 100.
[d]DPPC concentration = 20 mg/ml, DPPC/DOC = 100, injection rate = 3ug/min.
[e]Final protein to lipid ratio obtained after sucrose desity centrifugation.

$10^{-4}$ had no effect on the percent of palmitoyl antibody of palmitoyl antibody (by pipete) into the liposome suspension resulted in no antibody incorporation regardless of other incubation conditions. The lipid-to-deoxycholate ratio was kept as large as possible with a minimun of 60. Below this ratio, palmitoyl antibody incorporation and trapping efficiency decreased. A protein-to-lipid ratio less than or equal to 5 x incorporation. However, further increase in this ratio produced predominantly protein-lipid aggregates.

Release of Carboxyfluorescein from Liposomes by Heating

The effect of palmitoyl antibody incorporation on heat-sensitive release of entrapped contents was investigated by measuring the release of carboxyfluorescein from the immunoliposomes as a function of temperature. Carboxyfluorescein was used for these studies because its fluorescence is quenched at high concentrations (75 % at 50 mM) (58). A trapped concentration of 50 mM carboxyfluorescein inside the liposomes yields 80 % quenching of fluorescence. Upon heating, the dye diffuses out of the liposomes and is diluted to a concentration to where the flourescence is no longer quenched. Liposomes with entrapped carboxyfluorescein were heated from 25 to 45$^{o}$C at a scanning rate of 0.3$^{o}$C/min and the

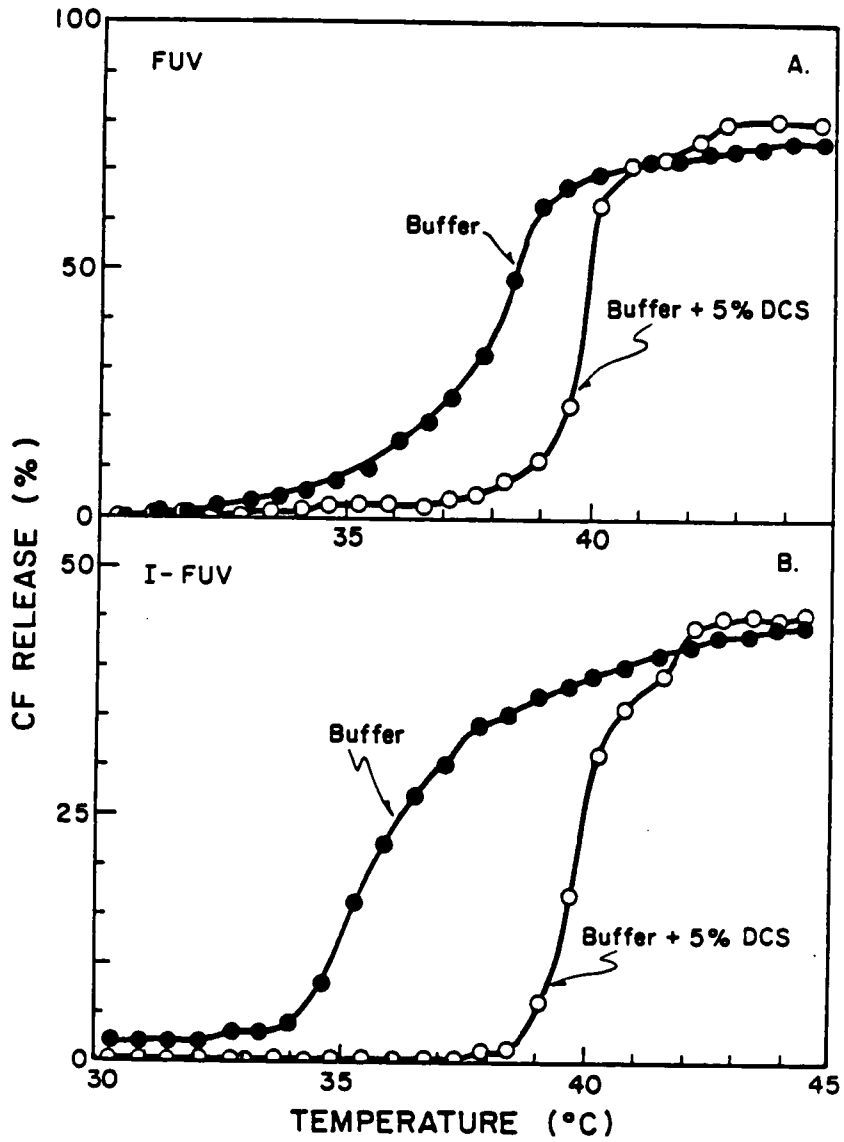


Figure 3. Release of Carboxyfluorescein by Heating from Bare Liposomes (A) and Immunoliposomes (B). Liposomes were suspended in phosphate buffered saline containing 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 13 mM glucose, pH 7.4. The temperature scanning rate was 0.3 $^{o}$C/min. Carboxyfluorescein release was monitored in the absence ( ● ) and presence ( ○ ) of 5 % donor calf serum (DCS).

increase in fluorescence was recorded. The results from these heating scans are shown in Fig.3. The percent of carboxyfluorescein release was calculated from the equation:

$$\% \text{ Carboxyfluorescein Release} = \frac{(F_{total} - F_T)}{(F_{total} - F_0)} \times 100$$

The fluorescence for the total dye release, $F_{total}$, was obtained by adding 5 % deoxycholate to a final concentration of $5 \times 10^{-3}$ M above the $T_M$ for DPPC; $F_0$ was the initial fluorescence at 25 °C, and $F_T$ was the fluorescence obtained at temperature T. No significant quenching of carboxyfluorescein fluorescence was observed by deoxycholate. Also, no significant temperature quenching of carboxyfluorescein fluorescence was observed.

The temperature scan for bare liposomes is shown in Fig. 3, panel A. In the absence of serum, the initial release of dye was observed at 33°C. The maximal rate of release was obtained between 38 and 39°C. In the presence of serum, the initial carboxyfluorescein release was not observed until 35°C and the temperature for the maximal rate of release was increased to 40°C. No quenching of carboxyfluorescein fluorescence was observed in the

presence of 5 % donor calf serum. Higher concentrations of donor calf serum were not tested. The reason being that higher concentraions of serum produced a high degree of light scattering, thus obscuring the carboxyfluorescein fluorescence.

The temperature scan for immunoliposomes is shown in panel B of Fig. 3. In the absence of serum, the initial dye release was observed at 34$^{o}$C and the temperature for the maximum rate of release was between 35 and 36$^{o}$C. In the presence of serum, the initial release was not observed until 39$^{o}$C and the temperature for the maximum rate of release was increased to 40$^{o}$C. The serum also increased the rate of release at this temperature.

The trapping protocol for carboxyfluorescein resulted in an 80 mosM hypoosmostic imbalance for the liposomes. Increase in the salt concentration of the external medium to a hyperosmotic condition in the absence of serum increased the maximal release temperature to 40$^{o}$C and decreased the rate of release at the maximal release temperature (data not shown). A similar observation has been made for small unilamellar DPPC liposomes (48).

