DEVELOPMENT OF AN EFFECTIVE DRUG DELIVERY SYSTEM USING LOADED PLATELETS

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1993

(Submitted May 4, 1993)

ACKNOWLEDGMENTS

A brief history....

One day my dad was playing golf, which isn't that unusual, and noticed his golf swing was slightly off, which also is not that unusual. However, on this occasion his leg was cramping and stiff, so he went to the doctor for an examination. Sure enough the doctor found an arterial blockage and dad underwent the conventional drug therapies. Within a few months he was back on the greens again.

Throughout this experience he encouraged me to develop a more effective therapy for golfers with blockages that impair golf swings. He was convinced that I could do it. Little did he realize that I had just graduated with a degree in physical chemistry, not medicine. (In my family, if you do science you are either a rocket scientist or are trying to cure cancer.) However, a short time later the faculty lectures to the first year graduate students began (during the baseball playoffs!). John gave his lecture about tumors in rats and curing cancer and clots, and I knew I was being offered a great opportunity. In addition to finding a thesis topic, I could cure dad and the myriad of golfers with his affliction. He was thrilled!

There are many people who I would like to thank for helping me attain my Ph. D. degree. I am sincerely grateful to John Baldeschwieler for his guidance and to all the people in the JDB group for their friendship and Tuesday lunches at restaurants too exotic for Carl. I would especially like to thank Van for being a mentor, colleague and friend.

As always, I am deeply grateful for my parents, sister and other family members, who have encouraged me and are a great source of happiness. I would also like to thank Carl, my fiancé for his warmth and understanding throughout my graduate years. To all my friends, I thank you all!

ABSTRACT

Liposomes have been used to deliver diagnostic and therapeutic drugs with moderate success. Current applications include targeting to tumors (1) , where the vasculature is leaky and allows liposome penetration, and Kupffer cells in the liver (2). Reticuloendothelial system (RES) uptake and lack of targeting specificity of the liposomes have been the major problems encountered. Several groups including Allen *et al.* (3) and Liu *et al.* (4) have increased the circulation times by attaching polyethylene glycol (PEG) or the ganglioside GM_1 to the liposome surface; the circulation half-lives for disteroylphosphatidylcholine (DSPC):cholesterol:PEG and GM_1 liposomes are 20.0 ± 3.5 and 16.4 ± 3.1 hours, respectively as compared to 6.7± 4.5 hours for DSPC:cholesterol (2:1 mole ratio) liposomes (3). In addition, some attempts have been made to target specific areas by the addition of ligands, such as human gamma globulin or aminomannose; however this increased targeting has been largely limited to increased Kupffer cell and RES uptake (5, 6). Addition of antibodies to the liposomes increases targeting specificity, but has met with limited success since the liposomes are still prone to RES uptake (7).

Reconstituted Sendai virus envelopes (RSVE) are essentially liposomes with binding and fusogenic glycoproteins on their surface. These vesicles have been used as vehicles for delivering molecules into cells *in vitro* and for the transfer of membrane proteins into the cells' plasma membranes. Similar to intact viruses, RSVE attach preferentially to cells having the appropriate receptors, usually gangliosides, and fuse with the cellular membranes, thus emptying their contents into the cell cytoplasm.

This also results in the implantation of the viral envelope components into the recipient cell plasma membrane.

We have studied the *in vitro* interactions of small unilamellar vesicles (SUV) and reconstituted Sendai virus (RSVE) with platelets with the objective of developing new drug delivery systems. Specifically, we have examined the kinetics and mechanisms of uptake of SUV, with and without covalently attached proteins, and RSVE. Liposomes that have been studied include: DSPC:cholesterol (2:1 mole ratio) liposomes (control) (8) and aminomannose (Am), human gamma globulin (HgG) and transferrin (Tf) labeled control liposomes (9). From our data we conclude that the mechanisms and kinetics of uptake and subsequent specific localization of the lipid and aqueous components of the liposomes within the cells are dependent on the type of liposome used.

Platelets have the unique ability to target to specific areas *in vivo* including areas of infection and inflammation, tumors and clots. Therefore we hoped to combine this *in vivo* targeting ability with the platelets' ability to take up liposomes and RSVE *in vitro* to create new drug delivery systems to deliver diagnostic and therapeutic reagents. Having a variety of systems from which to choose should offer the ability to optimize drug delivery parameters.

In vitro functional assays, including microaggregation, serotonin release and membrane integrity, conclude that platelet function is not inhibited by liposome uptake (8, 9). However, uptake of intact and reconstituted Sendai virus particles induce platelet aggregation and secretion. *In vivo* organ distribution studies in Sprague-Dawley rats

indicate that circulation times and RES uptake of liposome-loaded rat platelets are identical to control rat platelets (10).

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TABLE OF CONTENTS

CHAPTER ONE

INTRODUCTION

DRUG DELIVERY SYSTEMS:

In order to increase the targeting specificity and circulation times of lipophilic and hydrophilic drugs, we decided to investigate the possibility of loading both types of drugs in platelets via the use of liposomes and reconstituted Sendai virus envelopes (RSVE). Other techniques for loading platelets were available, but for one or more reasons were deemed unsuitable. Among these techniques are: microinjection by needles (1,2), electroporation (3) and electroinjection (4) and fusion with erythrocyte ghosts (5).

Microinjection has been used to inject a variety of hydrophilic drugs into the cytoplasm or the nucleus of living cells. This method is reproducible, but the number of cells that can be injected is limited. Electroporation techniques, which employ voltage discharges (2-8 kV/cm) or repetitive pulses of slightly lower field strengths to induce pores in the plasma membrane, severely impair cell viability due to cytoplasmic leakage (6). Electroinjection, a more recent technique, uses much lower field strengths (0.3-0.7 kV/cm) to reversibly permeabilize the cell membrane (4). Under carefully defined conditions, electroinjection is an efficient and reproducible method for injecting large numbers of cells (10^6 cells). As of now, the technique has been applied to loading cultured cells with great success; *i.e.* high loading efficiency and cell viability. However, electroinjection of non-cultured cells, including macrophages, has resulted in decreased cell function. Fusion of drug-loaded erythrocyte ghosts with target cells can be promoted by the addition of polyethylene glycol (5). However, the use of an added fusogenic agent complicates this method, reduces its efficiency and makes it less specific.

Loading drugs into cells by the use of liposomes and RSVE can be very efficient for cells that are capable of phagocytosis or contain receptors which can bind liposomes with specific ligands or RSVE. In addition, unlike the microinjection, electroporation or electroinjection methods, liposomes or RSVE can load lipophilic as well as hydrophilic drugs into cells.

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PLATELETS:

Platelet Formation

The first description of platelets was published in 1824 by Donné, who speculated that they might be derivatives from the chyle and perhaps were the percursors of leukocytes (1). Forty years later Bizzozero determined that platelets were a distinct type of blood cell (2). In 1906 Wright proposed that platelets were derived from megakaryocytes (3).

Megakaryocytes are very large cells, up to 160 μm diameter, which are derived from stem cells within the bone marrow and spleen. Basically there are three morphological stages of megakaryocyte maturation prior to platelet formation (4). Stage 1, the megakaryoblast, is readily recognized by its loose chromatin structure. During this stage the cell enlarges progressively with repeated endomitosis and is engaged in extensive protein and DNA synthesis. Stage 2, the basophil megakaryocyte, is initiated after DNA synthesis and endomitosis are mostly completed. During this stage the cell undergoes cytoplasmic maturation, which is characterized by intense RNA production and protein synthesis. At the commencement of stage 3, the cell is polychromatic but has a nucleus to cytoplasm ratio of greater than 1:3. As the cell matures the nucleus condenses and the nucleus to cytoplasm ratio falls. In addition the remaining basophilia disappear and the granules typical of platelets appear. The ratio of organelles to cytoplasm varies greatly with the degree of endomitosis. Those with 8N constitution contain a greater ratio of organelles to cytoplasm volume; those with 32N constitution contain a lesser ratio but a greater membrane content, having undergone more

extensive endomitosis and membrane synthesis. Presumably this heterogeneity accounts for the heterogeneity seen in platelet populations. After the completion of stage 3, the bone marrow megakaryocytes, which are primarily located within the bone marrow sinusoids (5), adopt an amoeboid form with the pseudopodia penetrating through the sinusoidal wall into the lumen. The stage of maturation between megakaryocyte pseudopodia formation and the earliest circulating platelet remains a matter of considerable controversy. It is uncertain whether proplatelet projections fragment in the marrow sinusoid to give rise to platelets or break off from the parent cell and are carried elsewhere in the circulation to undergo subsequent fragmentation.

Platelet Morphology

The average human adult has 250,000 platelets/μl (6) and approximately 35,000 platelets are produced per microliter of blood every day (7). The average lifetime of the platelet is approximately 9-12 days (8). In circulation the platelet is a disk shaped cell with an average size of 2-3 μm by 1 μm; however it can easily change to a spherical or irregular shape by shooting out pseudopodia of various lengths in response to a multitude of stimuli. Because the platelet is a cytoplasmic fragment of the megakaryocyte, it has no nucleus and the endoplasmic reticulum and Golgi complex are virtually absent.

The ultrastructural features of a platelet are illustrated in Figure 1. The anatomy of the platelet can be divided into three major regions: the peripheral, the sol-gel and the organelle zones. The peripheral zone is composed of the exterior coat, the membrane and the submembrane

regions. The exterior coat or glycocalyx covering the outer surface of the platelet is approximately 10-20 nm thick and contains various glycoproteins important for adhesion and aggregation processes. Underneath the exterior coat is the trilaminar unit membrane, approximately 7-9 nm thick, which provides a physiochemical separation between intracellular and extracellular constituents and processes. The core of the membrane is formed by a lipid bilayer to which peripheral proteins, such as cytoskeletal proteins, are attached. The lipid bilayer contains 70% phospholipid by weight; the remainder consists mainly of cholesterol plus a small amount of glycolipids. Five major phospholipid classes have been identified in the human platelet (9,10): phosphatidylcholine (38%), phosphatidylethanolamine (27%), sphingomyelin (19%), phosphatidylserine (10%) and phosphatidylinositol (5%). As in red blood cells, the phospholipids are asymmetrically distributed over the bilayer. Important components of the plasma unit membrane are the Na/K ATPase and the anion pump which help maintain the appropriate transmembrane ionic gradients. Another characteristic of the membrane is its invaginations which form a network of tortuous channels that burrow through the cytoplasm. This system of channels, the open channel system (OCS), greatly enhances the surface area of the cell and serves as a means for uptake of substances from the plasma and rapid extrusion of granule contents into the plasma. Intimately associated with the OCS is the dense tubular system (DTS) which White has proposed may be derived from the endoplasmic reticulum (11).

At least three cytoskeletal systems are present in the matrix of the platelet: the submembrane filaments, microtubules and microfilaments. All of these are believed to contribute in the maintenance of discoid shape and extrusion of pseudopodia.

Electron microscopy reveals several types of organelles in the cytoplasm of the platelet including mitochondria and three distinct types of granules. Electron dense bodies are distinctive because of their intensely opaque internal contents which are often separated from the enclosing membrane by a clear space. They are storage sites for ADP, ATP, serotonin and calcium (12,13) and number approximately 2-10 per platelet. Alpha granules are of moderate electron density, number between 20 to 200 per platelet and contain fibrinogen (14), factor V (15), von Willebrand factor (16,17) and other proteins. The lysosomal granules contain a number of acid hydrolases (18).

Haemostasis

Haemostasis is the collective noun for the processes that cause cessation of the flow of blood through a damaged vessel wall. The main components of the haemostatic system are: platelets, humoral coagulation enzymes, the layer of endothelial cells that line blood vessels, the subendothelial structures, and the smooth muscles that support the vessels.

When a blood vessel is damaged, the defect must be sealed through the coordinated action of the components of the haemostatic system. The relative contribution of the different components depends on the extent and location of the damage. Vasoconstriction may be effective in stopping

bleeding in the capillary bed, but is not sufficient in arterioles and venules. In these vessels, recruitment of platelets and activation of the coagulation system is necessary to achieve haemostasis. Haemostasis in arteries and large veins, in which the blood pressure is higher, generally requires outside intervention.

A number of coagulation enzymes circulate in the blood in an inactive zymogen form. These zymogens can be converted to their active proteolytic forms by cleavage of specific peptide bonds. Damage to the endothelium induces adhesion and aggregation of platelets and activation of the first enzyme of the coagulation cascade (Figure 2). Successive reactions ultimately result in the conversion of soluble fibrinogen into a fibrin network.

Platelet Involvement in Haemostasis:

The initial reaction to vascular damage is adhesion of platelets to the exposed subendothelial structures, which are composed of microfibrils of elastin, basement membrane-like amorphous material and collagen fibrils (19). Platelet adhesion is largely mediated by a high molecular weight protein, von Willebrand factor (20). Under normal physiological conditions von Willebrand factor does not readily interact with human platelets. However, interactions between von Willebrand factor and the subendothelium is thought to produce a conformational change in the protein which enables binding to the platelet surface and subsequent platelet adhesion. After adhering, platelets often undergo release reactions. In the primary release the contents of the dense bodies, which include adenine nucleotides and serotonin, are released into the

surrounding medium. The release of ADP stimulates new platelets to aggregate and serotonin is a mediator of vasoconstriction. Usually a second release reaction involving release from the α-granules, which contain fibrinogen and factor V, also occurs. Fibrinogen is an essential cofactor for platelet aggregation (21,22). Factor V and thrombin activated factor V bind to the platelet membrane and serve as a membrane receptor for coagulation factor X (23,24). In addition, adherent platelets produce metabolites of arachidonic acid, particularly thromboxane A_2, a very potent platelet-aggregating agent. This close cooperation between platelets and clotting factors results in the production of a fibrin-reinforced plug localized at the site of the vascular defect.

Tissue Repair and Fibrinolysis

As soon as the bleeding is stopped, the tissue repair process begins. The fibrin meshwork and cellular debris are removed by fibrinolytic and phagocytic processes; healthy cells are stimulated to undergo mitosis. Neutrophils and eventually macrophages are attracted to the damaged areas by chemotactic factors released during the haemostatic process (25-27). The phagocytic cells release lytic enzymes and take up cellular debris. The fibrinolytic system is activated by a tissue-type plasminogen activator released from the endothelium. Fibrinolytic enzymes which enter the circulation after resolution of the fibrin meshwork are rapidly inactivated by inhibitors present in the blood (28-32).

Endothelium Involvement in Haemostasis

The layer of endothelial cells that line the inner surface of the blood vessel is not "inert"; instead it is an active participant in both the haemostatic and fibrinolytic processes. After vessel wall injury, the stimulated endothelial cells synthesize thromboplastin which initiates the coagulation cascade (33,34). In addition, endothelial cells synthesize clotting factors V and VIII (35,37). Thrombomodulin, another cofactor present on the endothelial cell surface, binds thrombin which increases the activation rate of protein C (38,39). Protein C inactivates the coagulation factors V_a and $VIII_a$ thereby slowing down thrombin generation (40-42). Protein C also stimulates the fibrinolytic process (43,44).

Senescence of Platelets

A large body of evidence supports the idea that platelets undergo a process of senescence which results in their removal from the circulation. These senescent changes have been related to functional and structural parameters. As platelets age they become less adherent to collagen (45), less reactive to aggregating and releasing stimuli (46), less metabolically active (47) and less capable of shortening bleeding times *in vivo* (48,49). In addition, platelets lose sialic acid over time, presumably from surface glycoproteins and/or gangiosides.

Removal of sialic acid from the platelet surface by neuraminidase (50), removal of sialic acid containing glycopeptides by the proteolytic enzymes such as plasmin, chymotrypsin and trypsin (51) and alteration of sialic acid with sodium periodate (52) have been shown to result in

decreased platelet survival *in vivo*. These findings, in conjunction with the observation that platelets lose sialic acid with age, strongly indicate the role of desialization in senescent recognition. However, it is not clear by what mechanism the RES might recognize desialized platelets.

Some hypotheses suggest that such cells might be recognized by their decreased negative charge. Other data indicate that galactose, the penultimate residue of polysaccharide side chains of glycoproteins and gangliosides, might be recognized by a membrane receptor. Such a receptor has been identified on Kupffer cells (53). Recent findings suggest that clearance of desialized erythrocytes is dependent upon a plasma factor which possesses many characteristics of an immunoglobulin which may recognize a cryptic antigen exposed by desialization (54). The fact that desialization, modification of sialic acid and removal of glycopeptides all produce decreased platelet survival argues against the galactose receptor hypothesis. All of the manipulations reduce negative surface charge and could potentially expose cryptic antigens.

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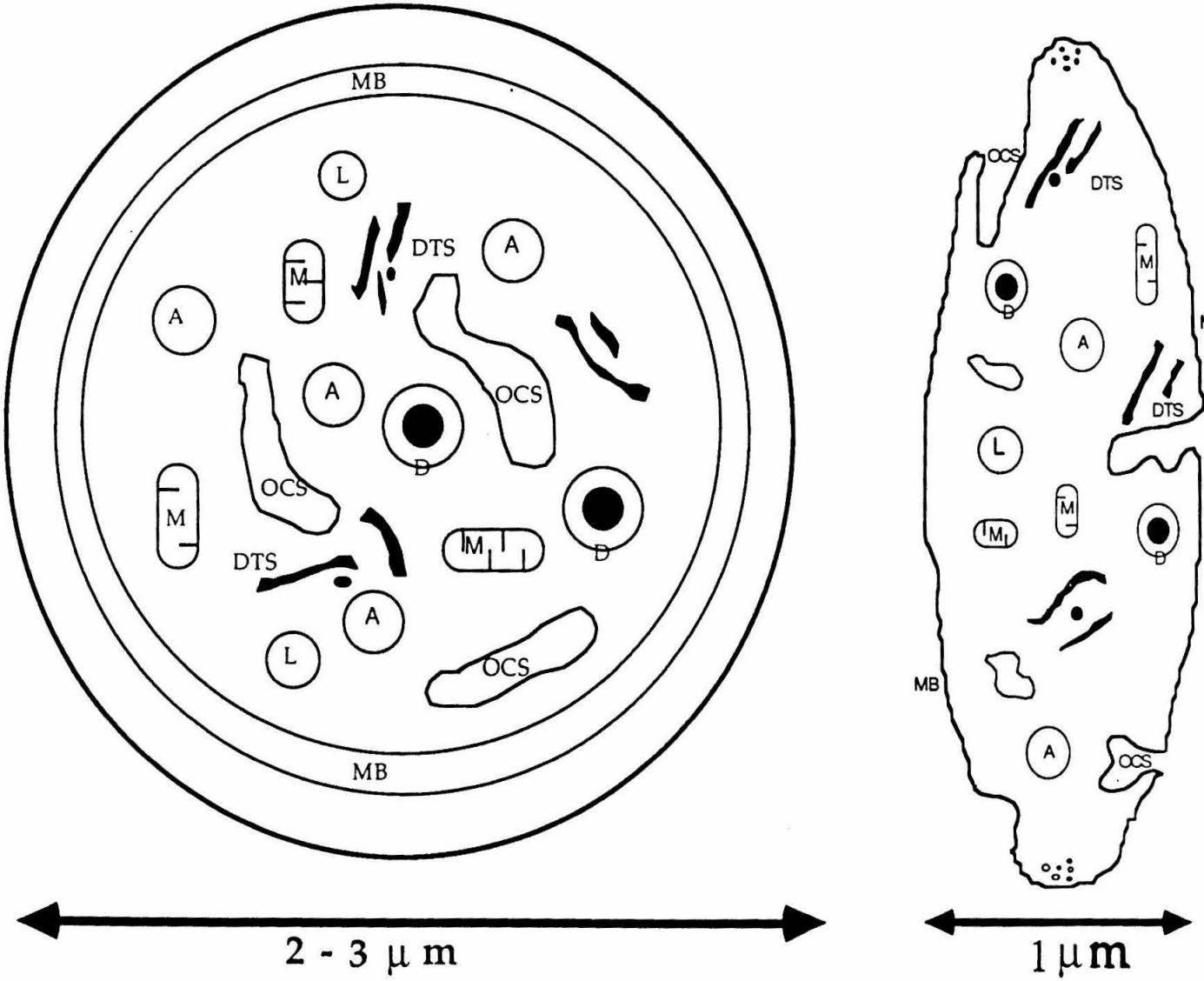
MB
L
DTS
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OCS
DTS
M
D
MB
A
DTS
L
M
M
D
MB
A
OCS
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Figure 1: The ultrastructure of a platelet. MB, microtubule. A, alpha granule. L, lysosome. D, dense body. M, mitochondria. OCS, open channel system. DTS, dense tubule system.

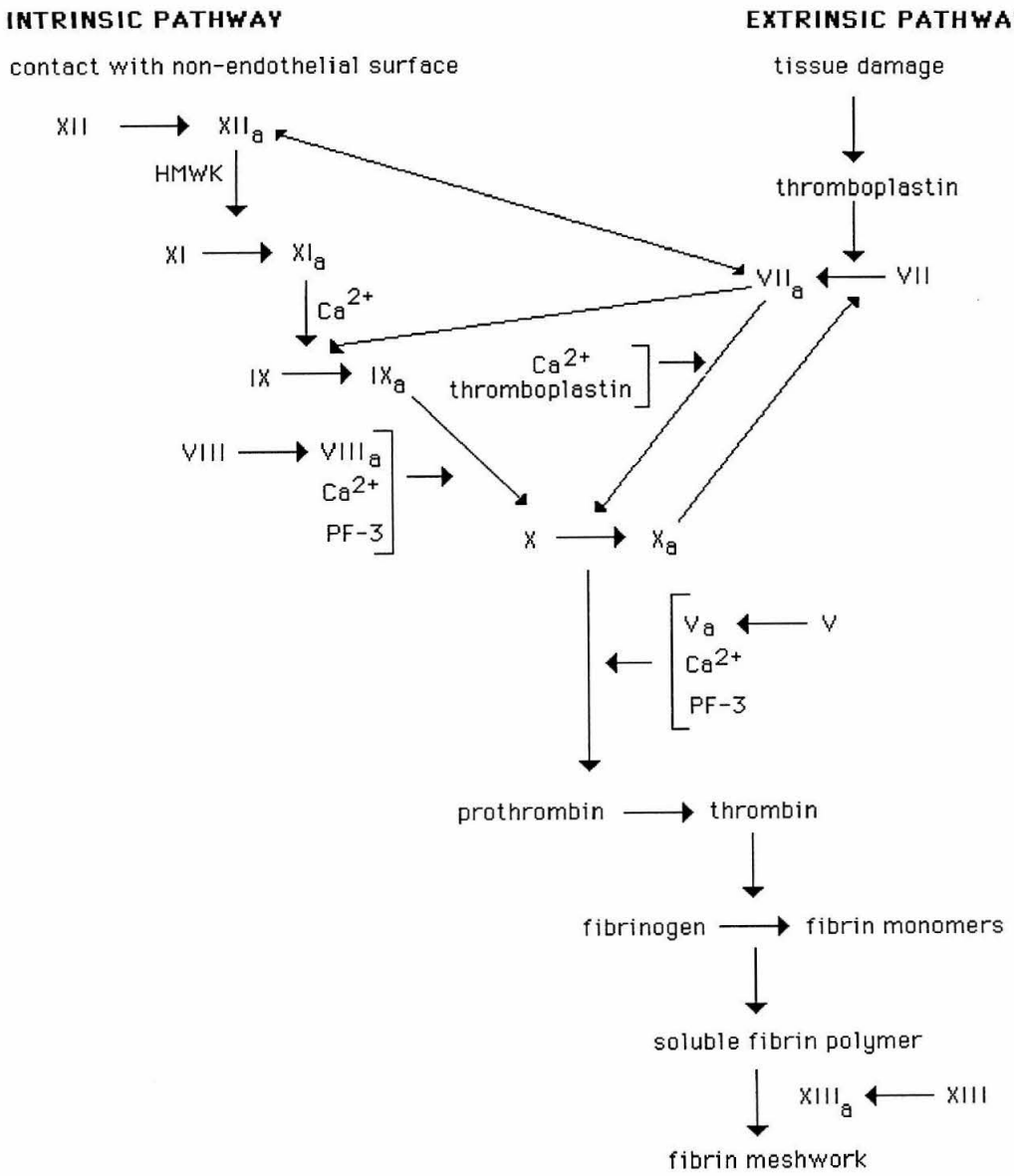
INTRINSIC PATHWAY
EXTRINSIC PATHWAY
contact with non-endothelial surface
tissue damage
XII
XIIa
HMWK
thromboplastin
XI
XIa
VIIa
VII
Ca2+
IX
IXa
Ca2+
thromboplastin
VIII
VIIIa
Ca2+
PF-3
X
Xa
Va
V
Ca2+
PF-3
prothrombin
thrombin
fibrinogen
fibrin monomers
soluble fibrin polymer
XIIIa
XIII
fibrin meshwork

Figure 2: The coagulation cascade. HMWK, high molecular weight kininogen. PF-3, platelet factor 3.

LIPOSOMES:

Introduction

In the past decade the use of liposomes as potential controlled drug delivery systems has gained wide interest (1,2). However, to date, in spite of some remarkable successes, the use of liposomes in this capacity has been limited.

Liposomes are microscopic structures consisting of one or more concentric lipid bilayers surrounding aqueous compartments. Liposomes form spontaneously when amphipathic lipids are dispersed in excess water (3). The lipid molecules arrange themselves such that their polar heads are towards the water phase and their apolar hydrocarbon constituents pack together to form a bilayer. Formation of stable liposomes from phospholipids is only possible at temperatures above the gel to liquid crystalline phase transition temperature (T_c). The T_c is dependent on the nature of the polar head group and the length and degree of unsaturation of the acyl chains. Above the T_c, phospholipids are in the liquid-crystalline phase which is characterized by an increase in the mobility of the acyl chains as compared to that in the gel-phase.

Multilamellar vesicles (MLV) are formed spontaneously upon hydration of dry phospholipid in excess water. These vesicles are very heterogeneous in diameter (up to several microns) and shape, but can be "sized" by extrusion through polycarbonate filters. Ultrasonication of MLV results in a population of significantly smaller (as small as 25 nm) homogeneous small unilamellar vesicles (SUV). Large unilamellar vesicles (LUV) of most lipid compositions can be prepared by

polycarbonate membrane extrusion or by reverse-phase evaporation as described by Szoka *et al.*(4). The relatively large aqueous volumes of MLV and LUV (~3 L/mol of lipid and 6 L/mol of lipid) versus SUV (~0.3 L/mol of lipid) make the former liposomes attractive for delivery of hydrophilic drugs. However, clearance of liposomes by the reticuloendothelial system (RES) is directly proportional to size.

Liposomes *In Vivo*

Three main routes of administration of liposomes exist: intravenous, intraperitonial and subcutaneous injection. Although the exact values of the circulation half-lives and organ distributions of the liposomes vary with the method of administration (5), the majority of liposomes are quickly cleared by the RES, especially by macrophages in the liver (Kupffer cells) and the spleen (6-8). Circulating monocytes have also been reported to take up significant numbers of liposomes (9). In addition to size, clearance is also dependent on the liposomes' composition. For example, neutral sphingomyelin/cholesterol vesicles have a circulation half-life of approximately 24 hours (10,11) whereas incorporation of a phosphatidylserine, a negatively charged lipid, increases the rate of RES uptake. The circulation times of liposomes can be improved by the addition of polyethylene glycol (PEG) or GM_1, a monosialic ganglioside, to the liposome formulation (12,13).

In addition to rapid clearance by the RES, the potential use of liposomes as drug delivery systems suffers from the instability of the liposomes in serum and the inability of liposomes to target to the desired cell types. During circulation within the bloodstream, liposomes may be

susceptible to the destabilizing effects of serum proteins and phospholipases, resulting in leakage of their encapsulated water-soluble compounds. High density lipoproteins (HDL) have been shown to penetrate the liposomal bilayer and induce leakage (14,15). This can be overcome to some extent by the addition of cholesterol, which improves retention by impeding penetration of the serum proteins (16). Targeting of liposomes to cells other than those in the RES and the blood has also presented several problems. This is primarily because the vesicles must first cross the capillary endothelium and the basement membrane and many cell types, including most tumor cells, display only very low endocytic capacities. Techniques to improve the targeting ability of liposomes include the addition of cell specific antibodies to the surface of the liposomes. However, use of these liposomes has met with limited success since they are still prone to RES uptake (17).

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SENDAI VIRUS:

Introduction

Sendai virus is a murine parainfluenza virus belonging to the genus *Paramyxovirus*, family Paramyxoviridae (1). Sendai virus particles are pleomorphic and range in size from 150 to 250 nm. The virions are bound by a lipid bilayer membrane which contains two surface glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F) which are anchored in the plasma membrane by hydrophobic transmembrane domains (2,3). The HN protein (M_r = 64000) is responsible for viral attachment and binds to cells via sialic acid containing receptors; predominantly gangliosides (4). HN also has hemagglutinin and neuraminidase activities, the clumping and aggregation of red blood cells and enzymatic cleavage of sialic acid, respectively. The F protein (M_r = 53000) penetrates the host cell and induces fusion.

In addition to the two surface glycoproteins, Sendai virus contains matrix protein (M), nucleocapsid protein (NP), and L and P proteins (Figure 1). M protein (M_r = 39500), a viral envelope protein, is thought to function in maturation and assembly of the virions in infected cells (5). NP, L and P are nucleocapsid proteins. NP (M_r = 57800) is the structural protein of the nucleocapsid and may play a role in packaging nucleocapsids into maturing virions (6,7). L (M_r = 255800) and P (M_r = 67500) appear to act cooperatively in viral RNA synthesis as well as in other enzymatic functions of the nucleocapsid including methylation, capping and polyadenylation of mRNA (6).

Reconstituted Sendai Virus Envelopes

Fusogenic reconstituted viral envelopes can be obtained by solubilization of the virus with Triton X-100 (8). Following addition of the detergent, the solubilized membrane is separated from the insoluble nucleocapsid by centrifugation. The remaining detergent is removed by dialysis or the addition of SM-2 Biobeads (8). Addition of aqueous compounds to the detergent solubilized-viral mixture results in their entrapment within the vesicles formed after the removal of the detergent. Lipophilic compounds added to the detergent-solbilized mixture are integrated into the reconstituted viral envelope. Subsequently, fusion of the RSVE with cells results in the injection of aqueous compounds into the cytoplasm and lipophilic compounds into the cells' plasma membranes (Figure 2). Compounds that have been successfully entrapped in RSVE and injected into cells include ferritin (9), ^{3}H-IgG (10) and DNA (9).

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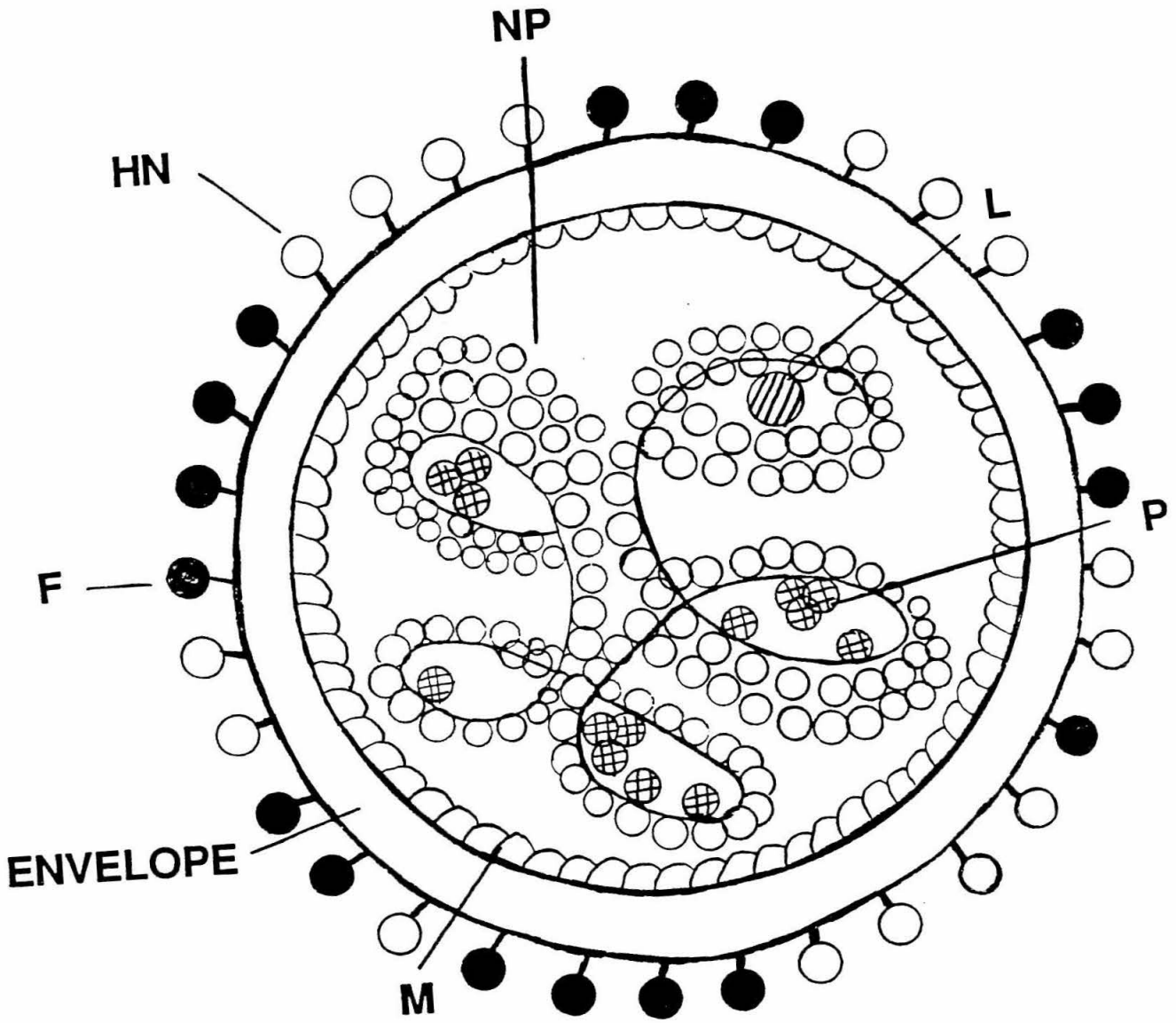
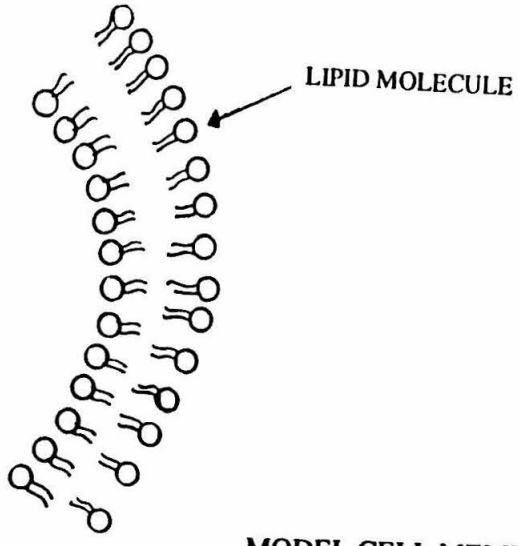
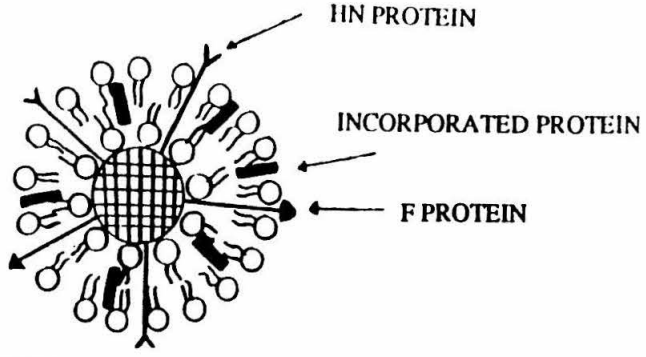

NP
HN
L
P
F
ENVELOPE
M

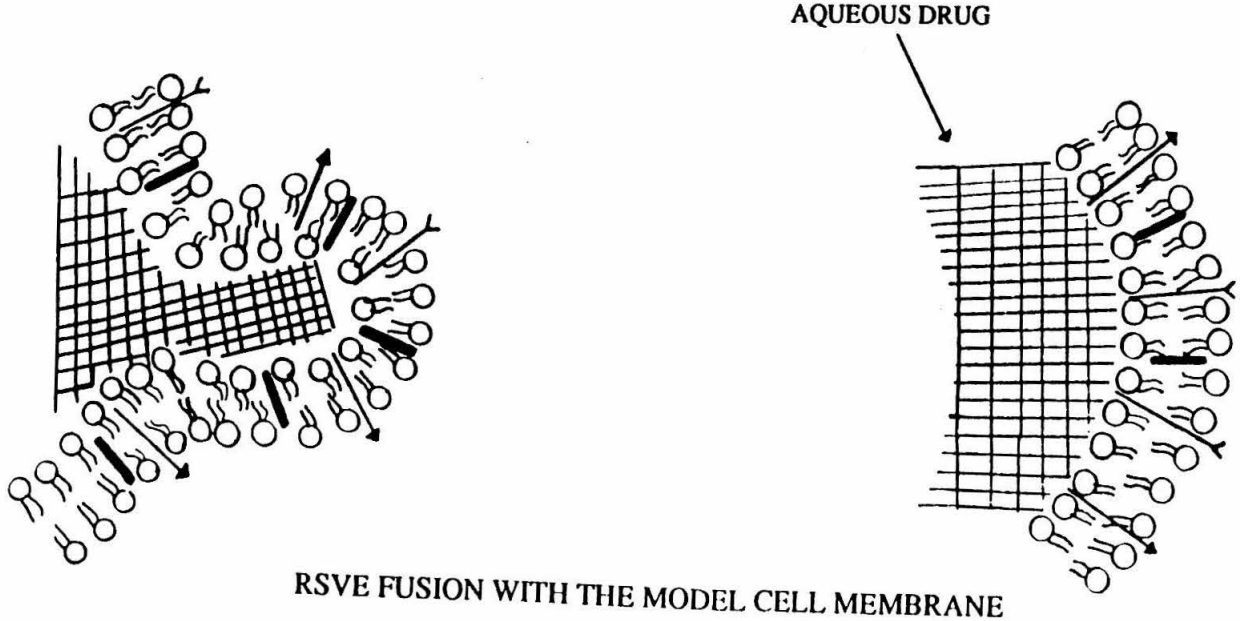
Figure 1: The ultrastructure of Sendai virus. NP, nucleocapsid protein. L, large protein. P, polymerase protein. M, matrix protein. F, fusion protein. HN, hemagglutinin-neuraminidase protein.