From the data shown in Fig. 3, it can be concluded that the interaction of palmitoyl antibody with the liposomes alters the heat-sensitive release of the entrapped

carboxyfluorescein. The temperature for the maximal rate of carboxyfluorescein release was lowered, and the amount of total released carboxyfluorescein decreased from 80 to 45 %. The time course for carboxyfluorescein release by the immunoliposomes incubated at 41°C in the presence of serum, showed a initial burst yielding 40 % release within the first minute of heating. This was followed by a slower release rate which reached a plateu after ten minutes yielding a final total release of 48 % (data not shown). These results suggest that once the temperature reached a point in which the permemability of the bilayer had increased, the osmotic imbalance resulted in the initial burst of carboxyfluorescein release. Once the osmotic imbalance was relieved, a much slower rate of equilibration between the internal aqueous compartment and the external medium resulted.

The presence of serum stabilized both bare liposomes and immunoliposomes at temperatures below the maximal release temperature. No leakage was observed prior to the onset of the maximal release temperature range. The serum components also increased the maximal release temperature and the rate of release at this temperature. Upon repeated cooling and heating scans, further release of carboxyfluorescein could be obtained at 40°C.

Binding of Immunoliposomes to Target Cells

Immunoliposomes were incubated with L-929 cells ($H2K^k$) or A-31 cells ($H2D^d$) at 4 $^oC$ for 2 hrs. and the association of both $^{125}I$-labeled antibody and $^3H$-labeled lipid were determined. The degree of binding was also determined for $^{125}I$-labeled underivatized antibody, bare liposomes, and immunoliposomes in the presence of a 50-fold excess of underivatized antibody. A summary of the results is shown in Table II.

Incubation of cells with immunoliposomes resulted in 4.7 % of the antibody added and 2.1 % of the lipid added bound to the L-929 cells whereas 0.2 % of the antibody added and 0.6 % of the lipid added bound to the A-31 cells. Immunoliposomes bound to L-929 cells had a higher protein-to-lipid ratio than that of the original immunoliposomes, indicating that only those liposomes containing sufficient antibody could bind to the target cells. The protein-to-lipid ratio of the immunoliposomes bound to A-31 cells, on the other hand, was significantly lower than that of the original liposomes, indicating that only those liposomes containing little or no antibody were non-specifically bound to the control cells. Bare liposomes yielded approximately the same percent bound to the L-929

TABLE II

BINDING OF IMMUNOLIPOSOMES TO TARGET CELLS

| Condition[a] | Cell Type | Bound | | |
|---|---|---|---|---|
| | | ug Ab | ug DPPC | pAb/DPPC[d] |
| Immunoliposome | L-929 | 0.045 | 0.74 | $3.02 \times 10^{-4}$ |
| Immunoliposome + Anti-H2K$^{k}$[b] | L-929 | 0.005 | 0.19 | $1.29 \times 10^{-4}$ |
| Immunoliposome + P3 IgG[c] | L-929 | 0.046 | 0.83 | $2.71 \times 10^{-4}$ |
| Immunoliposomes | A-31 | 0.002 | 0.20 | $4.89 \times 10^{-5}$ |
| $^{125}$I-Anti-H2K | L-929 | 0.097 | | |
| $^{125}$I-Anti-H2K + Anti-H2K | L-929 | 0.008 | | |
| $^{125}$I-Anti-H2K + P3 IgG | L-929 | 0.103 | | |
| $^{125}$I-Anti-H2K | A-31 | 0.002 | | |
| Bare Liposome | L-929 | | 0.26 | |
| Bare Liposome | A-31 | | 0.16 | |

[a]Cells (1-2 x $10^6$ cells/well) were incubated at 4 °C for 2 hours in growth medium containing immunoliposomes (1 ug pAb/ml, 34 ug DPPC/ml), $^{125}$I-anti-H2K (1ug Ab/ml), or bare liposomes (34 ug DPPC/ml). Protein to lipid ratio was $1.33 \times 10^{-4}$.

[b]50 ug anti-H2K$^{k}$

[c]50 ug P3 IgG

[d]Molar ratios

as was observed for immunoliposome binding to the A-31 cells. Also, underivatized antibody incubated with the A-31 cells showed the same degree of binding that was observed for the immunoliposomes. The results clearly illustrate the binding specificty of the immunoliposomes for the L-929 cells.

To show the immunoliposome-binding specificity was mediated by antigen-antibody binding, a 50-fold excess of the native underivatized antibody was coincubated with the immunoliposomes. This resulted in an 89 % inhibition of antibody binding and a 74 % inhibition of lipid binding. This would indicate that liposomes with no palmitoyl antibody or with a low palmitoyl antibody densitiy were non-specifically absorbed to the cell surface. Substitution of P3 IgG for anti-$H2K^k$ did not inhibit immunoliposome binding to the L-929 cells, ruling out the possibility of $F_C$ receptor-mediated binding. These data clearly illustrate the binding specificity of the immunoliposomes for L-929 cells was mediated by cell surface antigen-antibody binding. RDM4 lymphoma cells grown in suspension also express $H2K^k$ antigen. Immunoliposomes exhibited the same degree of binding specificity for these cells as observed for L-929 cells ( data not shown).

Release of Entrapped Carboxyfluorescein from Immunoliposomes Bound to Target Cells

Carboxyfluorescein release from immunoliposomes bound to RDM4 cells was brought about by heating the cell suspension from 20 to 45°C within 3 min. The purpose for the rapid heating rate was to minimize endocytosis of the immunoliposomes by the target cells. The effect of this rapid heating rate on carboxyfluorescein release from the immunoliposomes alone was also examined. The results are shown in Fig.4. The temperature for maximal release for immunoliposomes in the absence of serum was at 39°C. The increase in the temperature scanning rate resulted in an increase in the maximal release temperature by 4° compared to that obtained for the slower scanning rate (0.3°C/min) in Fig.3. In the presence of serum, the maximal release temperature was unchanged regardless of the temperature scanning rate. This shows the rate of carboxyfluorescein release in the absence of serum was dependent upon the temperature-scanning rate whereas the interaction of the liposomes with serum abolished this dependence. The maximal release temperature for carboxy-fluorescein release from immunoliposomes bound to target cells was very similar to a that obtained for unbound immunoliposomes in the presence of serum. However, the

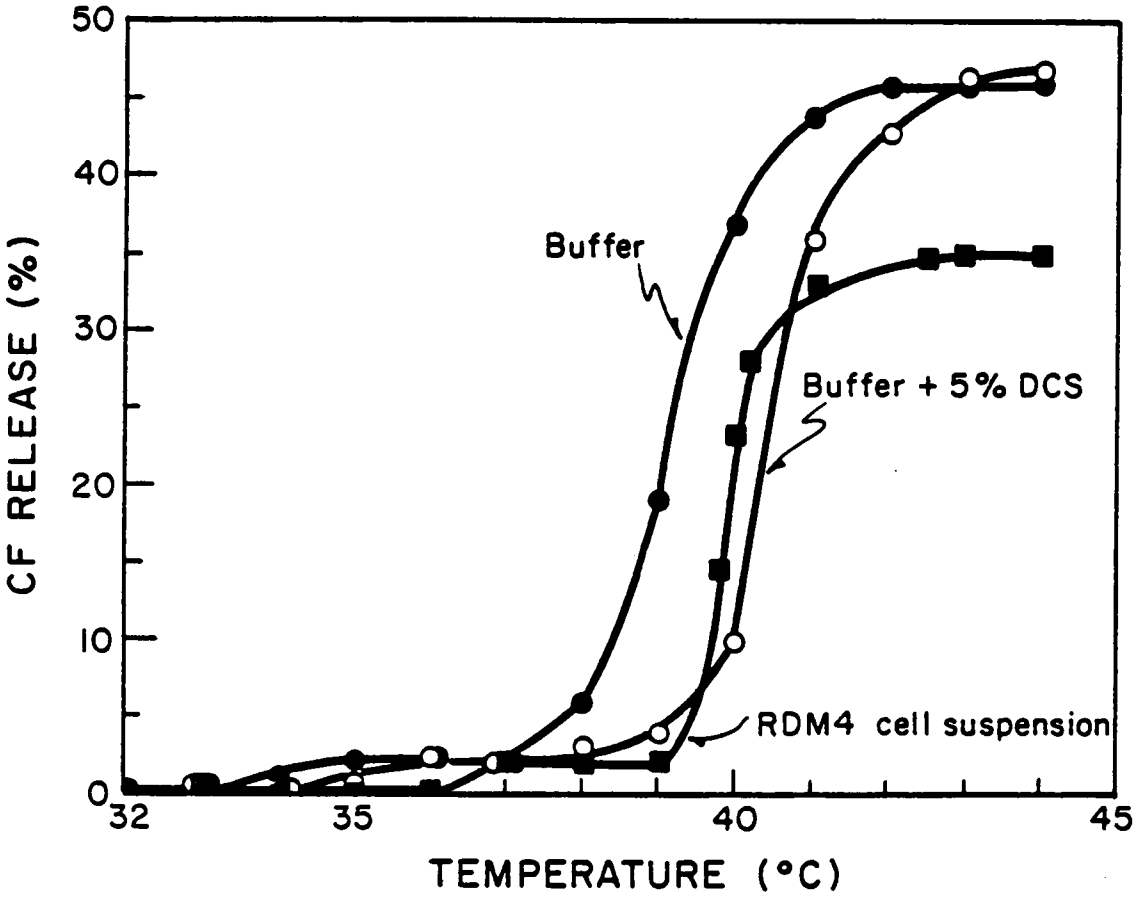


Figure 4. Release of Carboxyfluorescein by Heating from Free Immunoliposomes ( ○ and ● ) and from Immunoliposomes bound to RDM4 cells ( ■ ). Temperature was increased from 4°C to 45°C within 3 min. Carboxyflourescein release from free immunoliposomes was measured in the absence ( ● ) and presence ( ○ ) of 5 % donor calf serum (DCS).

immunoliposomes released their entrapped content to a lesser extent than did the free immunoliposomes. This may be the result of the immunoliposome-cell interaction. The same percent quenching of carboxyfluorescein fluorescence was observed for bound and free immunoliposomes, indicating no premature loss of marker as the result of cell binding. Centrifugation through 1 ml 6 % Ficoll-phosphate buffered saline for the heated sample and the sample maintained at room temperature showed the same degree of cell-associated antibody. This suggests heating of the immunoliposomes bound to target cells did not induce dissociation of the bound liposomes. These results illustrate that immunoliposomes bound to target cells retain their ability to release entrapped carboxyfluorescein upon heating.

## IV. DRUG DELIVERY PROPERTIES OF HEAT SENSITIVE IMMUNOLIPOSOMES

In the previous section, release of carboxyfluorescein from heat sensitive immunoliposomes bound to target upon heating was demonstrated. Carboxyflourescein has a very slow rate of cellular absorption. Consequently, the dye diffused away from the cell surface before a detectable amount had been taken up by the target cell. For this reason, carboxyfluorescein was a poor probe for examining the delivery properties of the heat sensitive immunoliposomes. In order to assess the effectiveness of this mode of drug delivery, the rate of cellular drug uptake must be competitive with its diffusion rate away from the target cell surface.

Several systems were investigated as potential model systems for studying the delivery properties of the heat sensitive immunoliposomes. These were: a.) delivery of deoxyglucose via the glucose transporter; b.) delivery of beta-adrenergic receptor agonists, c.) delivery of chemotactic peptide to polymorphonucleocytes, and d.) delivery of nucleosides via nucleoside transporter. Of these systems, nucleoside transport was the most suitable.

Nucleoside transport and metabolism are collectively refered to as uptake. Specifically, the uptake process involves transport of extracellular nucleosides across the plasma membrane via facilitated diffussion, followed by the intracellular phosphorylation of transported nucleosides. Uridine was selected as a model nucleoside because its transport properties and subsequent metabolism have bee well charecterized. With respect to future application, the kinetic parameters for uridine uptake closely resemble those for cytosine arabinoside (71), a cytotoxic nucleoside analog used in cancer chemotherapy. Also, it was cost prohibitory to use radiolabeled cytosine arabinoside. The $V_{Max}$ for uridine uptake ranges from 1-3 pmol/sec/$10^6$ cells and the $K_M$ ranged from 4-30 uM for rat hepatoma cells (49). These parameters are similar for other cell types as well (50,51). Uridine transport is extremely rapid. The intacellular pool and extracellular concentration equilibrate within 30 secs under saturating conditions (41). The $K_M$ and $V_{Max}$ for uridine kinase more closely resembles the kinetic parameters for uptake and therefore, phosphorylation must be the rate limiting step for uridine uptake.

Temperature Dependent Release of Uridine from Immunoliposomes

Heat sensitive immunoliposomes were prepared containing 50 mM $^{3}$H-uridine. Incubation with $10^{7}$ RDM4 cells yielded between 15 to 20 percent of palmitoyl antibody bound and between 5 to 8 percent of uridine bound. A 50 fold excess of underivatized antibody was able to inhibit palmitoyl antibody binding and uridine binding (data not shown). This demonstrates that cell associated uridine was the result of immunoliposome binding and not due to uptake of released uridine during the incubation. This was further shown by release of uridine from heat sensitive immunoliposomes bound to RDM4 cells shown in figure 5. This figure shows the temperature dependent release and cellular uptake of uridine. Below 41$^{o}$C, negligible amounts of uridine had been released from the immunoliposomes. It was only above 35$^{o}$C that the maximal amount of uridine release was observed. No further increase in percent release was observed above 41$^{o}$C. These results were very similar to those obtained for carboxyfluorescein release.