MODEL CELL MEMBRANE



RECONSTITUTED SENDAI VIRUS ENVELOPE (RSVE)
WITH INCORPORATED PROTEINS AND AQUEOUS DRUG



RSVE FUSION WITH THE MODEL CELL MEMBRANE

Figure 2: A model of the fusion of a reconstituted Sendai virus envelope with a cell membrane and the injection of incorported membrane proteins and aqueous drugs.

CHAPTER TWO

PHAGOCYTOSIS OF LIPOSOMES BY HUMAN PLATELETS

R. Male, W. E. Vannier and J. D. Baldeschwieler

ABSTRACT

We have shown that platelets are capable of phagocytosing liposomes rather than simply sequestering particles as previously postulated. Incubation of human platelets with small neutral unilamellar liposomes, ~ 74 nm, resulted in the uptake of the liposomes and retention of the lipid with rapid release of the aqueous phase components. The lipid label ^{3}H-cholesterylhexadecyl ether and water soluble ^{3}H-inulin were used to study the fate of the liposome components. Uptake of liposomes was proportional to the number of liposomes added and to the incubation time. Approximately 250 liposomes per platelet were taken up within a five hour incubation period. Uptake of the liposomes occurred through the open channel system, as evidenced by thin section electron microscopy, and was followed by accumulation and degradation in acid and esterase containing vesicles, as determined by changes in fluorescence of the pH-sensitive probe, pyranine (1-hydroxypyrene-3,6,8-trisulfonic acid), and hydrolysis of the cholesteryl[^{14}C]oleate membrane marker. Uptake was inhibited by the addition of EDTA, cytochalasin B or 2,4 dinitrophenol and iodoacetate to the media. Results from the serotonin release assay, microaggregation assay, fluorescein diacetate membrane integrity assay and electron microscopy indicate that neither the conditions for loading nor phagocytosis of liposomes significantly alters platelet function or morphology.

INTRODUCTION

Platelets have been shown to take up a variety of particles *in vitro* (1-6), and evidence is accumulating which suggests that removal of foreign materials from the blood may be an important physiologic role for these cells

(7-10). However, it remains to be definitively demonstrated whether platelets are truely phagocytic or simply sequester the particles within the open channel system.

In prior work, the platelet-latex sphere system has been used as a model for foreign particle ingestion by these cells (2,3,11-13). Various studies have shown progressive accumulation of latex spheres in the open channel system (OCS), a system of channels believed to be formed by invagination of the plasma membrane. Lewis *et al.* noted subsequent localization of the particles in acid phosphatase-positive electron-opaque vacuoles and concluded that platelets phagocytose latex particles (13). However, White *et al.* concluded that platelets sequester latex particles within membrane invaginations which are in communication with the exterior of the cell (12). We report the use of small neutral unilamellar liposomes to study the possible phagocytosis of particles by platelets. Liposomes have been used extensively to model microorganism interactions and, unlike latex beads, are more suitable for the study of uptake, metabolism and exocytosis.

MATERIALS AND METHODS

Purification of Platelets. Platelets were prepared using two methods; experiments measuring uptake using either platelet preparation gave similar results. Platelet-rich plasma packs obtained from the Red Cross were diluted in a 1:1 ratio with 0.9% saline solution. A volume of 4.5% dextran (molecular weight 250,000) in saline solution was added to an equal volume of the diluted packs and stirred; the red blood cells were allowed to sediment for 30 minutes at room temperature. Fifteen ml aliquots of the supernatant were pipetted into centrifuge tubes. Equal volumes of a solution containing 6.35%

Ficoll and 10% Hypaque w/w were gently layered on the bottom of the tubes. The tubes were spun at 400 g for 15 minutes at room temperature. The cloudy band containing the platelets and lymphocytes located directly above the Ficoll-Hypaque gradient was gently removed, diluted in a 1:1 ratio with saline solution and centrifuged at 300 g for 15 minutes at room temperature to sediment the lymphocytes. The supernatant was spun at 800 g for 20 minutes; the platelet pellet formed was washed twice in saline solution and finally resuspended in Hanks' Balanced Salt Solution (HBSS).

Platelets purchased from the American Blood Institute (Los Angeles) were centrifuged at 1000 g for 15 minutes in an IEC Centra-8 centrifuge. The pellet was resuspended in either 0.9% saline solution or Tyrodes buffer and spun at 110 g for 5 minutes to remove excess red blood cells. The supernatant was spun at 450 g for 10 minutes. The pellet was resuspended in HBSS or modified Tyrodes buffer (Tyrodes buffer plus 1.26 mM of $CaCl_2$).

The final purities of both platelet preparations were greater than 95% platelets as assessed by a Colter S+4 Cell Counter; the main contaminants were red blood cells. Platelets were used within 24 hours of blood collection.

Determination of the Number of Platelets per mg of Protein. Cell counts were performed with a Colter S+4 Cell Counter to determine the number of platelets. The Peterson modification of the Lowry protein assay (14) with a bovine serum albumin standard and without TCA precipitation was used to determine the amounts of protein in the same samples. A milligram of protein was found to correspond to 3×10^8 platelets.

Preparation of the Liposomes. *^{3}H-cholesterylhexadecyl ether Labeled Liposomes:* A 2:1 mole ratio mixture of distearoylphosphatidylcholine (Avanti Polar-Lipids, Inc.) and cholesterol (cell culture tested Sigma Chemical Co.) in

chloroform was made such that the total lipid content was approximately 20 mg. Five μCi of ^{3}H-cholesterylhexadecyl ether (New England Nuclear) in chloroform was also added. The solution was then taken to dryness in a 100 ml round bottom flask with a Büchler "Rotovap" apparatus and was dried under vacuum overnight. The resulting phospholipid mixture was resuspended in 5 mL of PBS (0.90% NaCl: 0.12% $Na_2HPO_4 \cdot 7H_2O$: 0.013% $NaH_2PO_4 \cdot H_2O$ w/w in water, pH 7.3), vortexed and 2.5 ml portions probe sonicated for 15 minutes using a Heat Systems-Ultrasonics, Inc. sonicator with a microprobe.

^{3}H-inulin Labeled Liposomes. The liposomes were prepared exactly as described except that no ^{3}H-cholesterylhexadecyl ether was added and the resuspension buffer contained 5 μCi of ^{3}H-inulin (Amersham, Corp.). Excess ^{3}H-inulin, (molecular weight 5,200) was separated from the liposomes by gel filtration with a Sephadex G-50-100 column.

Non-radiolabeled Liposomes. The liposomes were prepared as described but without the tritiated labels.

Pyranine (1-hydroxypyrene-3,6,8-trisulfonic acid, HPTS) Labeled Liposomes. The liposomes were prepared as described above except that the resuspension solution was 35 mM in HPTS (Molecular Probes) and 75 mM in NaCl. Excess HPTS was removed by gel filtration with a Sephadex G-50-100 column equilibrated with 150 mM NaCl.

Cholesteryl[^{14}C]oleate Labeled Liposomes. The liposomes were prepared as described for the ^{3}H-cholesterylhexadecyl ether labeled liposomes except 5 μCi of cholesteryl[^{14}C]oleate (Amersham, Corp.) in chloroform was added instead of ^{3}H-cholesterylhexadecyl ether.

Incubation Procedure. The platelets and liposomes were incubated at 37°C in 1.5 mL polypropylene centrifuge tubes for the desired incubation times after which the mixtures were centrifuged at 5700 g for 5 minutes in a Beckman Microfuge 11. The pellets were washed twice in buffer and finally resuspended in 0.5 M NaOH/0.5% SDS and incubated overnight at room temperature. The solution was transferred to liquid scintillation vials with 10 ml of Safety Solve (Research Products, Inc.), mixed and allowed to sit at least two hours prior to scintillation counting. For each experiment all samples were done in duplicate; the number of independent experiments, n, are noted in the figure captions or in the text. Bars on the graphs represent the range of the data.

Exocytosis Studies. Platelets and liposomes were incubated as above for 12 hours. The mixtures were centrifuged and the platelets washed by centrifugation twice in buffer to remove excess liposomes and further incubated at 37°C for varying periods of time. Both the pellets and supernatants were collected by centrifugation, solubilized in the NaOH/SDS solution overnight and counted.

Determination of Liposome Diameters and Estimation of Liposome Numbers. The liposome diameters for different preparations were determined by photon correlation spectroscopy at 480 nm using a Malvern Instruments PCS 100 system. The numbers of ^{3}H-cholesterylhexadecyl ether labeled liposomes were calculated by assuming that the counts per weight of lipid were constant for all preparations, the average surface area for a phospholipid molecule is 0.7 nm^2 (15) and the average molecular weight for the lipid is 656 g/mole. The numbers of ^{3}H-inulin labeled and non-radiolabeled liposomes were determined by performing Böttcher phosphate

assays (16) on the samples and assuming a 0.7 nm^2 phospholipid surface area. The light scattering data indicated that the liposomes diameters were 65 to 83 nm. The number of liposomes reported have all been normalized assuming that the diameters of the liposomes for all experiments were 74 nm.

Fluorimetry of HPTS. After incubation with HPTS labeled liposomes for various times, the cells were washed twice with buffer. Fluorescence excitation spectra (λ_{ex} 395-465 nm, 4 nm bandwidth) were measured at 510 nm emission (4 nm bandwidth) using a SLM 4800 (SLM Instruments, Inc.) outfitted with a stirred, temperature controlled cuvette (20°C). HPTS is a pH-dependent dye which exhibits two major fluorescence maxima (403 and 460 nm) which have a complementary pH dependence in the range 5-9; the peak at 403 nm is maximal at low pH values while the peak at 460 nm is maximal at high pH values. The fluorescence at 413 nm is relatively pH-independent and is used to standardize the concentration of dye associated with the cells. The fraction of liposomes taken up by phagocytosis and delivered to an acidic, pH 6.0, environment was calculated using the 460/413 nm ratio and the equation:

Fraction phagocytosed = $(ratio_{pH\ 7.4} - ratio_{meas.})/(ratio_{pH\ 7.4} - ratio_{pH\ 6.0})$

where $ratio_{meas.}$ is the 460/413 ratio of the liposome treated cells and $ratio_{pH\ 7.4}$ and $ratio_{pH\ 6.0}$ are the 460/413 ratios of liposomes in buffer and acidified buffer respectively (17). In the original paper by Daleke *et al.*, the second peak occurred at 450 nm; however, according to our data the peak maximum is at 460 nm.

Intralysosomal Degradation of the Cholesteryl[^{14}C]oleate Labeled Liposomes. 3 x 10^8 Platelets were preincubated with 7 x 10^{11} of ^{3}H-cholesterylhexadecyl ether or cholesteryl[^{14}C]oleate labeled liposomes for 0.5

hour at 37°C. The mixtures were washed twice in buffer by centrifugation. The pellets were collected, resuspended in buffer and incubated for 10 to 60 minutes at 37°C. Following incubation, the mixtures were centrifuged to collect the pellets that were then solubilized in NaOH/SDS solution overnight and counted.

Upon intralysosomal degradation cholesteryl[^{14}C]oleate is hydrolysed to form [^{14}C]oleate which is released from cells. Conversely, ^{3}H-cholesterylhexadecyl ether is retained within the cells. Therefore, by monitoring the ^{3}H/^{14}C ratio of the pellets, the degree of degradation can be determined (18).

Thin Section Electron Microscopy. Samples of 3 x 10^{8} platelets were incubated with 7 x 10^{11} non-radiolabeled liposomes for 6 hours at 37°C , fixed in standard fashion and embedded in LR white.

In addition, platelets suspended in HBSS or modified Tyrodes buffer incubated with or without liposomes for 1 hour at 37°C, prepared as above, were studied to determine changes in platelet morphology under the conditions used for studying the liposome interaction.

***In vitro* Studies to Assess Platelet Function.** *Serotonin Release Assay.* The serotonin assay was performed with 3 x 10^{8} platelets, suspended in HBSS or modified Tyrodes and incubated with or without 1.4 x 10^{12} liposomes for one to nine hours, as described (19).

Microaggregation Assay. Platelets suspended in HBSS or modified Tyrodes solution were incubated at 37°C, with or without liposomes, for one to nine hours and then fixed with 1% glutaraldehyde. The percentages of aggregated platelets were determined by examining the cells in a hemacytometer.

Fluorescein Diacetate Assay. After incubation of platelets suspended in HBSS or modified Tyrodes solution at 37°C, with or without liposomes, for one to nine hours, fluorescein diacetate (Sigma Chemical Co.) was added and incubated (final concentration 4 μg/mL) with the cells for 10 minutes at room temperature. The platelets were then kept on ice for 1 minute and pelleted at 4°C. The fluorescence was measured at 520 nm emission while exciting at 489 nm.

FDA is a non-fluorescent probe which readily crosses the cell membrane. Within the cell the probe is hydrolysed by esterases to form fluorescent fluorescein which does not readily traverse the cell membrane at 4°C but can leak out of the cells through membrane lesions. Therefore the leakage of fluorescein is a measure of loss of membrane integrity (20).

RESULTS

^{3}H-cholesterylhexadecyl ether Labeled Liposome Uptake. In order to model the liposome uptake by platelets, ^{3}H-cholesterylhexadecyl ether labeled liposome uptake was studied for varying incubation times and incubation concentrations of both the liposomes and platelets. Platelet uptake of ^{3}H-cholesterylhexadecyl ether labeled liposomes increased with increasing incubation times (Fig. 1). After incubation of 3×10^8 platelets with 7×10^{11} liposomes for five hours, approximately 250 liposomes were associated with each platelet. Liposome uptake by platelets also increased with increasing numbers of incubated liposomes; two incubation times are shown (Fig. 2). Incubation for 12.5 hours resulted in greater uptake values than the 2.0 hour incubation. As the number of platelets incubated with a constant amount of liposomes was increased, the overall number of liposomes incorporated

increased; however the ratio of liposomes to platelets remained constant at approximately 475 liposomes/platelet for a 12.0 hour incubation period (Fig. 3).

^{3}H-inulin Labeled Liposome Uptake. Uptake of the aqueous components of the liposomes was studied by following the uptake of ^{3}H-inulin labeled liposomes versus varying incubation times and liposome concentrations. Although the number of ^{3}H-inulin labeled liposomes taken up increased with time (Fig. 4), the kinetics of uptake were different than that for the ^{3}H-cholesterylhexadecyl ether labeled liposomes. Incubation of 2×10^{12} liposomes with 3×10^{8} platelets resulted in the uptake of approximately 150 liposomes per platelet after five hours. The uptake of ^{3}H-inulin labeled liposomes versus the concentration of liposomes incubated is shown for two different incubation times (Fig. 5). Uptake was proportional to both the number of liposomes incubated and the incubation time. The difference in kinetics of uptake as assessed by the lipid label and the aqueous label suggests that there is a difference in the fate of the lipid and aqueous components after platelet uptake. Therefore the values reported for the uptake of the ^{3}H-inulin labeled liposomes are given as "apparent liposomes per platelet".

Release of the Tritiated Radiolabels. To study the possible exocytosis of the liposomes, the amounts released of the tritiated radiolabels, ^{3}H-cholesterylhexadecyl ether and ^{3}H-inulin probes were examined (Fig. 6). After 12 hours no membrane associated probe was released. However, up to 60% of the aqueous phase probe was released during this period.

HPTS Labeled Liposome Uptake. In order to determine the percentage of liposomes phagocytosed and delivered to acidic compartments, the uptake of

fluorescently labeled (HPTS) liposomes was measured by fluorimetry versus time (Fig. 7). Initially the liposomes were associated with the platelets, either bound to the surface or contained in the OCS, but were not phagocytosed. Within minutes the liposomes began to accumulate in acid-containing vesicles. The percentage of platelet associated liposomes phagocytosed increased to 80% within one hour.

Cholesteryl[^{14}C]oleate Labeled Liposome Degradation. Liposome degradation was monitored by studying the time dependence of the relative abundances of ^{3}H and ^{14}C within the platelets after preincubating the cells with ^{3}H-cholesterylhexadecyl ether or cholesteryl[^{14}C]oleate labeled liposomes for half an hour, washing the pellets twice and resuspending in buffer (n=10). During a sixty minute time period, the ^{3}H/^{14}C ratios within the cells increased linearly from 1.00 at 0 min., 1.028 at 10 min., 1.130 at 20 min., 1.212 at 30 min, 1.246 at 50 min. to 1.294 at 60 min. ^{3}H-cholesterylhexadecyl ether was retained within the platelets; however the cholesteryl[^{14}C]oleate was hydrolysed to form [^{14}C]oleate which was released by the cells. Therefore the increase in the ^{3}H/^{14}C ratio in the cells was indicative of degradation of the liposomes. The amount of liposome degradation is probably underestimated since the incubations were done in the absence of serum which is unfavorable for the release of oleate (21).

Electron Microscopy. Electron microscopy was performed to study the effects of HBSS and modified Tyrodes solution and the phagocytosis of liposomes on platelet morphology (n=4). The effects of incubation of up to 2.1×10^{12} liposomes were studied.

Platelets maintained a discoid shape when suspended in modified Tyrodes buffer. However, platelets suspended in HBSS tended to have

irregular shapes and approximately half had pseudopodia. Therefore we believe that incubation in modified Tyrodes buffer is superior. Incubation with liposomes did not significantly alter platelet morphology.

Competitive Assay. A competitive assay was performed to establish whether liposome uptake could be saturated. Preincubation of platelets with 0 to 3.4 x 10^{12} non-radiolabeled liposomes prior to two centrifugation washes and incubation with 7.9 x 10^{11} tritiated liposomes for preincubation times of 0.5, 2 and 6 hours and incubation times of 0.17, 0.5, 2 and 6 hours gave similar results (data not shown, n=4) The amount of ^{3}H-cholesterylhexadecyl ether liposomes taken up was independent of the amount of non-radiolabeled liposomes previously added. In addition, uptake was relatively independent of the preincubation time while uptake increased with increasing incubation times. Simultaneous incubation of non-radiolabeled and ^{3}H-labeled liposomes resulted in a linear decrease in uptake of the ^{3}H-labeled liposomes as expected.

Inhibition of Liposome Uptake. The inhibitory effects of a variety of reagents known to decrease platelet uptake of latex spheres were examined during platelet incubation with liposomes. Incubation for 12 hours in the presence of 5mM EDTA inhibited the uptake of liposomes by 48±9%; however, incubation for 1 hour showed no significant inhibition of uptake (n=10). The addition of 10 μg/ml of cytochalasin B (n=8) or 2.5 x 10^{-4} M 2,4-dinitrophenol with 5 x 10^{-5} M iodoacetate (n=8) also inhibited uptake by 46±16% and 66±12% respectively.

***In vitro* Assays to Assess Platelet Function.** Although the numbers of liposomes phagocytosed by platelets suspended in HBSS or modified Tyrodes buffer are identical, the *in vitro* assays showed that platelets, incubated with

or without liposomes, spontaneously secrete serotonin, aggregate and lose membrane integrity more while suspended in HBSS. However, phagocytosis of liposomes did not significantly alter platelet secretion of serotonin or induce additional platelet aggregation or loss of membrane integrity. This was not surprising since it had been previously shown that the uptake of "Intralipid", a lipid emulsion used in treatment of patients with deficient caloric intake, does not increase platelet aggregation or induce morphological changes (22).

DISCUSSION

We have determined that platelets are capable of phagocytosing small unilamellar liposomes rather than sequestering particles as previously postulated (12). Liposomes are taken up through the OCS, localize within acid and esterase containing vesicles and are degraded. The lipid is retained; the aqueous components are exocytosed. Therefore all uptake values reported using ^{3}H-inulin labeled liposomes really reflect a consecutive first order kinetic scheme of uptake and exocytosis.

The values reported in this paper for platelet uptake do not differentiate between incorporated and bound liposomes. However, according to EM micrographs, the vast majority of the liposomes appear to be within the OCS. In addition the use of a pH-dependent fluorescent liposome probe confirms that a substantial portion of the liposomes are phagocytosed and are in acid-containing compartments. These data suggest that the liposomes are incorporated in surface invaginations which pinch off their connections to the cell membrane and migrate into the cytoplasm as sealed vesicles, rather than being sequestered in the OCS and in contact with the outside media.

Degradation of the liposomes, which probably occurs in the acid-phosphatase and esterase containing vacuoles, has also been demonstrated by utilizing cholesteryl[^{14}C]oleate labeled liposomes. The mechanism for the release of the aqueous components to the outside of the cell probably involves the migration of these vacuoles to the periphery of the cell and exocytosis of their contents; however, this has not been confirmed.

Although the space available for uptake within the cell must be limited, the exocytosis of the aqueous components of the liposomes increases the amount of liposome lipid capable of being stored as detected by the ^{3}H-cholesterylhexadecyl ether label. With the amounts of liposomes and platelets used and on the time scale of the preincubation experiments, the space available for uptake is not saturable. The uptake is independent of the amount of non-radiolabeled liposomes added and the preincubation times. Simultaneous incubation of non-radiolabeled and radiolabeled liposomes demonstrates a dilution effect, which results in decreased uptake of the radiolabeled liposomes.

According to Mustard *et al.* the addition of EDTA inhibits phagocytosis of particles and platelet aggregation, but does not interfere with particle adherence (3). Liposome uptake is inhibited by the presence of EDTA at long time intervals but not at short intervals when the predominant process of uptake in the absence of Ca^{2+} may be binding. This evidence suggests that divalent cations are involved in the phagocytosis process; however, the effects of different divalent cations have not been investigated. The inhibitory effect of cytochalasin B with respect to platelet uptake has been attributed to its effect on microfilaments and the plasticity of the platelet membrane (23). The addition of 2,4-dinitrophenol and iodoacetate prior to incubation blocks

aggregation and phagocytosis by interfering with glycolysis and oxidative phosphorylation (2). Liposome uptake is Ca^{2+} dependent; however, platelets tend to aggregate and secrete more in the presence of Ca^{2+}. Therefore we studied the effects of HBSS and modified Tyrodes buffer on the morphology and function of platelets which both contain 1.26 mM $CaCl_2$. The serotonin release assay, microaggregation assay and FDA membrane integrity assay all show that platelet function and morphology are better preserved in modified Tyrodes than in HBSS. However, even in modified Tyrodes buffer, the loss of membrane integrity is significant. This loss may be due to incubation of the platelets in small polypropylene centrifuge tubes at 37°C. It is preferable to store platelets at room temperature, but phagocytosis is maximized at 37°C. The assays also indicated that phagocytosis of liposomes does not adversely affect the platelets.

Although we began this research to define the mechanisms involved in platelet-liposome interactions, we realize the ability to encapsulate a variety of drugs or radioactive labels *in vitro* in platelets and to target specific locations *in vivo* such as areas of inflammation or infection, neoplastic tumor cells or thrombosis would be of great medical importance. The exocytosis process is comparatively rapid, the half life of the aqueous compartment calculated assuming a first order reaction is 3.9 hours, therefore it may be necessary to use inhibitors which would slow the digestion of the liposomes or the release of the liposomes' aqueous components. Alternatively, lipid analogs of the drugs could be used for longer platelet retention. In some cases rapid release of drugs from the aqueous compartments may be desirable. An example of this might be the delivery of thrombolytic agents to a coronary thrombus in the initial treatment of myocardial infarction.

ACKNOWLEDGMENTS

We are indebted to Dr. Dudley Moon, at Albany College of Pharmacy, and Dr. Pramod Lad and his staff, at the Kaiser Regional Research Laboratory, Los Angeles, for their advice during the course of the work. We thank Dr. G. L. Scherphof for suggesting the use of the cholesteryl[^{14}C]oleate hydrolysis method. Funding for this work was provided by Army Research Office grant #DAAL-03-87-K-0044, and the Caltech Consortium in Chemistry and Chemical Engineering; Founding Members: E.I. du Pont de Nemours and Company, Inc., Eastman Kodak Company, Minnesota Mining and Manufacturing Company, Shell Oil Company Foundation.

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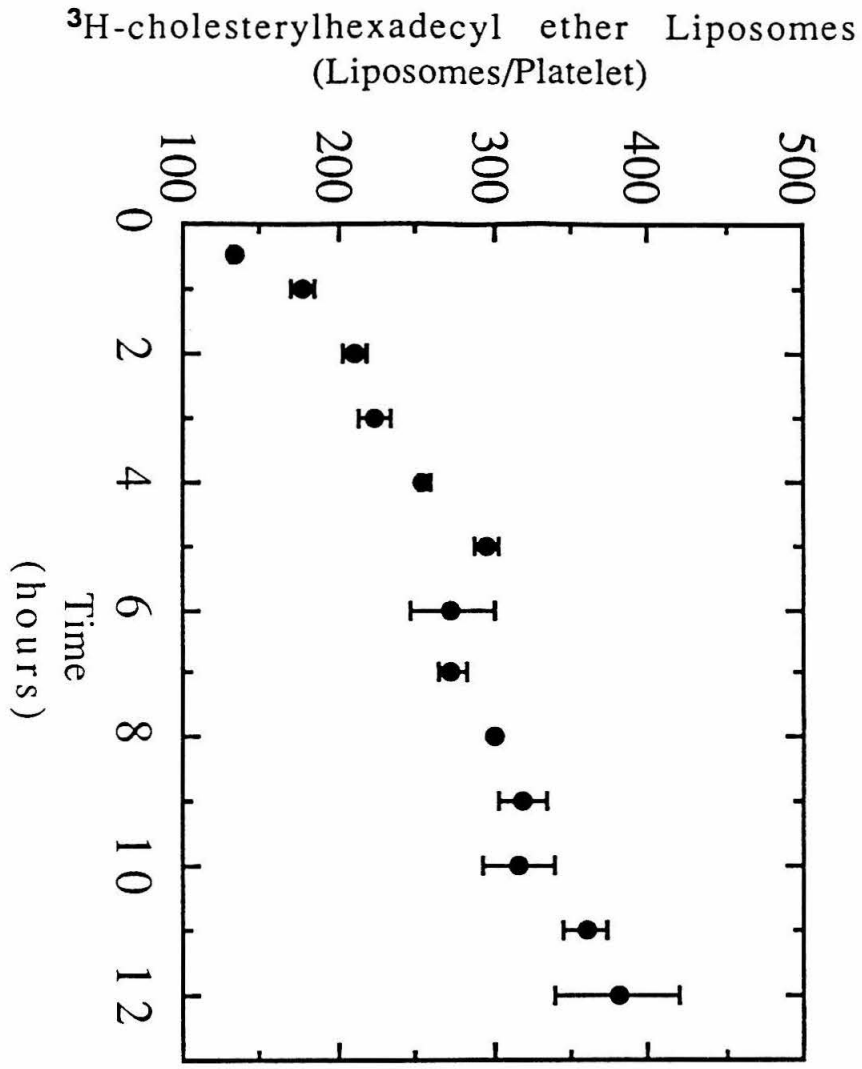
^{3}H-cholesterylhexadecyl ether Liposomes
(Liposomes/Platelet)
100
200
300
400
500
0
2
4
6
8
10
12
Time
(hours)

Figure 1: Incubation time dependence of platelet uptake of ^{3}H-cholesterylhexadecyl ether labeled liposomes. 3 x 10^{8} platelets were incubated with 7 x 10^{11} ^{3}H-cholesterylhexadecyl ether labeled liposomes at 37^{o}C for varying incubation times (n=8).

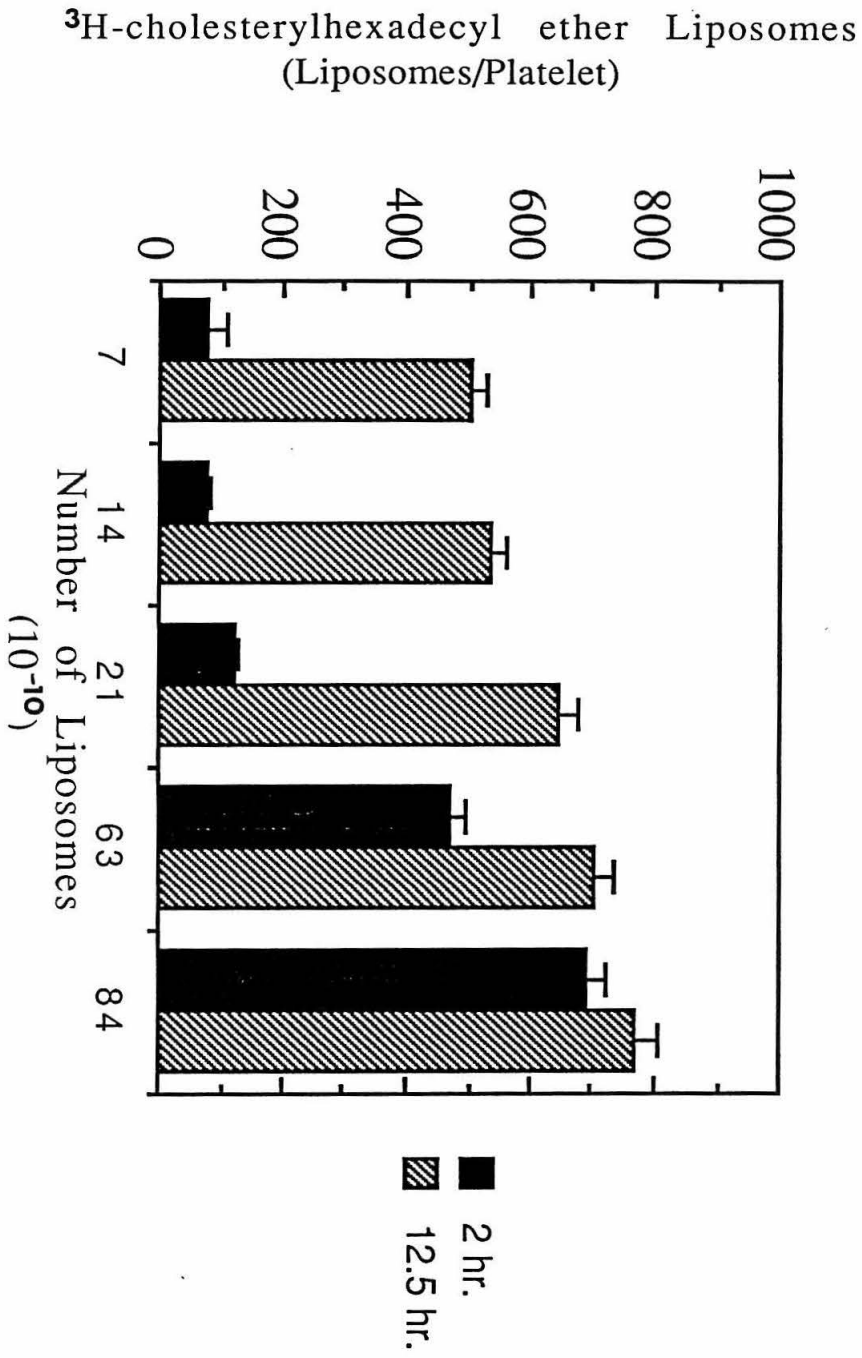
^{3}H-cholesterylhexadecyl ether Liposomes
(Liposomes/Platelet)
0
200
400
600
800
1000
7
14
21
63
84
Number of Liposomes
(10^{-10})
2 hr.
12.5 hr.

Figure 2: Liposome concentration dependence of platelet uptake of ^{3}H-cholesterylhexadecyl ether labeled liposomes. 3 x 10^{8} platelets were incubated with varying amounts of ^{3}H-cholesterylhexadecyl ether labeled liposomes at 37°C for 2 hours (■) or 12.5 hours (❑) (n=10 for 2 hr, n=6 for 10 hr).

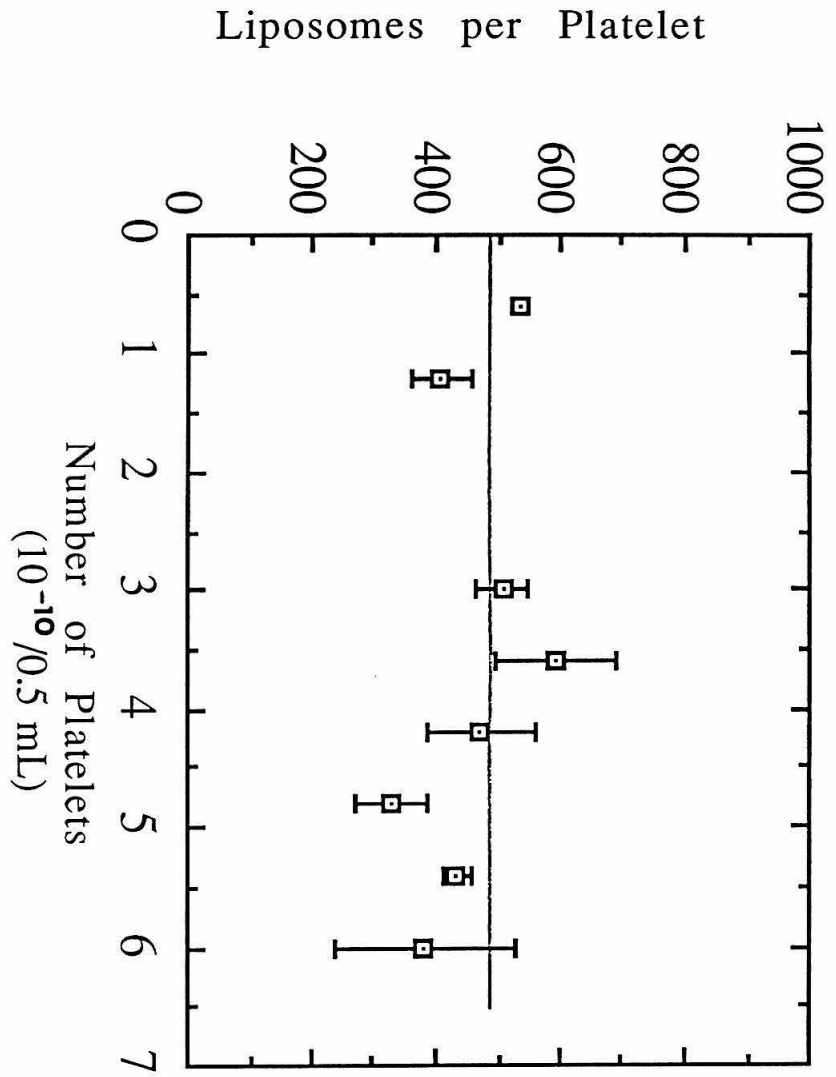
Liposomes per Platelet
0
200
400
600
800
1000
0 1 2 3 4 5 6 7
Number of Platelets
(10-10/0.5 mL)

Figure 3: Platelet concentration dependence of platelet uptake of ^{3}H-cholesterylhexadecyl ether labeled liposomes. 8.3 x 10^{11} ^{3}H-cholesterylhexadecyl ether labeled liposomes were incubated with varying numbers of platelets (0.5 mL final volume) at 37°C for 2.0 hours. The line representing 475 liposomes per platelet is shown (n=6).

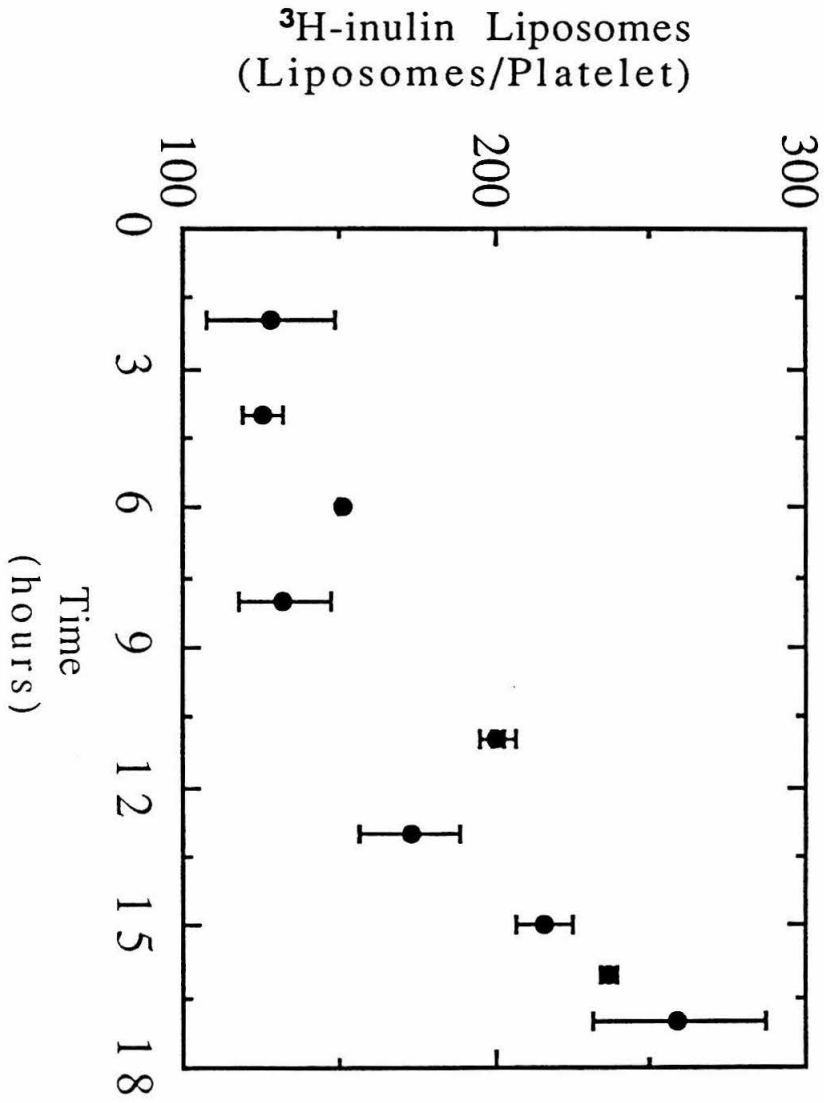
^{3}H-inulin Liposomes
(Liposomes/Platelet)
100
200
300
0
3
6
9
12
15
18
Time
(hours)

Figure 4: Incubation time dependence of platelet uptake of ^{3}H-inulin labeled liposomes. 3×10^{8} platelets were incubated with 2×10^{12} ^{3}H-inulin labeled liposomes at 37°C for varying incubation times (n=6).