Phosphorylation of uridine only occurs intracellularly

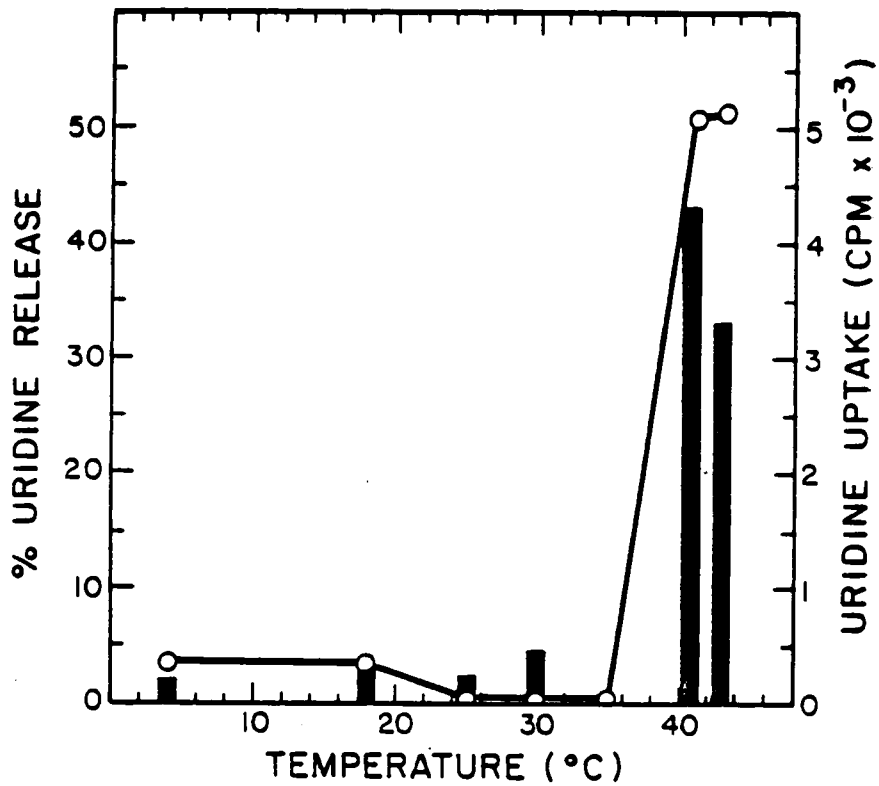


Figure 5. Temperature Dependent Liposome Release and Cellular Uptake of Uridine. 1 ug of Liposome associated palmitoyl antibody was incubated with $10^7$ RDM4 cells in a volume of 1 ml for 1 hr. at 4°C. The cell suspension was incubated at 18°C for 10 min. followed by a 5 min. incubation at the designated temperature. The cell suspension was analyzed for percent uridine release from immunoliposomes ( ● ) and uridine uptake (bar graph).

and is therefore a good criteria for examining the efficiency of uridine delivery. An assay was developed which collectively quantitated all phosphorylated uridine metabolites, including UMP, UDP, UTP, UDP-glucose and RNA. Phosphorylated uridine metabolites were collected on an anion exchange filter and unphosphorylated uridine was washed away. Comparison of this assay with paper chromatographic analysis of perchloric acid soluble material (52) yielded comparable results (data not shown).

Determination of Uridine Uptake Rate for Free Uridine and Uridine Released from Immunoliposomes and Bare Liposomes

We tested uridine uptake for uridine released from immunoliposomes and bare liposomes, and free uridine. Figure 6 shows the percent uptake as a function of time for uridine released from heat sensitive immunoliposomes, heat sensitive bare liposomes and free uridine. When first placed in the water bath, a 1 minute lag period was observed before the cell suspension reached the release temperature. Hence, the zero time point represents one minute after the cell suspension was placed in the water bath. For the free uridine control, uridine was added to the cell suspension one minute after the cell suspension was placed in the water bath. The rate of uridine uptake

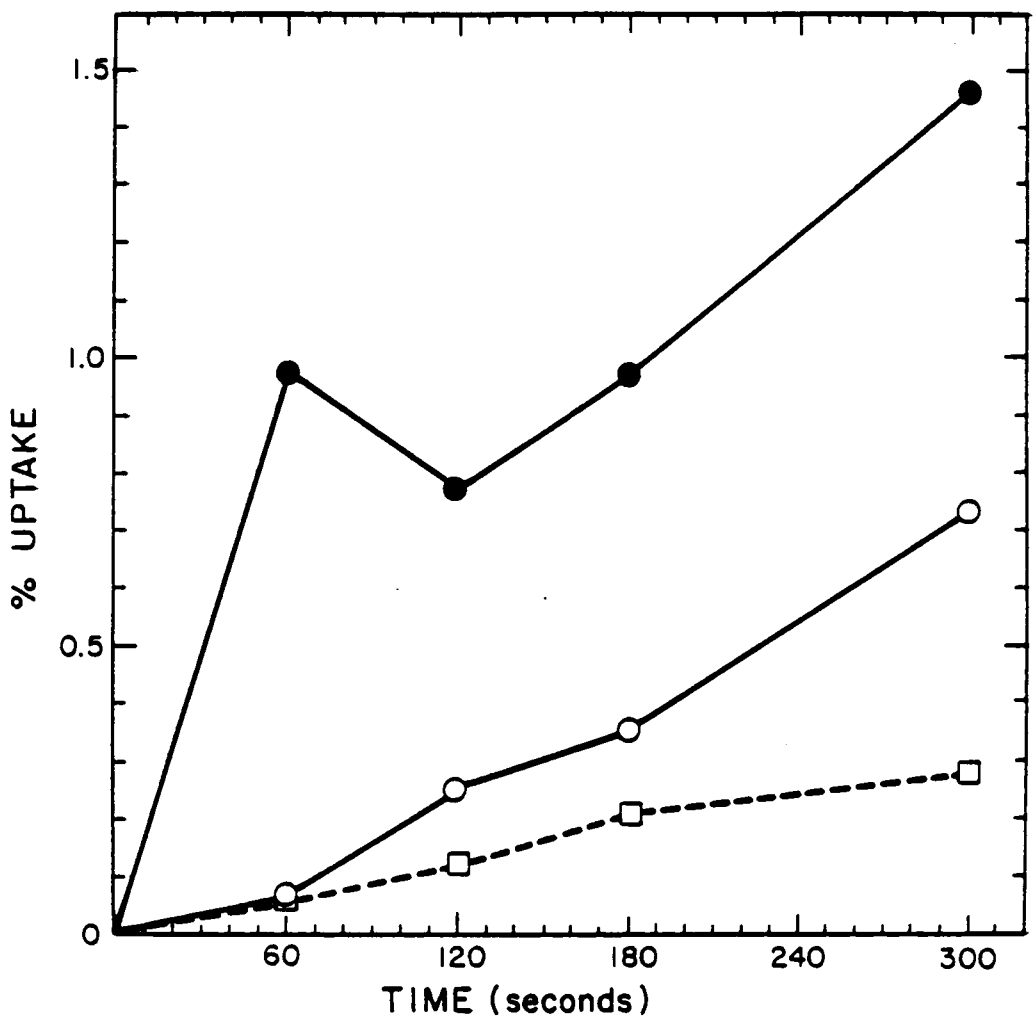


Figure 6. Kinetics of Uridine Uptake. 1 ug of Liposome associated palmitoyl antibody was incubated with $10^7$ RDM4 cells in a volume of 1 ml at 4°C for 1 hr. The cell suspension was incubated for 10 min. at 18°C, diluted to 4 mls with cell medium, and heated to 41°C. Samples were analyzed at various time points, after the cell suspension had reached 41°C, for uptake of uridine released from immunoliposomes ( ● ) and bare liposomes ( ○ ), and free uridine. □