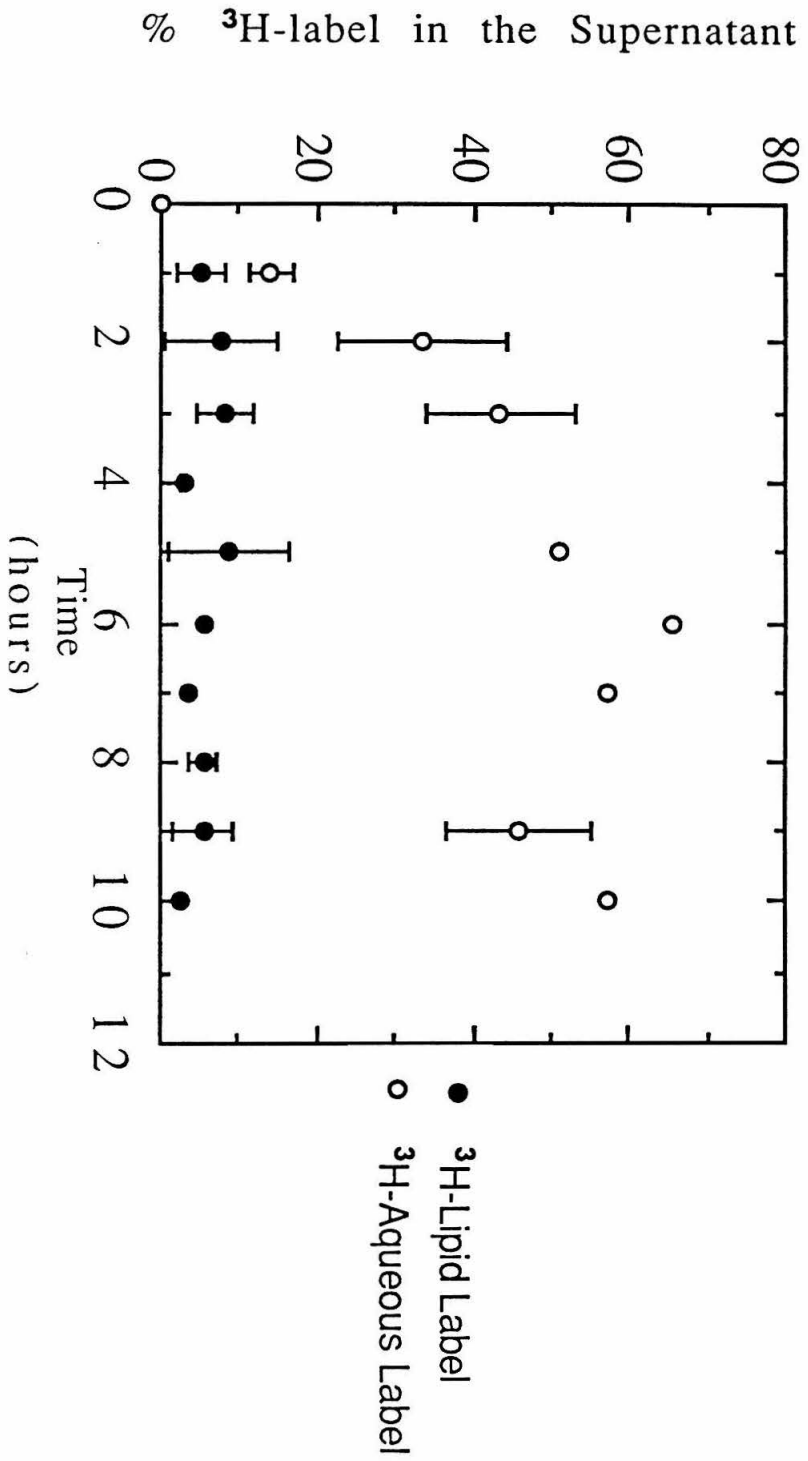
% ³H-label in the Supernatant
0
20
40
60
80
0
2
4
6
8
10
12
Time
(hours)
³H-Lipid Label
³H-Aqueous Label

Figure 5: Liposome concentration dependence of platelet uptake of ^{3}H-inulin labeled liposomes. 3 x 10^{8} platelets were incubated with varying amounts of ^{3}H-inulin labeled liposomes at 37°C for 6.0 hours (■) and 12.5 hours (❑) (n=6).

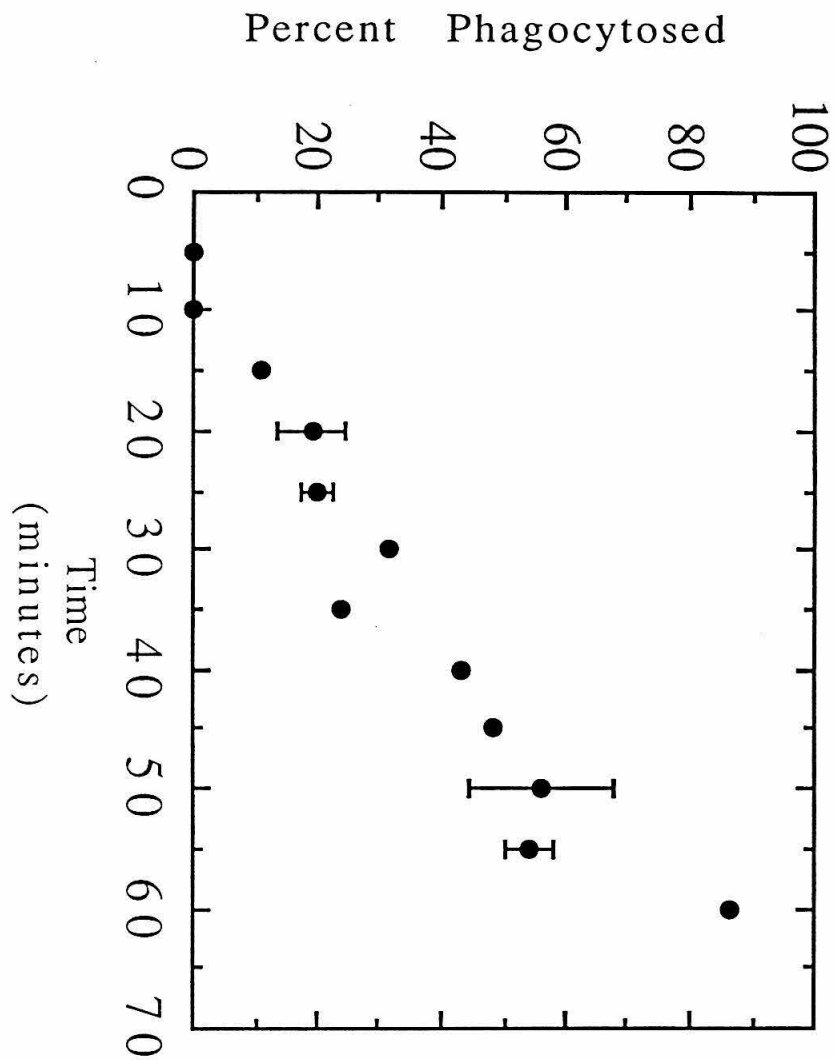
Percent Phagocytosed
0
20
40
60
80
100
0 10 20 30 40 50 60 70
Time
(minutes)

Figure 6: Release studies of the lipid and aqueous probes. 3×10^8 platelets were preincubated with 8.3×10^{11} ^{3}H-cholesterylhexadecyl ether labeled (●) or 2×10^{12} ^{3}H-inulin labeled (○) liposomes at 37°C for 12.0 hours. The cells were then pelleted, washed twice in buffer, resuspended in buffer and incubated for various incubation times at 37°C. The percents of the ^{3}H-cholesteryl- hexadecyl ether and the ^{3}H-inulin labels in the supernatants were determined (n=6).

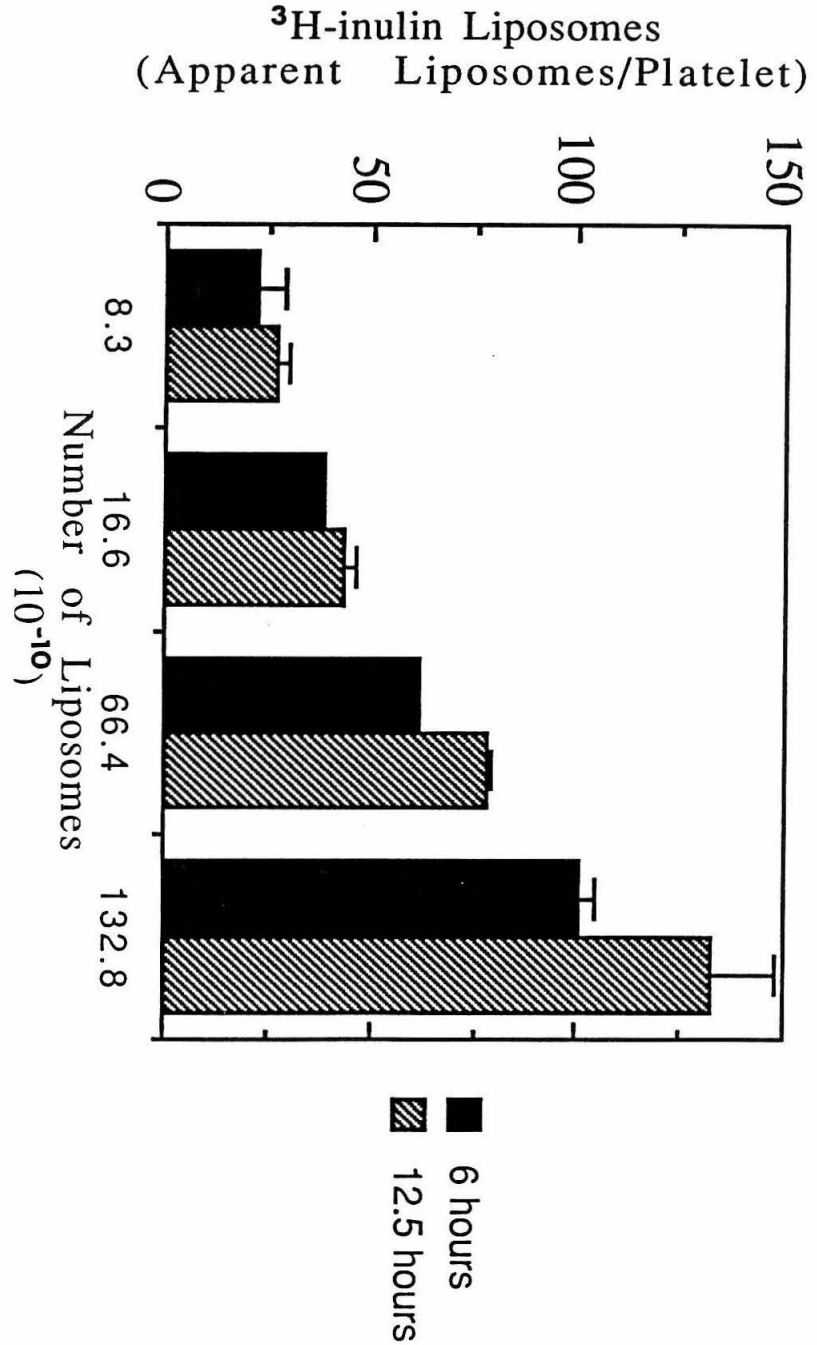
^{3}H-inulin Liposomes
(Apparent Liposomes/Platelet)
0
50
100
150
8.3
16.6
66.4
132.8
Number of Liposomes
(10^{-10})
6 hours
12.5 hours

Figure 7: Incubation time dependence of the percentage of platelet associated HPTS labeled liposomes phagocytosed. 3×10^8 platelets were incubated with 7×10^{11} HPTS labeled liposomes at 37°C for varying incubation times. The cells were then pelleted, washed twice in buffer and resuspended in buffer. The percentages of platelet-associated liposomes phagocytosed were calculated (n=6).

CHAPTER THREE

KINETICS OF LIPOSOME UPTAKE BY HUMAN PLATELETS

R. Male, W. E. Vannier, J. D. Baldeschwieler

ABSTRACT

Distearoylphoshatidylcholine:cholesterol (2:1 mole ratio) small unilmallar vesicles are taken up intact by human platelets with a rate constant of $k_1 = 7.1 \times 10^{-3}$ hr^{-1} M^{-1}. The lipid is retained; the aqueous components are exocytosed with a rate constant of $k_2 = 1.8 \times 10^{-1}$ sec^{-1}.

INTRODUCTION

The uptake of ^{3}H-cholesterylhexadecyl ether labeled (lipid label) and ^{3}H-inulin labeled (aqueous-phase label) DSPC:cholesterol (2:1 mole ratio) liposomes by human platelets was studied (see Chapter 2). Uptake of both types of liposomes was linear with time and liposome incubation concentration. However, uptake of the ^{3}H-cholesterylhexadecyl ether labeled liposomes was greater than that of the ^{3}H-inulin labeled liposomes. These data suggested that the intracellular fates of the lipid and aqueous components might be different. Therefore the uptake of pyranine labeled and cholesteryl[^{14}C]oleate labeled liposomes was examined to detect possible lysosomal uptake and degradation of the liposomes. Pyranine is a pH-dependent dye that exhibits two major fluorescence maxima, which have a complementary pH dependence in the range pH=5-9 (1). Upon intralysosomal degradation, cholesteryl[^{14}C]oleate is hydrolyzed to form [^{14}C]oleate, which is released from cells (2). From these data it was apparent that the liposomes were taken into acid-containing vacuoles and degraded. The presence of ^{3}H-inulin in the supernatant following preincubation of ^{3}H-inulin labeled liposomes with platelets, removal of free liposomes by centrifugation, resuspension in buffer and incubation confirmed that the aqueous components were exocytosed. Similar studies with ^{3}H-

cholesterylhexadecyl ether labeled liposomes demonstrated that the lipid is retained. The overall mechanism of DSPC:cholesterol (2:1 mole ratio) liposome uptake by platelets is summarized in Figure 1. The kinetics of uptake of DSPC:cholesterol (2:1 mole ratio) liposomes and exocytosis of the aqueous components, based on the data presented in Chapter 2, is discussed.

MATERIALS AND METHODS

The methods for preparation of the liposomes and platelet preparation are given in Chapter 2.

RESULTS

Uptake of ^{3}H-cholesterylhexadecyl ether labeled liposomes. Platelet uptake of ^{3}H-cholesterylhexadecyl ether labeled liposomes increased with increasing incubation time (Figure 2). In addition, the amount of uptake was independent of the number of non-radiolabeled liposomes preincubated with the platelets prior to the addition of the radiolabeled liposomes and the preincubation time (data not shown). Therefore the uptake of the ^{3}H-cholesterylhexadecyl ether labeled liposomes can be modeled as follows:

$$\text{Liposomes}_{\text{free}} + \text{Platelets} \Rightarrow \text{Liposomes}_{\text{phagocytosed}}$$

$$\frac{d[\text{phagocytosed}]}{dt} = k_1[\text{free}]$$

$$[\text{phagocytosed}] = k_1[\text{free}]t; \text{ where } [\text{free}] \sim 7 \times 10^{11} \text{ liposomes}/0.5 \text{ ml.}$$

The number of ^{3}H-cholesterylhexadecyl ether labeled liposomes phagocytosed, as compared to the number added, is less than 10%, therefore the number of free liposomes is approximately constant. Since platelet uptake is independent of the amount of previously phagocytosed liposomes, the

platelet reaction sites are represented as part of the constant k_1. Using this model and the data in Figure 2, k_1 is determined to be 7.1×10^{-3} hr^{-1} M^{-1}.

^{3}H-inulin labeled liposome exocytosis. To study the possible exocytosis of the liposomes, the amounts released of the tritiated radiolabels ^{3}H-cholesterylhexadecyl ether and ^{3}H-inulin following preincubation of the ^{3}H-labeled liposomes with platelets, removal of free liposomes by centrifugation, resuspension in buffer and incubation were examined (Figure 3). After 12 hours no membrane-associated probe was released. However, up to 60% of the aqueous-phase probe was released during this period. The release kinetics are modeled as shown below.

$$\text{Liposome}_{\text{phagocytosed}} \Rightarrow {}^3\text{H-inulin}_{\text{free}}$$

$$\frac{d[\text{phagocytosed}]}{dt} = -k_2[\text{phagocytosed}]$$

$$\ln\left(\frac{[\text{phagocytosed}]}{[\text{phagocytosed}]_0}\right) = -k_2 t$$

Using this model and the data in Figure 3, k_2 is determined to be $1.8 \times 10^{-1} hr^{-1}$.

Uptake of ^{3}H-inulin labeled liposomes. DSPC:cholesterol (2:1 mole ratio) liposomes are phagocytosed intact; however, the intracellular fate of the lipid and the aqueous components are different. The exocytosis of the ^{3}H-inulin label after liposome degradation, is reflected in the apparent decrease in the uptake values for the ^{3}H-inulin labeled liposomes. Therefore the values reported for the uptake of the ^{3}H-inulin labeled liposomes are given as "apparent liposomes per platelet". The model below describes the kinetics of the platelet associated ^{3}H-inulin probe which is dependent on both the uptake of the ^{3}H-inulin labeled liposomes and the exocytosis of the free probe.

$$\text{Liposomes}_{\text{free}} + \text{Platelets} \Rightarrow \text{Liposomes}_{\text{phagocytosed}} \Rightarrow {}^{3}\text{H-inulin}_{\text{free}}$$

$$\frac{[\text{phagocytosed}]}{[\text{free}]_0} = \frac{k_1}{(k_2 - k_1)}\{\exp(-k_1 t) - \exp(-k_2 t)\}$$

Figure 4 shows the uptake of ^{3}H-inulin labeled liposomes. The curve is derived from the above equation using the values of k_1 and k_2.

DISCUSSION

The uptake of DSPC:cholesterol (2:1 mole ratio) liposomes occurs through the OCS and the lysosomal pathway. The liposomes are taken up intact with an uptake rate of $k_1 = 7.1 \times 10^{-3}$ hr^{-1} M^{-1} and are then degraded, presumably in the acid-containing vacuoles. The lipid is retained; the aqueous components are exocytosed. The exocytosis also appears to follow a first order reaction scheme with $k_2 = 1.8 \times 10^{-1}$ hr.

The potential of using liposome-loaded platelets as a new drug delivery system relies greatly on the kinetics of uptake and exocytosis of the liposomes. Lipophilic drugs introduced into platelets by this method will remain within the cell indefinitely. Therefore, liposome-loaded platelets, which contain lipophilic diagnostic drugs, might be ideal for imaging platelet rich tumors or clots. Aqueous drugs will be exocytosed from the platelets with a half-life of 4 hours. Therefore, aqueous drugs, which are insensitive to low pH, might be used for therapeutic applications that require the release of the drug.

1. Daleke, D. L., Hong, K. and Papahodjopoulos, D. (1990) *Biochim. Biophys. Acta* **1024**, 352-366.
2. Derksen, J. T. P., Morselt, H. W. M. and Scherphof, G. L. (1988) *Biochim. Biophys. Acta* **971**, 127-136.

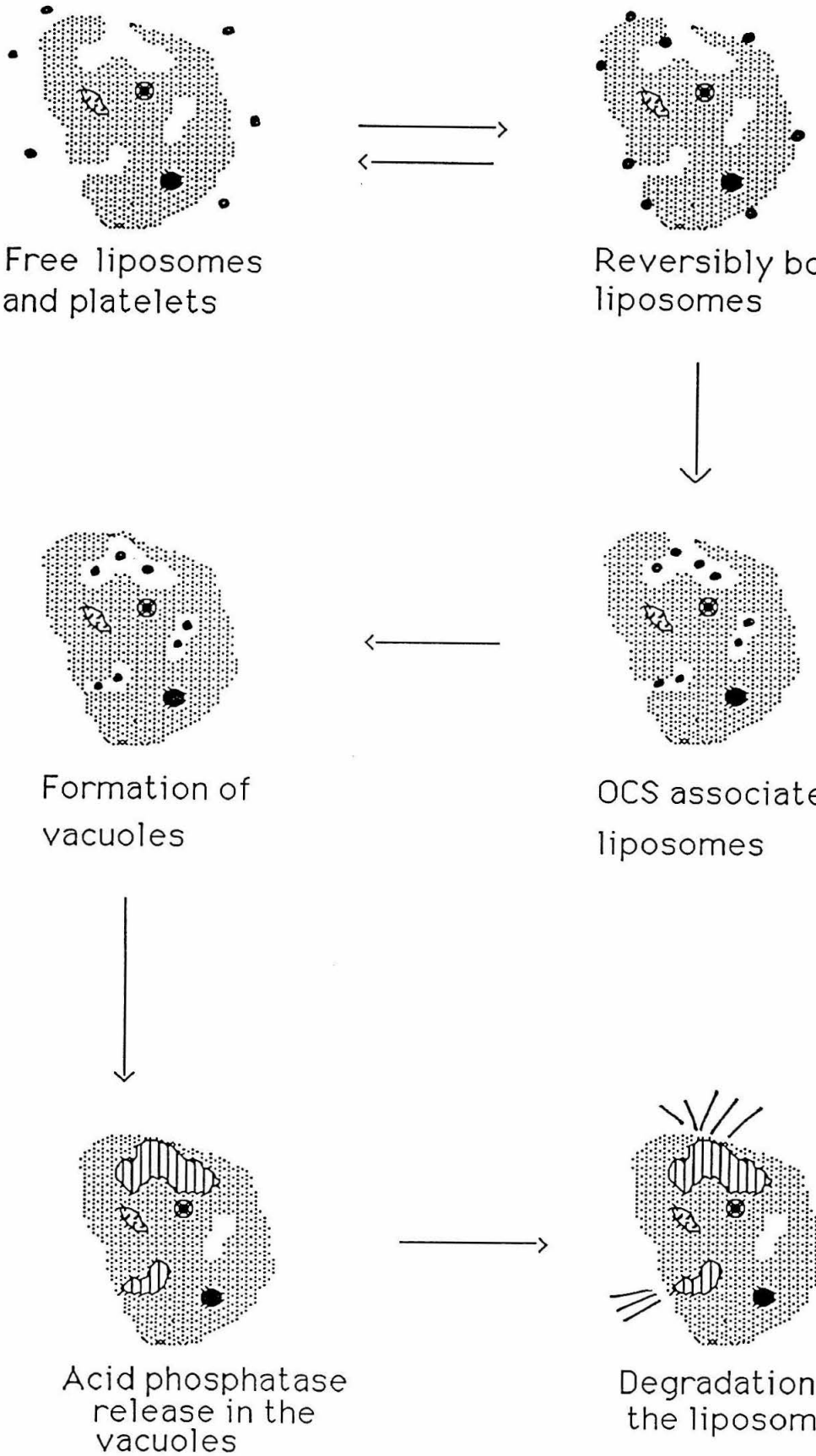
Free liposomes and platelets
Reversibly bound liposomes
OCS associated liposomes
Formation of vacuoles
Acid phosphatase release in the vacuoles
Degradation of the liposomes

Figure 1: A schematic representation of the uptake of DSPC:cholesterol (2:1 mole ratio) liposomes by human platelets.

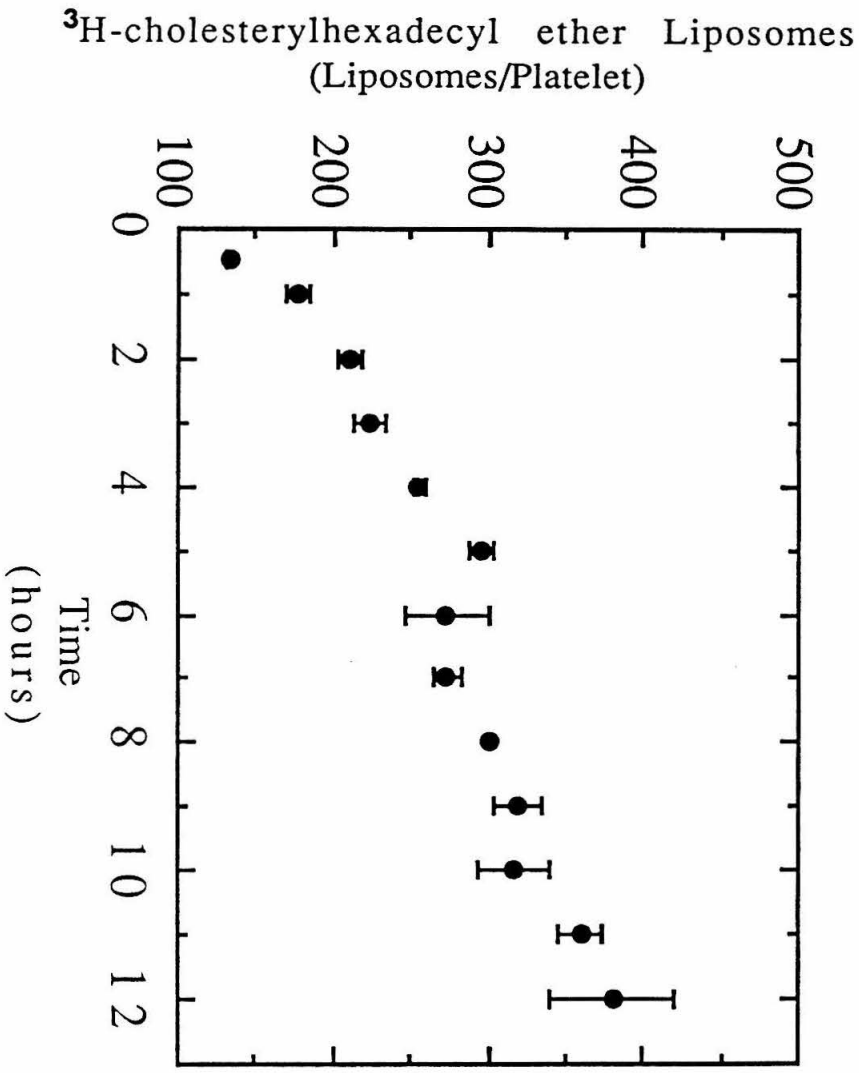
^{3}H-cholesterylhexadecyl ether Liposomes
(Liposomes/Platelet)
100
200
300
400
500
0
2
4
6
8
10
12
Time
(hours)

Figure 2: Incubation time dependence of platelet uptake of ^{3}H-cholesterylhexadecyl ether liposomes. Platelets (3×10^{8}) were incubated with the liposomes (7×10^{11}) for various incubation times.

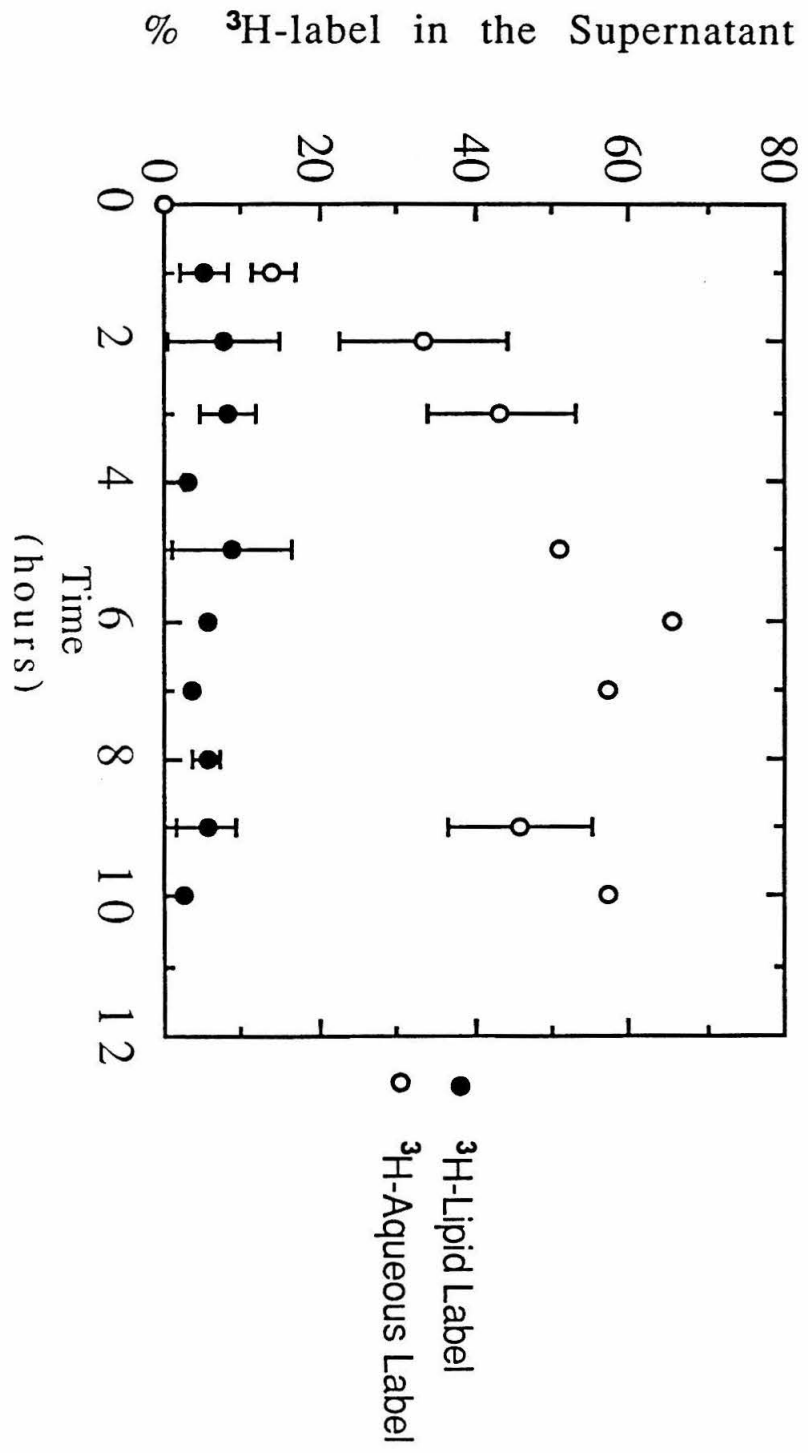
% ³H-label in the Supernatant
0
20
40
60
80
0
2
4
6
8
10
12
Time
(hours)
³H-Lipid Label
³H-Aqueous Label

Figure 3: Release studies of lipid and aqueous probes. Platelets (3×10^8) were preincubated with 8.3×10^{11} ^{3}H-cholesterylhexadecyl ether liposomes (●) or 2×10^{12} ^{3}H-inulin labeled liposomes (○) at 37°C for 12 hours. Cells were then pelleted, resuspended in buffer, and incubated for various incubation times at 37°C. Percentages of the ^{3}H-cholesterylhexadecyl ether and ^{3}H-inulin labels in the supernatant were determined.

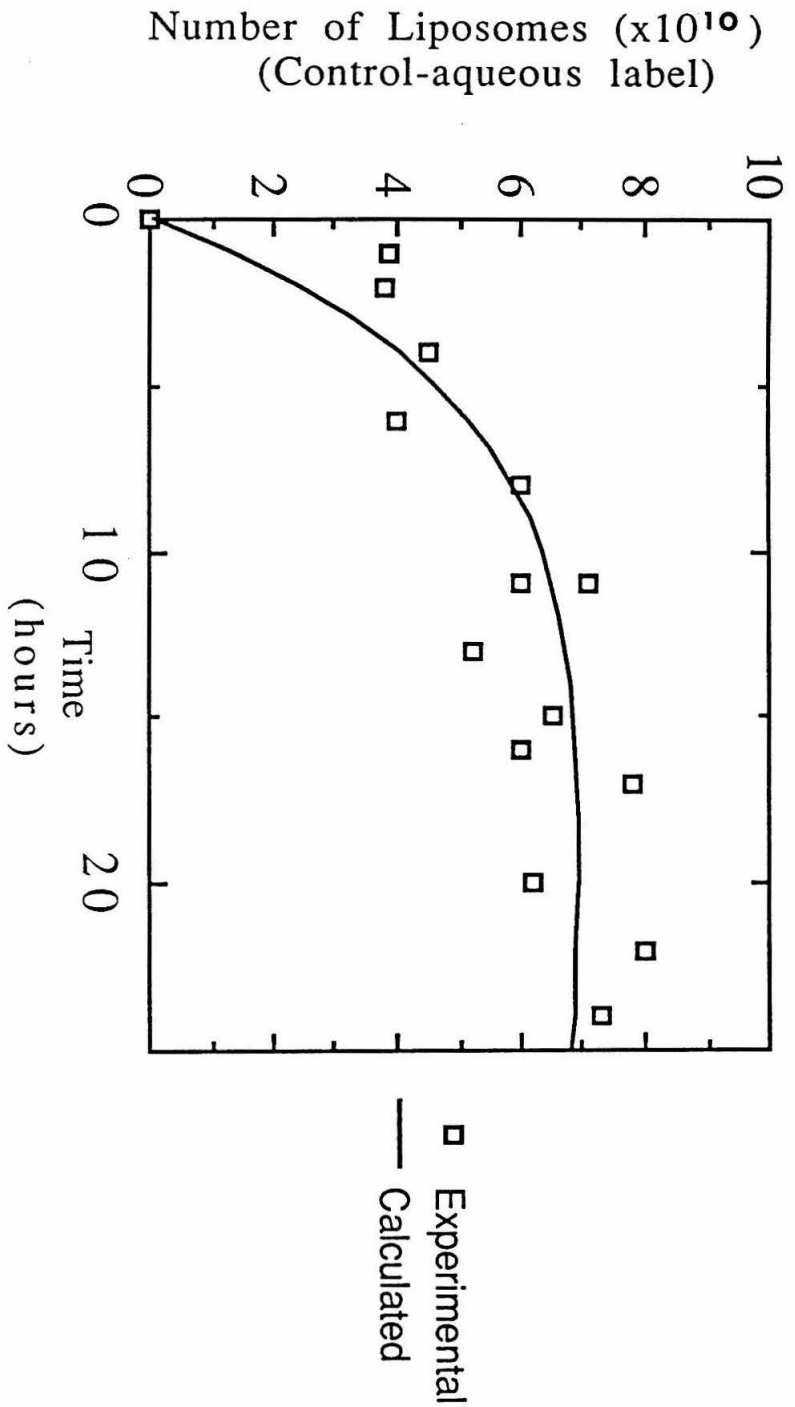
Number of Liposomes (x10¹⁰)
(Control-aqueous label)
0
2
4
6
8
10
0
10
20
Time
(hours)
Experimental
Calculated

Figure 4: Incubation time dependence of platelet uptake of ^{3}H-inulin labeled liposomes. Platelets (3×10^{8}) were incubated with 2×10^{12} liposomes at 37°C for various incubation times.

CHAPTER FOUR

ORGAN DISTRIBUTIONS OF LIPOSOME-LOADED RAT PLATELETS

R. Male, D. G. Moon†, J. S. Garvey, W. E. Vannier, and J. D. Baldeschwieler

Department of Chemistry, California Institute of Technology

†Albany College of Pharmacy, Albany, New York

ABSTRACT

Past *in vitro* functional assays have demonstrated that platelet function is not inhibited by liposome uptake. In the present study, the organ distributions of control and liposome-loaded Sprague-Dawley rat platelets were examined to determine whether liposome uptake enhances reticuloendothelial system (RES) uptake. Platelets were isolated using STRactan density gradient centrifugation, incubated with small unilamellar liposomes *in vitro* for 1 hour, labeled with ^{51}Cr and injected into a cohort group of rats. One hour post-injection the spleen, liver, lungs, blood and bladder contents were removed and percentages of the recovered dose localized per total organ (%RD) were determined. The RES index, defined as $\%RD_{liver} + \%RD_{spleen}$, were 24.8 ± 4.5 and 20.5 ± 5.0 for the control platelets and liposome-loaded platelets, respectively. These results indicate that liposome uptake does not enhance RES uptake.

INTRODUCTION

Liposomes have been used to deliver diagnostic and therapeutic drugs with moderate success. Reticuloendothelial system (RES) uptake and lack of targeting specificity of the liposomes have been the major problems encountered. Several groups have increased the circulation times by attaching either polyethylene glycol (PEG) or the ganglioside GM_1 to the liposome surfaces (1,2); the circulation half-lives for distearoyl-phosphatidylcholine (DSPC): cholesterol:PEG and GM_1 liposomes are 20.0 ± 3.5 and 16.4 ± 3.1 hours, respectively, as compared to 6.7 ± 4.5 hours for DSPC:cholesterol (2:1 mole ratio) liposomes (1). There have been some

attempts to target specific tissue sites by the addition of ligands, such as human gamma globulin or aminomannose; however this increased targeting has been largely limited to increased Kupffer cell and reticuloendothelial system (RES) uptake (3,4) Addition of antibodies to the liposomes increases targeting specificity, but success has been limited since the liposomes are still prone to RES uptake (5).

Recently we studied the *in vitro* interactions of small unilamellar vesicles (SUV) with platelets with the objective of developing an effective drug delivery system. Specifically, we have examined the kinetics and mechanisms of uptake of SUV, with and without covalently attached ligands. Liposomes that have been studied include: DSPC:cholesterol (2:1 mole ratio) control liposomes (6) and aminomannose- , human gamma globulin- and transferrin-labeled control liposomes (7). From our data we have concluded that attachment of these surface ligands increases the targeting specificity and efficiency of *in vitro* platelet uptake. In addition the mechanisms of uptake and subsequent specific localization of the liposomes and their contents within the cells are dependent on the type of liposome used.