for immunoliposome released uridine was approximately 10 pmol/min/$10^7$ cells, whereas uptake of uridine released from bare liposomes yielded a rate of approximately 2 pmol/min/$10^7$ cells and free uridine yielded a rate of approximately 1 pmol/min/$10^7$ cells. The difference in the rate of uptake for bare liposome released uridine and free uridine can be attributed to the non-specfic absorption of the bare liposomes to the target cell. These results clearly illustrate the improved delivery efficiency of the immunoliposomes over that of the bare liposomes and free uridine.

The temperature dependence for this enhanced rate of uptake of immunoliposome released uridine is shown in Figure 5. Below 35$^{o}$C, negligible amounts of uridine were released from the immunoliposomes. Consequently, only backround levels of uridine uptake were observed. Between 35$^{o}$C and 41$^{o}$C, maximal uridine release from the bound immunoliposomes was observed. The largest accumulation of phosphorylated uridine metabolites was also observed between this temperature range. In a control experiment, free uridine uptake showed a gradual increase in the uptake rate as a function of temperature, but no abrupt increase was observed between 35$^{o}$C and 41$^{o}$C (data not shown). A decrease in immunoliposome released uridine was observed at 43$^{o}$C. This decreased rate of

uridine uptake was also observed for free uridine. These results show the enhanced rate of uptake took place between 35°C and 41°C. Furthermore, this enhanced rate of uptake was due to uridine release from the immunoliposomes and not due to enhanced cellular metabolic activity in this temperature range.

Inhibition of Uridine Uptake

The initial premise for which this project was based is that release at the cell surface would be the driving force for an increase in the delivery efficiency. Examination of the mode of entry for the relaesed uridine was examined using three types of nucleoside uptake inhibitors: nitrothiobenzylinosine ($K_I$ = 1 nM), dipyramidole ($K_{I,int}$ = 12 uM, $K_{I,slope}$ = 3 uM) (41), and uridine. The results are shown in Figure 7. Panel A represents inhibition by uridine, panel B represents inhibition by nitrothiobenzylinosine, and panel C represents inhibition by dipyridamole. Inhibitor was added during the dilution of the cell incubation mixture to 4 ml and allowed to incubate with the cells an additional 10 minutes at 18°C. The additional incubation time did not increase the percent of immunoliposomes bound to the target cells. Estimation of the concentration necessary to yield 50 %

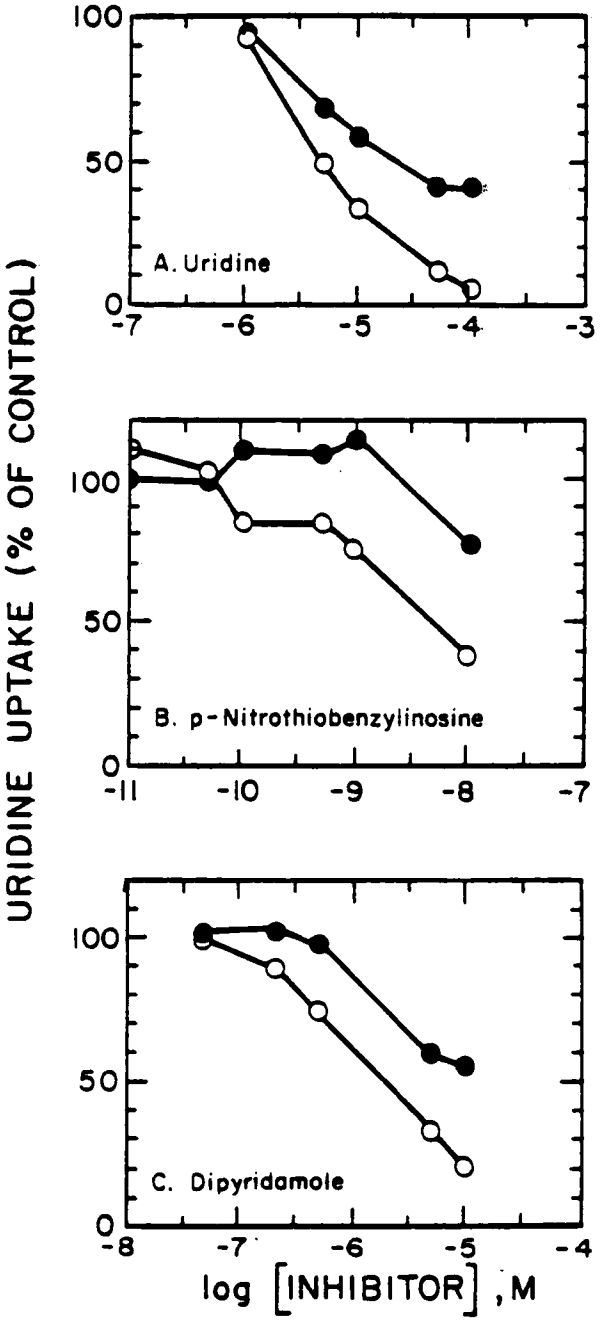


Figure 7. Inhibition of Immunoliposomes Released Uridine Uptake ( ● ) and Free Uridine uptake ( ○ ) by Nucleoside Uptake Inhibitors. Cells were incubated under the same conditions as for figures 1 and 2. After the 10 min., 18°C incubation, the cell suspension was diluted to 4 ml with culture medium containing inhibitor and analyzed for uridine uptake after a 5 min. incubation.

inhibition of uptake was obtained by 35 uM uridine for immunoliposomes and 5 uM for free uridine; 79 nM of nitrothiobenzylinosine for immunoliposomes and 5 nM for free uridine; and 16 uM of dipyridamole for immunoliposomes and 2 uM for free uridine. In summary, these results show that uptake of uridine released from immunoliposomes can be inhibited by various nucleoside uptake inhibitors. Also, the concentration of inhibitor necessary to produce 50 % inhibiton was conciderably greater for the immunoliposomes than for free uridine with nitrobenzylthioinosine exhibiting the largest concentration differential.

## V. DISCUSSION

The development of liposomes as a site directed drug delivery system has had to deal with many technical problems to have achieved its present stage of development. A major breakthrough came with the development of techniques for attaching antibodies to the liposomes (16-24). This improved the target specificity of the liposomes and also increased the diversity of the target cell. In our laboratory, a model system has been developed to study the interaction of antibody targeted liposomes with their target cells.