Platelets have the unique ability to target specific sites *in vivo* including areas of infection and inflammation, tumors and clots. Therefore we expect to combine this *in vivo* targeting capability with the propensity of the platelets to take up liposomes *in vitro* in order to create an effective drug delivery system for diagnostic and therapeutic agents. Having a variety of trial liposome systems should be advantageous in the optimization of parameters for drug delivery.

In vitro functional assays, including microaggregation, serotonin release and membrane integrity, suggest that platelet function is not inhibited by liposome uptake (6, 7). The present study was designed to determine whether uptake of liposomes enhances rat platelet RES clearance.

MATERIALS AND METHODS

Control Liposome Preparation. A chloroform solution of a 2:1 mole ratio of DSPC (Avanti Polar-Lipids, Inc.) and cholesterol (cell culture tested Sigma Chemical Co.) was prepared such that the total lipid content was approximately 20 mg. The solution was taken to dryness in a 100 ml round bottom flask with a Büchler "Rotovap" apparatus and was dried in a dessicator under vacuum overnight. The resulting phospholipid mixture was resuspended in 5 ml of PBS (0.90% NaCl: 0.12% $Na_2HPO_4 \cdot 7H_2O$: 0.013% $NaH_2PO_4 \cdot H_2O$ w/w in water, pH=7.3), vortexed and 2.5 ml portions sonicated for 15 minutes using a Heat Systems-Ultrasonics, Inc. sonicator with a microprobe. In the studies examining liposome uptake versus incubation time and liposome concentration, five μCi of [^{3}H]cholesterylhexadecyl ether (New England Nuclear) were also added to the lipid prior to drying.

Determination of Liposome Diameters and Estimation of Liposome Numbers. Liposome diameters for different preparations were determined by photon correlation spectroscopy at 480 nm with a Malvern Instruments PCS 100 system. The numbers of [^{3}H]cholesterylhexadecyl ether labeled liposomes were calculated by assuming that the counts per

weight lipid were constant for all preparations, the average surface area for a phospholipid molecule is 0.7 nm^2 (8), and the average molecular weight for the lipid is 656 g/mol. The numbers of non-radiolabeled liposomes were determined by performing Böttcher phosphate assays (9) on the samples and assuming a 0.7 nm^2 phospholipid surface area. The light scattering data indicated that liposome diameters were 68-80 nm. The numbers of liposomes reported have all been normalized assuming that the diameters of the liposomes for all experiments were 74 nm.

Platelet Rich Plasma Isolation. Male Sprague-Dawley rats weighing approximately 250 g were anesthetized with ether. Blood was collected using a 10 ml syringe with an 18 gauge needle that contained 0.2 ml of a 19% citrate solution from the posterior vena cava at kidney level. The needle was removed and the blood was dripped slowly into a centrifuge tube containing 2 ml of BSG-citrate solution (6.83 g NaCl, 4 g sodium citrate, 2.0 g dextrose, 0.218 g H_2KPO_4 and 1.22 g HNa_2PO_4 adjusted to 1 liter H_2O, pH 7.4). After slow inversion of the tube twice, an additional 2 ml of BSG-citrate was added. Each blood sample was kept separate throughout the following centrifugation: 150 x g for 3 minutes to obtain the platelet rich plasma (PRP). The remaining red blood cell fraction was resuspended in 9 ml BSG-citrate and centrifuged again under the same conditions. The PRP samples from each rat were pooled.

^{51}Chromium Labeling. 0.5 mCi of sodium ^{51}chromate was added to the PRP and incubated at room temperature for 30 minutes.

Platelet Isolation. STRactan gradients were prepared as follows. A stock solution of 30% STRactan (arabinogalactan, St. Regis, Tacoma, WA) was prepared in BSG-citrate and kept frozen until used. 10% and 20% solutions were prepared immediately before use by dilution with BSG-citrate. Three ml of the 10% STRactan solution were added to 15 ml polypropylene centrifuge tubes. Four ml of the 20% STRactan solution were carefully layered on the bottom of the tubes. Three ml of the ^{51}Cr labeled PRP were layered on top of the other layered solutions and the tubes were centrifuged at 700 x g for 20 minutes. The cloudy layers of platelets were removed, pooled, resuspended in 9 ml of BSG-citrate and centrifuged at 500 x g for 10 minutes to wash off the remaining STRactan. The cells were resuspended in modified Tyrodes solution (1.0 g dextrose, 1.0 g $NaHCO_3$, 0.2 g KCl, 8.0 g NaCl, 0.05 g Na_2HPO_4 and 0.14 g $CaCl_2$ in 1 lit H_2O, pH 7.4).

Incubation with Liposomes. Platelets were incubated with liposomes at 37°C for 1 hour. One hour allows significant uptake while minimizing platelet exposure to 37°C which has been shown to induce platelet membrane lesions (6). Throughout the experiments we attempted to incubate a constant ratio of 2.1×10^{12} liposomes to 2.0 mg platelet protein (0.81×10^9 rat platelets/mg protein (10)). After incubation, 9 ml of BSG-citrate was added to each tube prior to centrifugation at 500 x g for 10 minutes to inhibit aggregation. Centrifugation was repeated and the cells were finally resuspended in BSG-citrate. The Peterson modification of the Lowry protein assay with a bovine serum albumin standard and without

trichloroacetic acid precipitation was used to estimate the amounts of protein in the samples (11).

Organ Distribution. The rats were anesthetized with 30 mg nembutal/kg rat weight injected intraperitonially. Approximately 2 mg protein (0.5 ml) of control or liposome-loaded platelets were injected into each rat via the saphenous vein using a 1 ml syringe with a 23 gauge needle. Sufficient control and liposome-loaded platelets were retained to determine the total injected dose. Platelet localization in the spleen, liver, lung and blood were determined one hour post-injection. Small amounts of tissue were repeatedly washed in cold isotonic saline, weighed and gamma counted. Preliminary studies have shown that repeated washings in 200-300 ml of saline is as effective as the whole organ perfusion in removal of blood-borne radioactivity (12). The total volume of blood per rat was determined by assuming an 8% blood volume (ml) per kg rat body weight. Organ uptake of platelets is expressed as the mean percent of the recovered dose localized per total organ (%RD) ± the standard deviation and was compared by the *t* test utilizing a 95% confidence level. Platelet organ localization was evaluated in groups of six rats.

RESULTS

In vitro uptake of liposomes by rat platelets. 1.6×10^9 rat platelets (~0.5 mL) were incubated with 7.1×10^{11}, 2.1×10^{12} and 3.5×10^{12} liposomes for 1 hour at 37°C. Uptake increased with increasing numbers of incubated liposomes (Figure 1).

Organ distributions. The %RD for the spleen, liver, lungs and blood were 1.10 ± 0.28, 23.73 ± 4.21, 1.87 ± 0.36, and 52.83 ± 6.98 for the control platelets and 0.93 ± 0.22, 19.62 ± 4.82, 1.55 ± 0.40, and 59.18 ± 7.96 for the liposome-loaded platelets (Figure 2). The RES indexes, defined as the combined %RD of the liver and spleen, were 24.8 ± 4.5 and 20.5 ± 5.0 for the control platelets and liposome-loaded platelets, respectively.

The data are reported as the percentage of the recovered dose rather than the injected dose to avoid possible misleading results due to the presence of unincorporated ^{51}Cr. By combining the counts recovered from the bladder and kidneys plus those in the spleen, liver, lungs and blood (recovered dose), we account for greater than 75% of the injected dose.

DISCUSSION

The requirements for liposome-loaded platelets to be a successful drug delivery system are efficient loading without loss of platelet function and targeting capability. The *in vitro* function assays with human platelets, including microaggregation, serotonin release and membrane integrity suggest that platelet function is unimpaired by liposome uptake (6).

The current study has demonstrated that liposome uptake does not enhance platelet RES uptake or decrease circulation. Initial uptake by the liver and spleen within the first 15 minutes is not uncommon. It is hypothesized that during this time platelets that are minimally damaged undergo repair and are rereleased (13). However, subsequent accumulation of platelets in the RES is often indicative of irreversible platelet damage (14, 15). Localization within the lungs can indicate platelet aggregation and formation of microemboli (12). No significant differences

in the organ distributions or the RES indexes have been demonstrated in our studies. Therefore, we conclude that liposome uptake does not impair platelet function or circulation.

The ability to target might be dependent on factors other than those responsible for normal circulation. Therefore future studies will focus on experiments that assess the ability of the liposome-loaded platelets to target thrombi and tumors. Additionally, imaging thrombi and tumors using liposome-encapsulated imaging agents, including contrast dyes or radiolabels, will be examined and compared to conventional techniques. The efficacy of delivery of thrombolytic and anti-cancer drug using this method will be addressed in detail.

ACKNOWLEDGMENT

Funding for this work was provided by the Army Research Office, Grant DAAL-03-87-K-0044 and the Caltech Consortium in Chemistry and Chemical Engineering (founding members: E. I. du Pont de Memours and Company, Inc., Eastman Kodak company, Minnesota Mining and Manufacturing Company, Shell Oil company Foundation).

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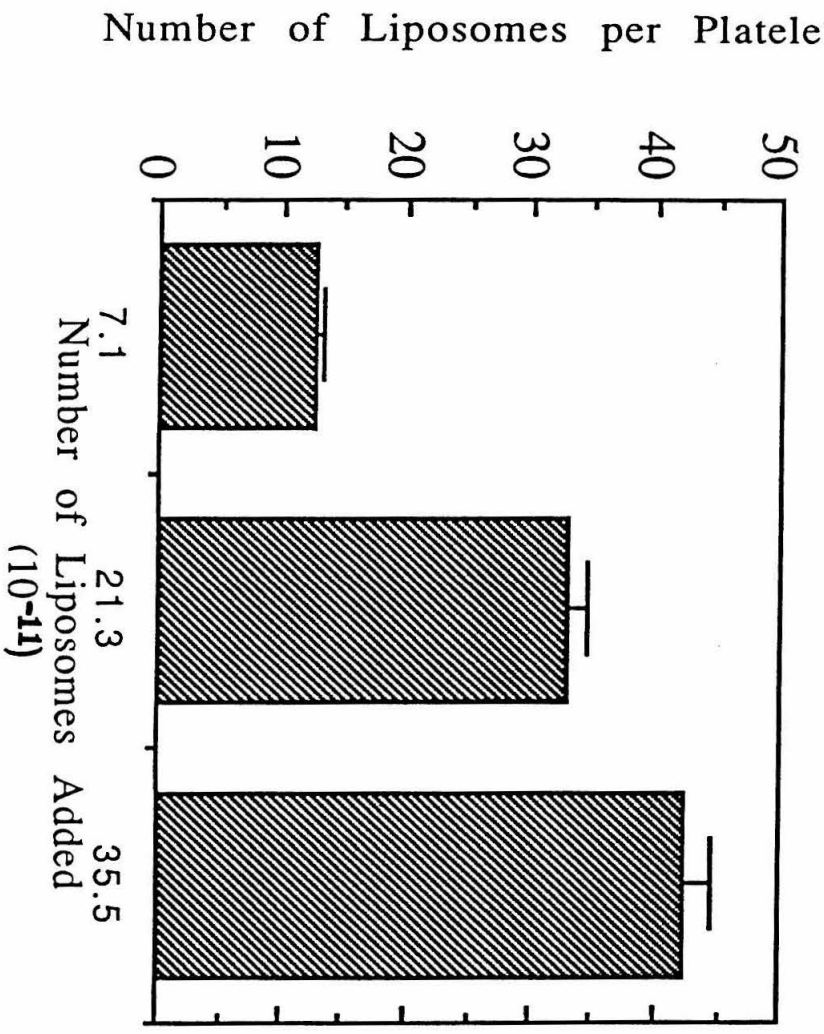
Number of Liposomes per Platelet
0
10
20
30
40
50
7.1
21.3
35.5
Number of Liposomes Added
(10-11)

Figure 1. Liposome concentration dependence of Spraque-Dawley rat platelet uptake of [^{3}H]cholesterylhexadecyl ether labeled liposomes. 1.6 x 10^9 platelets were incubated with varying amounts of [^{3}H]cholesterylhexadecyl ether labeled liposomes at 37^{o}C for 1 hour.

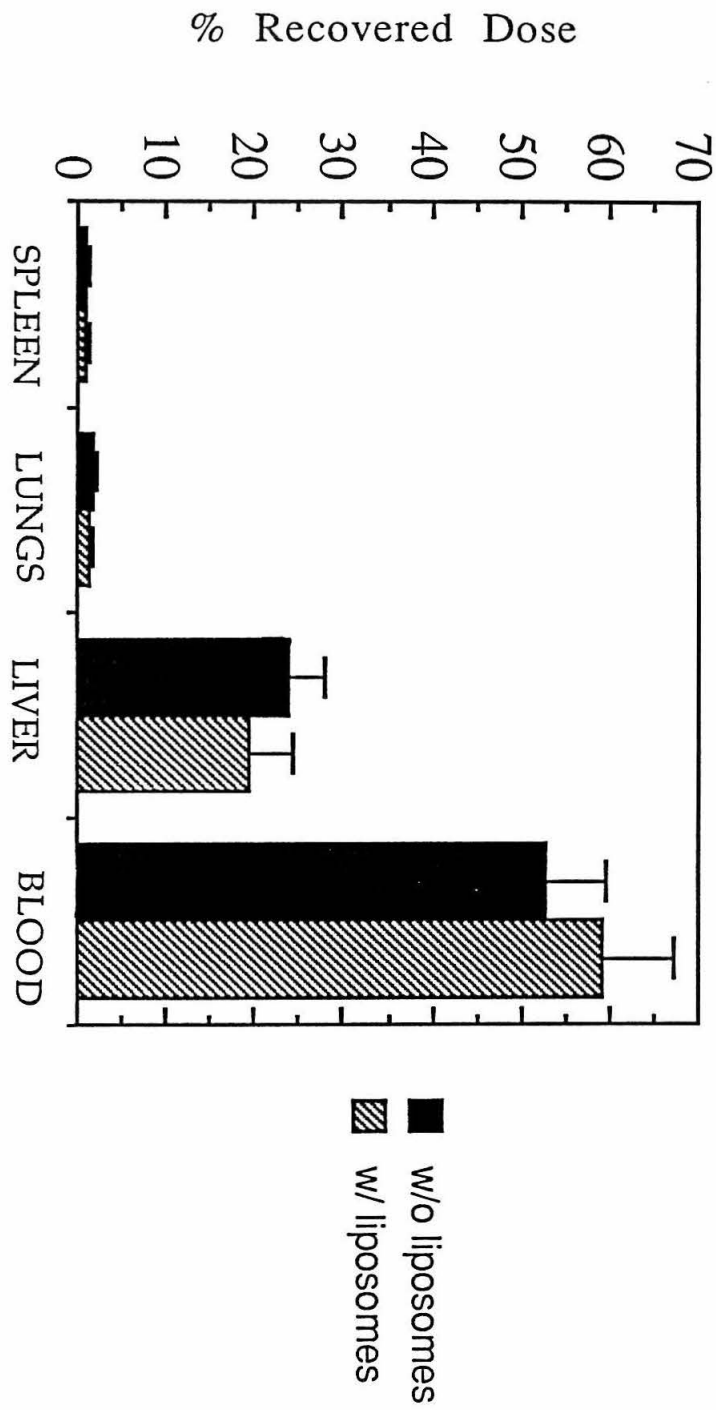
% Recovered Dose
0
10
20
30
40
50
60
70
SPLEEN
LUNGS
LIVER
BLOOD
w/o liposomes
w/ liposomes

Figure 2. Organ distributions of control and liposome-labeled platelet one hour post-injection. The data are represented as the percentage of the recovered dose localized per total organ (%RD) ± SD.

CHAPTER FIVE

UPTAKE OF LIPOSOMES WITH COVALENTLY ATTACHED LIGANDS BY HUMAN PLATELETS

R. Male, W. E. Vannier, T. Derrington and J. D. Baldeschwieler

ABSTRACT

We have shown that the addition of human gamma globulin (HGG), transferrin (Tf) and aminomannose (Am) to the liposome surface enhances platelet uptake. Incubation of human platelets with HGG and Tf liposomes resulted in the uptake of the liposomes and retention of the lipid and release of the aqueous-phase components. The lipid label ^{3}H-cholesterylhexadecyl ether and water-soluble ^{3}H-inulin were used to study the fate of the liposome components. Uptake of the HGG and Tf liposomes was proportional to the incubation time and liposome concentration. Approximately 1400 HGG and 550 Tf liposomes were taken up within a 5 hr incubation period. Uptake of the Am liposomes increased initially, but then decreased with time. Uptake was proportional to the number of liposomes added. Approximately 1000 liposomes were associated with each platelet after a 5 hour incubation period.

INTRODUCTION

Previous studies have demonstrated the platelets' ability to phagocytose small unilamellar distearoylphosphatidylcholine (DSPC): cholesterol (2:1 mole ratio) vesicles (1). Uptake of the liposomes occurred through the open channel system (OCS), and was followed by accumulation and degradation within acid-containing vacuoles. The lipid was retained; the aqueous components were exocytosed with a half-life of 4 hours. Approximately 250 liposomes per platelet were taken up within a 5 hour incubation period. Uptake of the liposomes did not inhibit platelet function, as assessed by the serotonin release, microaggregation and fluorescein diacetate assays.

Although several therapeutic and diagnostic drugs would be well suited to this liposome-loaded platelet delivery system, delivery of other drugs via this method might not be ideal. Exposure to low pH may inactivate the drugs and the release times from the platelets following uptake may be too fast or slow. Therefore we developed new liposomes which are taken up more avidly than the DSPC:cholesterol liposomes and have different uptake and exocytosis mechanisms and possibly different intracellular fates. Specifically, we have studied the uptake of human gamma globulin (HGG), transferrin (Tf) and aminomannose (Am) liposomes (Figure 1). These ligands were chosen because previous studies with macrophages have demonstrated enhanced uptake, as compared to DSPC:cholesterol liposomes (2-4), and their ability to undergo receptor-mediated endocytosis, which might offer alternative uptake and exocytosis pathways as compared to platelet phagocytosis of the ligand-free liposomes. HGG, the principle antibody in the serum, binds to foreign materials and results in their recognition by Fc receptors on phagocytic cells and complement proteins. Tf receptors bind transferrin-bound iron and endocytose the complex; the iron is internalized and the receptor is recycled within minutes. The receptor(s) for Am has not been determined.

MATERIALS AND METHODS

Purification of Platelets. Platelets purchased from the American Blood Institute (Los Angeles) were centrifuged at 1000 g for 15 minutes in an IEC Centra-8 centrifuge. The pellet was resuspended in either 0.9% saline solution or Tyrodes buffer and spun at 110 g for 5 minutes to remove excess red blood cells.