This system utilizes a monoclonal antibody raised against the major histocopatability antigen, H-2K$^{k}$. The antibody has been derivatized with NHSP yielding palmitoyl groups covalently attached to the antibody. The molar ratio of NHSP to antibody yields an average of four chains per antibody molecule wtih the heavy chains being predominantly derivatized. The palmitoyl chains increase the hydrophobicity of the antibody molecule allowing it to incorporate into the liposome bilayer. The exact configuration of this incorporated antibody is presently uncertain.

Liposomes containing the palmitoyl anti-H2K$^{k}$ were

shown to bind specifically to cells expressing the cell surface antigen (24-26). Upon binding to the target cell, these immunoliposomes were endocytosed as shown by the latency of cell surface removal
by protease treatment and inhibition of internalization by endocytosis inhibitors (30). Delivery of cytotoxic drugs to the target cells by these immunoliposomes showed the main route of delivery to be endocytosis.

Two types of drugs were used to demonstrate this mode of delivery, methotrexate (MTX) and cytosine arabinoside (araC). MTX is stable in the lysosomal enviornment but the N-glycosidic linkage of araC is labile to lysosomal hydoxylases. Immunoliposome encapsulated MTX showed enhanced killing over that of free MTX whereas immunoliposo encapsulated araC showed no enhancement in cytotoxicity over that of free drug. Similar observations were made by Heath et al. (31) using the same model system but the method of immunoliposome preparation was different and a natural analog of MTX which is not transported by the folate transporter was used in place of MTX. In both cases, the immunoliposomes showed enhanced cytotoxicity over that obtained for free drug; however both systems proved to be inefficient in delivering cytotoxic drugs to

target cells. These results sparked investigation into mechanisms for improved delivery efficiency.

One type of strategy has taken advantage of the endocytosis process. An intermediate step between internalization delivery of internalized material to the lysosome is the formation of endosome. The internal enviornment of the endosome becomes acidified before it delivers its entrapped contents to the lysosomes. Liposomes have been prepared which under acidic enviornments become fusion competent (33-35). Upon acidification of the endosomes, the liposomes are expected to fuse with the endosomal membrane releasing their entrapped contents to the cytosol and avoid delivery to the lysosomes.

This scheme has been highly sucessful for cells which actively endocytose, but the process is ineffective for those cells which do not actively endocytose. For these cells, we have developed immunoliposomes which can release their entrapped contents upon heating a few degrees above physiological temperature. After the immunoliposomes are bound to the target cells, heat is applied ($41^{o}C$) resulting in release of entrapped contents at the cell surface. The transiently localized, high concentration of drug at the cell surface results in an increased rate of

uptake over that of the same amount of free drug offered in the bulk medium.

For heat sensitive immunoliposomes, palmitoyl monoclonal antibody was incorporated into pre-formed heat sensitive liposomes. The method of preparation is unique. The reason is that antibody incorporation and entrapment of drug can be achieved simultaneously. Several factors were crucial in the development of this system.

Derivatization of the antibody with NHSP increases the hydrophobicity of the antibody such that it is no longer soluble in an aqueous medium. Detergent (such as deoxycholate) micelles are an absolute requirement for the antibody to remain in solution. Dilution of detergent below the critical micellar concentration results in antibody aggregation and the rate of dilution controls the degree of aggregation. In the initial development of these liposomes, antibody was added directly to the liposome suspension yielding erratic results with respect to percent antibody incorporation and liposome integrity. By introducing the antibody slowly into the liposome suspension, reproducible results were obtained. This finding allowed for the optimization of antibody incorporation to be determined. The optimal conditions were obtained from the results summarized in Table I, page 23.

Another finding essential to the development of these liposomes was the following. Fused unilamellar DPPC liposomes analyzed by 5 to 20 % analytical sucrose density gradient centrifugation yielded three bands. The first sedimented three quarters of the way into the gradient, the second band sedimented one quarter of the way into the gradient, and the third band remained at the 5 % sucrose-phosphate buffered saline interface. Addition of derivatized antibody to this liposome suspension produced lipid-protein aggregates sedimenting to the bottom of the gradient and the first band disappeared. Further increase in palmitoyl antibody concentration resulted in the depletion of the second band. Removal of these two bands by a preprative sucrose density gradient prior to addition of derivatized antibody resulted in association of the palmitoyl antibody with the third band remaining at the sucrose-phophate buffered saline interface. Only under palmitoyl antibody to lipid ratios greater that $1 \times 10^{-3}$ were protein-lipid aggregates observed.

In characterizing the formation of fused unilamellar liposomes, incubation at room temperature yielded 75 nm diameter liposomes and incubation at $4^{\circ}C$ yielded 95 nm diameter liposomes (46). The 75 nm diameter liposomes are believed to be a transient intermediate between the 35

nm diameter liposomes obtained from the probe sonication and the 95 nm diameter liposomes (conversation with Dr. M. Wong, Abbott Laboratories). The two bands sedimenting into the gradiest are believed to be the unfused 35 nm diameter liposomes and the 75 nm diameter liposomes.

Other factors which determine antibody incorporation are: 1.) lipid concentration during antibody injection, 2.) maintenance of the DPPC to deoxycholate molar ratio above 60, and 3.) 1mM EGTA in all buffers except the cell incubation medium to inhibit the trace phospholipase activity which co-purified with the anti-H-$2K^k$. The optimal set of conditions obtained from the results in table I (section 3) routinely yielded 40 to 50 % palmitoyl antibody incorporation and the trapping efficiency ranged from 3 to 5 %.

Immunoliposomes targeted to cells which do not actively endocytose should remain bound to the cell surface. The only mechanism for drug delivery in this case is slow release of the drug from the bound liposomes. This process is effective for those drugs which saturate the drug trasporter at a low concentration. However, for those drugs which do not have this characteristic, the process is inefficient. The concentration of drug diffusing out of the bound liposomes is well below the

saturation level and therefore the transport rate is not competitive with the diffusion rate. Controlling the release of the drug from bound immunoliposomes would create a transiently localized, high concentration capable of saturating the transporter and increasing the rate of drug uptake. Heat sensitive liposomes become permeable to small water soluble compounds near the gel-to-liquid phase transition temperature with the highest degree of permeability occuring at the phase transition temperature (37,53). Attachment of antibody to these heat sensitive liposomes would yeild a target specific immunoliposome capable of releasing entrapped drug when bound to its target cell.

In Section 3, Figure 3 shows heat release of carboxyflourescein from bare and immunoliposomes in the absence and presence of serum. Comparison of the release properties of bare and immunoliposomes, showed the immuno-liposomes to release at a lower temperature. A further distinction was noted in the shape of the release curves. The immunoliposome release curve had a less sharper rise at the release temperature than that of the bare lipo-somes. The conditions under which carboxyfluorescein was trapped produced a hypoosmotic imbalance for the lipo-somes. The palmitoyl antibody-DPPC interaction may have

sensitized the bilayer in a localized fashion to the osmotic pressure imbalance such that the permeability was greater at a lower temperature. Alternatively, the palmitoyl antibody may alter the thermotropic behavior of the bulk lipid in such a manner as to broaden the phase transition and/or decrease the $T_M$. Acylated proteins and membrane proteins have been shown to alter the phase behavior of lipid bilayers in this manner (54,55), but at much higher protein-to-lipid molar ratios than that of our immunoliposomes. For this reason this latter mechanism is less favored.

In the presence of serum proteins, the differences in release temperature for bare and immunoliposomes were abolished; the rate of release at the maximal release temperature was increased; and release of dye prior to the maximal release temperature was diminished, especially in the case of the immunoliposomes. These results were highly favorable considering the information available for fluid state liposome serum- component interactions (56,57). Serum components, specifically high density lipoproteins, have been shown to induce liposome leakage and in some cases completely abolish the liposome structure (58). These deleterious effects were particularly pronounced for small unilamellar liposomes in the fluid state. Interac-

tion of serum proteins with gel state liposomes produces a large enhancement in the rate of release at the $T_M$ (48). This same effect was obvserved for immunoliposomes and bare liposomes.

The carboxyfluorescein release results show the serum components interacted with the liposomes. However, this interaction did not affect the binding specificity of the immunoliposomes as shown in Table II, page 27. Immunoliposomes bound only to cells expressing the correct cell surface antigen. The specificity of immunoliposome binding was further demonstrated by the inhibition of liposomal antibody and lipid binding to the L-929 cells by underivatized antibody. The sucrose gradient velocity centrifugation results shown in Figure 1 implied a heterogeneous distribution of the antibody existed amongst the liposomes. Comparison of the protein to lipid ratios for immunoliposomes free in solution and bound to target cells showed the bound immunoliposomes to be enriched with antibody. This has also been observed for immunoliposomes prepared by detergent dialysis using the palmitoyl anti-$H2K^k$ (24). The implication is that those liposomes with a higher palmitoyl antibody surface density had a higher affinity for the target cells than those liposomes with a

lower surface density. This hypothesis has received stronger experimental support from another system which utilizes an acylated ligand, i.e. α-bungarotoxin, incorporated into liposomes. The toxin specifically binds to acetylcholine receptor, and acylated toxin retains this binding specificity. The interaction of these toxin liposomes with plasma membrane vesicles enriched with acetylcholine receptor was used as a model system to study membrane-membrane interactions (59). Low toxin-to-lipid ratios yielded a higher apparent $K_D$ than obtained from liposomes with a high toxin-to-lipid ratio. The acylated toxin also appeared to be heterogeneously distributed amongst the liposomes. Analysis of the bound toxin-to-lipid molar ratios showed the bound liposomes to be enriched with the acylated toxin in comparison to unbound liposomes. Thus, our results are in complete agreement with those of this work.

Once bound to the target cells, cellular uptake of released drug from the heat sensitive immunoliposomes is dependent upon the drug concentration at the cell surface. As stated earlier, carboxyfluorescein is a poor choice for examing drug delivery properties due to its slow rate of uptake by cells. To examine cellular uptake, nucleoside transport and subsequent metabolism was chosen as a model

system. Nucleoside transport possesses the kinetic parameters necessary to take advantage of this transiently localized, high concentration of drug released from the heat sensitive immunoliposomes. Secondly, there are many nucleoside and base analogs which are used as anticancer agents (60). Information obtained from the model nucleoside uptake system could be directly applied to the use of these agents for enhanced therapeutics and decrease the side effects.

Uridine was chosen because its kinetic parameters for uptake are best charecterized. Secondly, uridine uptake resembles that of cytotoxic nucleosides, such as cytosine arabinoside, and is therfore a suitable model for examining the delivery properties of the immunoliposomes. Uridine was able to be released from heat sensitive immunoliposomes in a temperature dependent manner and the target cells were able to rapidly take up the released uridine as shown in Figure 5.

The main thrust of this research was to show an enhanced rate of cellular uptake of uridine released at the cell surface as opposed to release at a point distant from the cell surface or simply free in solution. This point was strongly made by the design of the assay in that the majority of immunoliposomes were unbound and not

removed from the cell suspension prior to heating. Both unbound and bound immunoliposomes have the same release properties and therefore, upon heating, most of the uridine was released into the bulk medium; however, an enhanced rate of uptake was still observed for the immunoliposome released uridine over that of bare liposomes and free uridine. The results shown in figure 6 clearly show that the rate of uridine uptake of uridine released from immunoliposomes was 10 fold greater than that obtained for the same amount of free uridine and 5 fold greater than that obtained for uridine released from bare liposomes. These results show a local, high concentration of uridine released from a small number of bound immunoliposomes made a significant improvement in cellular uptake of uridine.

A minor point to be addressed concerns the difference in the uptake rates of bare liposome released uridine and free uridine. The rate of bare liposome released uridine was 2 fold greater than free uridine. A small percentage of the bare liposomes were non-specifically adsorbed to the target cells (ten fold less than immunoliposomes-data not shown). Upon heating, uridine released at the cell surface may have resulted in the increase in the rate of uptake over that obtained for free uridine.

The mode of the enhanced uptake rate was examined using the following uridine uptake inhibitors: Nitrobenzylthioinosine, dipyridamole, and uridine. Nitrobenzylthioinosine is an inosine analog and a potent competitive inhibitor of nucleoside transport. The $K_I$ for nucleoside transport in erythocytes is 1 nM (61) and the $K_I$ for other cell types is similar (50,51). Dipridamole, or sometimes referred to as persantine, is a non-competitive inhibitor which yields mixed type inhibition for uridine transport. The $K_{I,int}$ is 12 uM and the $K_{I,slope}$ is 3 uM (41). It has been proposed that dipryidamole inhibits nucleoside transport by binding directly to the transporter, and also by altering the membrane fluidity thereby affecting the recycling of the transporter (52). Addition of unlabled uridine inhibited the rate of uptake by decreasing the specific activity of the entrapped radiolabeled uridine upon release from the immunoliposomes. Nucleoside kinases are regulated by product inhibition (27). Addition of uridine may have inhibited the kinase activity in this manner, to some extent, but the majority of inhibition was probably due to the decrease in specific activity.

The results in Figure 7 showed both free uridine and immunoliposome released uridine uptake were inhibited by these compounds. Estimation of inhibitor concentrations

necesaary to yeild 50 % inhibition of uridine uptake showed approximately an 8 fold greater concentration was required for uridine or dipyridamole to inhibit the uptake of immunoliposome released uridine. A 16 fold concentration difference was obtained for nitrothiobenzylinosine. Nitrothiobenzylinosine is strictly a competitive inhibitor of nucleoside transport (61) whereas uridine and dipyridamole are not (41). Release of uridine at the cell surface would create a higher concentration at the cell surface compared to concentration of uridine in the media and would therefore require a higher concentration of inhibitor. Alternative possibilities would include a.) decreased the accessability of the transporter by bound immunoliposome, or b.) adsorption of the inhibitors by the immunoliposomes thereby decreasing the concentration available for inhibition of uptake. However, empty immunoliposomes bound to the target cells did not alter free uridine uptake or inhibition of free uridine uptake (data not shown). These results support the premise that enhanced drug delivery by heat sensitive immunoliposmes was due to release at the cell surface.

Several factors must be optimized to improve the efficiency with which the heat sensitive immunoliposomes deliver entrapped contents to their respective target

cells. The first factor is the pre-equilibration step directly following the 4°C incubation. This 10 min., 18°C incubation was found to be essential in order to obtain an enhanced rate of uridine uptake. Binding analysis showed the pre-equilibration step to increase the number of bound immunoliposomes 2 fold and these immunoliposomes had a lower palmitoyl antibody density than those bound at 4°C. The binding was specific because underivatized antibody inhibited the binding of immunoliposomes to RDM4 cells. At 18°C, internalization of bound immunoliposomes takes place by recpetor mediated endocytosis (30). Intracellular release due to internalization of immunoliposomes can be ruled out for two reasons. First, replacement of the 4°C incubation with an 18°C incubation resulted in a decrease in the rate of uptake for immunoliposome released uridine (data not shown). Secondly, dilution of the cell suspension at 4°C to 4 ml prior to the 18°C pre-equilibration step did not produce an increase in the number of bound immunoliposomes and also did not yield an increase in the rate of immunoliposome released uridine uptake. A simple explanation would be that the enhanced uptake rate was due to an increase in the concentration of the transient localized high concen-

tration at the cell surface as the result of an increase in the number of bound immunoliposomes to the cell surface. Possibly, internalization of the cell bound immunoliposomes reduced the concentration of released uridine at the cell surface thereby resulting in a decrease in the rate of uptake. The exact mechanism by which this step causes more immunoliposomes to bind is uncertain. However, obtaining the exact temperature optimum and incubation time may further increase the number of bound immunoliposomes ultimately resulting in a further increase in the rate of uptake for uridine released from cell bound immunoliposomes.

The results discussed above imply that increasing the amount of cell associated immunoliposomes should result in an increased rate of uptake. Another viable parameter to investigate would be the protein-to-lipid ratio. There is not much leeway in varying this parameter for, as previously stated, too low of a protein-to-lipid ratio will result in lipid-protein aggregate formation. Too high of a protein-to-lipid ratio may be insufficient to confer immunoliposomes binding specificity. Optimizing the palmitoyl antibody density may improve two factors: a.) reduce non-specific binding, b.) yield a more homogeneous distribution of antibody amongst the liposomes. This will

have to be examined by varing the lipid concentration, deoxycholate to lipid ratio, antibody injection concentration and finally, the protein to lipid ratios.

Other parameters to examine would be the effect of the entrapped dose, heating strategy, and decrease in the intracellular nucleoside pool. The heat sensitive immunoliposomes can stably trap up to 100 mM uridine (data not showm). An increase in the entrapped drug concentration would increase the effective concentration of drug to be released at the cell surface and ultimately increase the rate of uptake.

The existing assay for measuring the delivery properties of the heat sensitive immunoliposomes employs a single heat step. Concievably more drug could be introduced into the target cells by using multiple heat steps. Prior to each heat step, additional immunoliposomes would be allowed to bind to the target cells. The unbound immunoliposomes would be removed and followed by heating of the cells. Attached cells may be better suited for this heating strategy because unbound immunoliposomes can be removed more easily. An alternative method for heating the cells would need to be designed as well. The duration between heatings would be dependent upon the cell surface density and rate of turnover of the antigen. This heating

strategy would increase the delivery efficiency of the cytotoxic drug.

Another modification to enhance drug uptake would be to decrease the intracellular concentration of the nucleoside pool. In the case of araC, a slow rate of uptake was observed in comparison to the uptake rate obtained by pretreating the cells with thymidine or hydroxyurea (57). Deoxycytidine kinase is under feedback regulation by deoxycytidine-5'-triphosphate (dCTP). Inhibition of de novo synthesis of dCTP was shown to enhance uptake of cytidine and also araC. Pretreatment of target cells with hydroxyurea or thymidine prior to incubation with immunoliposomes should enhance the uptake of araC by the target cells.

The therapeutic potential for this type of drug delivery is limited to sites accessible to localized heating. The administration routes can be intravenous, subcutaneous, and topical. Intravenous injection of liposomes has resulted in the liposomes to accumulate in the liver, spleen and lung (66-68). If delivery to other areas of the body were intended, heating would be restricted to sites away from these organ systems, especially the liver and spleen. Secondly, liposomes have

been shown to be unable to escape the circulatory system into the interstitial spaces of the organs. This restricts the type of target cell to those of the blood or to endothelial cells of the vascular system servicing the tumor, assuming a specific antibody can be obtained to specifically direct the immunoliposomes to that site.

Subcutaneous injections of liposomes has resulted in a.) rapid clearance from the site of injection and, b.) concentration of the liposomes in the local lymph nodes. Recycling of these liposomes from the lymph nodes into the circulation was dependent on size (64) with the small unilamellar liposomes being the most rapid. Lymph nodes are accessable to the external enviornment to a certain extent and are therfore potentially accessible to local heating. For this reason subcutaneous administration of heat sensitive immunoliposomes presents a very viable administration route.

Topical application of liposome encapsulated drugs to the skin has been shown to increase the retention time of a localized concentation and, at the same time minimize systemic absorption (65). The efficiency of delivery of this localized concentration may be enhanced by regulating the release of entrapped drug by temperature. Topical

application of liposomes encapsulated drugs have been extended to treatment of eye infections (69). Here, the advantage lies in a single liposome application whereas in other methods of application, the drugs must be applied several times due to natural continual bathing of the eye. Treatmet of eye infections have been extended to site directed drug delivery by intraocular injection as well (70) but the therapeutic advantage is still under investigation.

This work demonstrates that heat sensitive immunoliposomes are capable of delivering drugs more efficiently than free drug and drug released from untargeted liposomes. Further optimization of the system should be carried out using cytotoxic nucleosides such as cytosine arabionside, 5-fluorouracil, formycin A, etc. Direct monitoring of drug uptake should be accompanied by the development of a cytotoxicity assay to fully demonstrate the worth of this type of drug delivery.

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# VITA

Sean M. Sullivan was born in Elizabeth, New Jersey, on February 19, 1953. He graduated from Roselle Catholic High School in June of 1970. The following September, he entered Maryvill College in Maryville, Tennessee, and in June 1974 received a Bachelor of Arts degree in Chemistry.

In August of 1974, he was employed by Hoffman-La Roche to work in their Quality Control Division in Nutley, New Jersey, as a bench chemist. In June of 1975, he was transferred to the Roche Diagnostics Division in Sommerville, New Jersey. There he was commissioned to work with their radioimmunoassay systems.

In September of 1977, Mr. Sullivan enrolled at the University of Tennessee, Knoxville as a post baccalaureate student. In June of 1978, he was accepted to the Master's degree program in Biochemistry Department, and in March 1981 he received the degree of Master of Science.

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