The supernatant was spun at 450 g for 10 minutes. The pellet was resuspended in modified Tyrodes buffer (Tyrodes buffer plus 1.26 mM of $CaCl_2$).

The final purities of the platelet preparations were greater than 95% platelets as assessed by a Colter S+4 Cell Counter; the main contaminants were red blood cells. Platelets were used within 24 hours of blood collection; this time was required for HIV and hepatitis testing.

Determination of the Number of Platelets per mg of Protein. Cell counts were performed with a Coulter S+4 Cell Counter to determine the number of platelets. The Peterson modification of the Lowry protein assay (5) with a bovine serum albumin standard and without TCA precipitation was used to determine the amounts of protein in the same samples. A milligram of protein was found to correspond to 3×10^8 platelets. In addition the Lowry protein assay was used to determined the number of HGG and Tf ligands per liposome.

Preparation of the Liposomes. *MBPE liposomes.* A mixture of 16 mg distearoylphosphatidylcholine (Avanti Polar-Lipids, Inc.), 3.9 mg cholesterol (cell culture tested Sigma Chemical Co.) and 0.94 mg maleamidobutyryl phosphatidyl ethanolamine (MBPE) (Avanti Polar-Lipids, Inc.) in chloroform was made. The solution was then taken to dryness in a 100 ml round bottom flask with a Büchler "Rotovap" apparatus and was dried in a dessicator under vacuum overnight. The resulting phospholipid mixture was resuspended in isotonic saline or 5 ml of PBS (0.90% NaCl: 0.12% $Na_2HPO_4 \cdot 7H_2O$: 0.013% $NaH_2PO_4 \cdot H_2O$ w/w in water, pH 7.3), heated to 60°C, vortexed and 2.5 ml portions probe sonicated for 15 minutes using a Heat Systems-Ultrasonics, Inc. sonicator with a microprobe.

HGG and Tf coupling. Fifteen mg of heated aggregated HGG, prepared by heating a 30-50% HGG solution at 62°C for 30 minutes, or Tf was thiolated with 4

μL of N-succinimidyl-5-acetylthioacetate (SATA), 2.7 mg/50 μL DMF. The resultant thiolated compounds were separated from free SATA by gel filtration on a Sephadex G-25 column. The liposome coupling reaction was performed by addition of 5 mg of the SATA-HGG or SATA-Tf to 5 ml of the sonicated MBPE-liposomes and 0.2 ml of 0.5 M hydroxylamine, pH 7.2. The mixture was incubated with gentle mixing at room temperature for 1 hour. The reaction was terminated by the addition of 0.2 ml of 1 mg/ml N-ethylmaleimide. Free HGG or Tf was separated from the liposomes by gel filtration on a BioGel A 5m column (Figure 2). The number of ligands per liposome were approximately 140 and 210 or 420 μg HGG/μmole PO_4 and 260 μg Tf/μmole PO_4 for the HGG and Tf liposomes, respectively, assuming a molecular weight of 150,000 for the heat aggregated HGG and 90,000 for Tf.

Aminomannose Liposomes. 3.36 mg of an aminomannose cholesterol derivative was added to 16 mg DSPC and 1.96 mg cholesterol. Liposomes were prepared as for the MBPE liposomes. The number of Am ligands per liposome surface was approximately 5,000.

^{3}H-cholesterylhexadecyl ether Labeled Liposomes. Five microcuries of ^{3}H-cholesterylhexadecyl ether (New England Nuclear) in chloroform was added to the lipid mixture prior to drying and desiccation. Liposomes containing ^{3}H-cholesterylhexadecyl ether were resuspended in PBS.

^{3}H-inulin Labeled Liposomes. Two humdred fifty microcuries of ^{3}H-inulin (Amersham, Corp.) was added to the resuspension buffer after desiccation. Liposomes containing ^{3}H-inulin were resuspended in isotonic saline. Excess ^{3}H-inulin, (molecular weight 5,200) was separated from the liposomes by gel filtration with a Sephadex G-50-100 column. The percent encapsulation was approximately 15%.

Non-radiolabeled Liposomes. The liposomes were prepared as described but without the tritiated labels.

Incubation Procedure. The platelets and liposomes were incubated at 37°C in 1.5 mL polypropylene centrifuge tubes for the desired incubation times after which the mixtures were centrifuged at 5700 g for 5 minutes in a Beckman Microfuge 11. The pellets were washed twice in buffer and finally resuspended in 0.5 M NaOH/0.5% SDS and incubated overnight at room temperature. The solution was transferred to liquid scintillation vials with 10 mL of Safety Solve (Research Products, Inc.), mixed and allowed to sit at least two hours prior to scintillation counting. For each experiment all samples were done in duplicate; the number of independent experiments, n, are noted in the figure captions or in the text. Bars on the graphs represent the range of the data.

Exocytosis Studies. Platelets and liposomes were incubated as above for 3 hours. The mixtures were centrifuged and the platelets washed by centrifugation twice in buffer to remove excess liposomes and further incubated at 37°C for varying periods of time. Both the pellets and supernatants were collected by centrifugation, solubilized in the NaOH/SDS solution overnight and counted.

Determination of Liposome Diameters and Estimation of Liposome Numbers. The liposome diameters for different preparations were determined by photon correlation spectroscopy at 480 nm using a Malvern Instruments PCS 100 system. The numbers of ^{3}H-cholesterylhexadecyl ether labeled liposomes were calculated by assuming that the counts per weight of lipid were constant for all preparations, the average surface area for a phospholipid molecule is 0.7 nm^2 (6) and the average molecular weight for the lipid is 656 g/mole. The numbers of ^{3}H-inulin labeled and non-radiolabeled liposomes were determined by performing Böttcher phosphate assays (7) on the samples and assuming a 0.7

nm^2 phospholipid surface area. The light scattering data indicated that the HGG, Tf and Am liposomes diameters were 200-300 nm, 110-130 nm and 90-110 nm, respectively. The number of liposomes reported have all been normalized assuming that the diameters of the liposomes for all experiments were 74 nm, so that the data could be compared with previous data involving 74 nm DSPC:cholesterol (2:1 mole ratio) liposomes (1).

***In vitro* Studies to Assess Platelet Function.** *Serotonin Release Assay.* The serotonin assay was performed with 3 x 10^8 platelets, suspended in modified Tyrodes and incubated with or without 1.4 x 10^{12} liposomes, for one to nine hours as described (8). 0.5 μM ^{14}C-serotonin was preincubated with the platelets for 20 minutes at 37°C; 1 μM imipramine was then added to prevent reuptake of secreted serotonin. Subsequent release from the alpha granules, an indication of degranulation, was measured.

Microaggregation Assay. Platelets suspended in modified Tyrodes solution were incubated at 37°C, with or without 1.4 x 10^{12} liposomes, for one to nine hours and then fixed with 1% glutaraldehyde. The percentages of microaggregated platelets were determined by examining the cells in a hemacytometer.

RESULTS

HGG Liposomes. *^{3}H-cholesterylhexadecyl ether Labeled Liposome Uptake.* Platelet uptake of ^{3}H-cholesterylhexadecyl ether labeled liposomes increased with increasing incubation times (Figure 3). After incubation with 3 x 10^8 platelets for 5 hours, approximately 1400 ^{3}H-cholesterylhexadecyl ether labeled liposomes were associated with each platelet. ^{3}H-cholesterylhexadecyl ether labeled HGG liposome uptake by platelets also increased with increasing numbers of incubated

liposomes; two incubation times are shown (Figure 4). Incubation for 12 hours resulted in greater uptake than the 2.0 hour incubation.

^{3}H-inulin Labeled Liposome Uptake. Although the number of ^{3}H-inulin labeled liposomes taken up increased with time (Figure 3), the kinetics of uptake were different from that for the ^{3}H-cholesterylhexadecyl ether labeled liposomes. Incubation of 3×10^8 platelets with 7.1×10^{11} liposomes resulted in the uptake of approximately 500 liposomes per platelet after 5 hours. The uptake of ^{3}H-inulin labeled liposomes versus the concentration of liposomes incubated is shown for two different incubation times (Figure 5). Uptake was proportional to both the number of liposomes and incubation time. The difference in kinetics of uptake as assessed by the lipid label and the aqueous label suggests that there is a difference in the fate of the lipid and aqueous components after platelet uptake.

Release of Tritiated Radiolabels To study the possible exocytosis of liposomes, the amounts released of the tritiated radiolabels ^{3}H-cholesterylhexadecyl ether and ^{3}H-inulin were examined (Figure 6). After 7 hours no membrane associated probe was released. However, up to 34% of the aqueous-phase probe was released during this period.

Free HGG and mAb IV.3 Inhibition. HGG is known to interact with Fc receptors on cells. Therefore, to determine if uptake of the HGG liposomes was via a Fc receptor, free HGG or mAb IV.3 (Medarex, Inc. NH), an antibody to platelet Fc receptor, were incubated with the platelets for 20 minutes prior to incubation with the liposomes. Incubation with 40 or 100 μg HGG decreased uptake by an average of 14.0 ± 7.5 % and 21 ± 7.3 % respectively (n=2). Addition of 5 μg of mAb IV.3 decreased uptake by 13.7 ± 6.7 % (n=2). Platelets are known to contain at least two different Fc receptors (9,10); therefore it is not surprising that addition of an antibody, which is specific to only one of the receptors, decreased

uptake by only 13%. Previous studies demonstrated that binding of fluorescently labeled HGG to platelets was inhibited 25% by the addition of a hundred-fold excess of free HGG or a 3-fold excess of mAb IV.3 (11).

Tf Liposomes. *^{3}H-cholesterylhexadecyl ether Labeled Liposome Uptake.* Platelet uptake of ^{3}H-cholesterylhexadecyl ether labeled liposomes increased with increasing incubation times (Figure 7) and increasing number of incubated liposomes (Figure 8). After incubation of 7.1×10^{11} liposomes with 3×10^{8} platelets for 5 hours, approximately 550 ^{3}H-cholesterylhexadecyl ether labeled liposomes were associated with each platelet. Incubation for 12 hours resulted in greater uptake than the 2.0 hour incubation.

^{3}H-inulin Labeled Liposome Uptake. Uptake of ^{3}H-inulin labeled liposomes increased with time (Figure 7), but was less than that for the ^{3}H-cholesterylhexadecyl ether labeled liposomes. Incubation of 3×10^{8} platelets with 7.1×10^{11} liposomes resulted in the uptake of approximately 350 liposomes per platelet after 5 hours. The uptake of ^{3}H-inulin labeled liposomes versus the concentration of liposomes incubated is shown for two different incubation times (Figure 9). Uptake was proportional to both the number of liposomes and incubation time. Similar to the HGG liposomes, the difference in uptake of the lipid and the aqueous labels suggests that the fate of the Tf liposome lipid and aqueous components after platelet uptake are different.

Release of Tritiated Radiolabels To study the possibe exocytosis of liposomes, the amounts released of the tritiated radiolabels ^{3}H-cholesterylhexadecyl ether and ^{3}H-inulin were examined (Figure 10). After 7 hours no membrane associated probe was released. However, up to 32% of the aqueous-phase probe was released during this period.

Free Tf Inhibition. Some cells contain Tf receptors; as of now, the presence of Tf receptors on platelets has not been demonstrated. The addition of 25, 50 or 100 μg free Tf, which is sufficient to block 10^6 receptors per platelet, does not inhibit uptake (n=2). It is possible that the Tf liposomes are going through the OCS and phagocytic pathway like the non-ligand DSPC:cholesterol (2:1 mole ratio) liposomes (1) and/or a Tf-independent receptor.

Aminomannose Liposomes. *^{3}H-cholesterylhexadecyl ether Labeled Liposome Uptake.* Platelet uptake of ^{3}H-cholesterylhexadecyl ether labeled Am liposomes decreased with increasing incubation times after an initial uptake period; three incubation concentrations are shown (Figure 11). Overall, uptake increased with increasing numbers of incubated liposomes (Figure 12). Incubation of 7.1×10^{11} liposomes with 3×10^8 platelets for 1 hour resulted in the uptake of approximately 1400 liposomes per platelet. After 5 hours, 1000 liposomes are associated with each platelet.

^{3}H-inulin Labeled Liposome Uptake. Uptake of ^{3}H-inulin labeled liposomes also decreased with time (Figure 13), and was approximately the same as for the ^{3}H-cholesterylhexadecyl ether labeled liposomes. The uptake of ^{3}H-inulin labeled liposomes versus the concentration of liposomes incubated is shown for two different incubation times (Figure 14).

***In vitro* Assays to Assess Platelet Function.** Incubation with 14×10^{11} HGG, Tf or Am liposomes with 3×10^8 platelets for 0 to 9 hours did not significantly alter platelet secretion of serotonin or induce additional platelet aggregation (n=3).

DISCUSSION

In our original study we examined the uptake of DSPC:cholesterol (2:1 mole ratio) small unilamellar liposomes by human platelets (1). We were able to demonstrate that the liposomes entered the open channel system, accumulated in acid-containing vacuoles and were degraded. The lipid was retained; the aqueous components were exocytosed. The purpose of this study was to incorporate ligands on the surface of the liposomes with the hope of enhancing platelet uptake and modifying the intracellular fates of the liposomes. It is likely, in addition to other mechanisms, that a fraction of the ligand-liposomes are internalized by phagocytosis.

Uptake at 5 hours for 74 nm non-ligand liposomes is approximately 250 liposomes per platelet as compared to 1400, 550 and 1000 for the HGG, Tf and Am liposomes, respectively. These numbers were normalized, assuming that the diameters of the liposomes for all experiments were 74 nm, to allow comparison of the data between the two studies. The size of the ligand liposomes actually range between 200-300 nm for the HGG liposomes, 110-130 nm for the Tf liposomes and 100-120 nm for the Am liposomes. Therefore, we prepared 120 nm and 250 nm non-ligand liposomes and studied their uptake by platelets. The uptake of 120 nm and 250 nm non-ligand liposomes are 2 and 4 times greater than the uptake of 74 nm non-ligand liposomes. Therefore more accurate comparisons are 1400 and 1000 HGG and non-ligand liposomes per platelet, respectively, and 550, 1000 and 500 Tf, Am and non-ligand liposomes per platelet, respectively. The above uptake values, as well as all others in this paper, represent both incorporated and bound liposomes.

Without further experiments, the mechanisms of HGG, Tf and Am liposome uptake can only be hypothesized. The fact that the aqueous probe is selectively

released while the lipid is retained, strongly suggests that the HGG and Tf liposomes are internalized and degraded. Our data also strongly suggest that the HGG liposomes bind and are possibly endocytosed via a Fc receptor pathway since the addition of free HGG or mAb IV.3, an antibody directed against the Fc receptor, inhibits uptake. The uptake of Tf liposomes is not inhibited by the addition of free Tf and may be internalized via platelet phagocytosis or another Tf-independent pathway.

The mechanism of the aminomannose liposome interaction with platelets is not readily apparent. Since the uptake, as measured by the lipid and aqueous probes are identical, it would appear that the fates of the lipid and aqueous probes are identical. If the liposomes bind and then unbind, the concentration of the bound liposomes versus time should increase and then reach a steady state. However, the number of platelet associated liposomes does not plateau. If the liposomes are internalized intact and are released in such a state that they can not rebind or the receptor is altered to prevent rebinding, then the uptake could be described as a consecutive first order reaction kinetic scheme with $[\text{liposome}]_{\text{bound}} = \frac{k_1 [\text{liposomes}]_0}{(k_2 - k_1)}(\exp(-k_1 t) - (\exp(-k_2 t))$. The uptake profiles for 17.8 x 10^{11} and 35.5 x 10^{11} incubated liposomes are identical in shape and vary in amplitude as expected from the above equation, but the uptake for 7.1 x 10^{11} liposomes is very different in shape as well as amplitude. Another possible explanation for the data is that the liposomes are interacting with more than one type of receptor. Possible receptors include a mannose receptor (12), known to exist on macrophages and other cells, or a positive charge dependent receptor. Perhaps at lower concentrations the liposomes bind to the receptor with the highest binding constant; at higher concentrations other receptors with lower binding constants become involved. Although it seems unlikely that a factor of

2.5 change in the initial liposome concentration could result in the binding to an additional receptor, the uptake versus concentration data also support the idea that the mode of uptake changes between the concentrations of approximately 14 x 10^{11} and 19 x 10^{11} liposomes. Below 14 x 10^{11} liposomes the uptake at 2 hours exceeds that at 12 hours; above 19 x 10^{11} liposomes the uptake at 12 hours is greater than that at 2 hours.

The ability to encapsulate a variety of therapeutic and diagnostic *in vitro* and to target specific locations *in vivo* such as areas of inflammation or infection, neoplastic tumor cells, or thrombosis would be of great medical importance. The efficacy of using liposome-loaded platelets will be highly dependent on the concentration of drug per platelet, which is related to the number of liposomes per platelet, and the intracellular location of the drug, which is related to the mechanism of uptake. In addition, it may be possible to achieve sufficient platelet targeting specificity by manipulation of the liposome surface ligands so that the liposomes can be injected directly and load platelets *in vivo* .

ACKNOWLEDGMENTS

We would like to thank Vestar, Inc. for supplying the aminomannose used in this study. Funding for this work was provided by ARO grant #DAAL-03-87-0044, and the Caltech Consortium in Chemistry and Chemical Engineering; Founding Members: E. I. du Pont de Nemours and Company, Inc., Eastman Kodak Company, Minnesota Mining and Manufacturing, Shell Oil Company Foundation.

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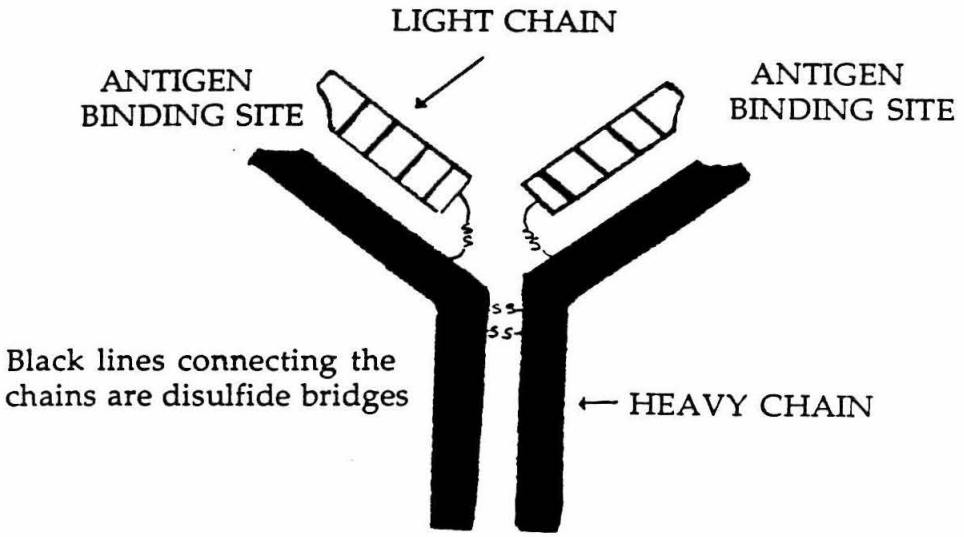
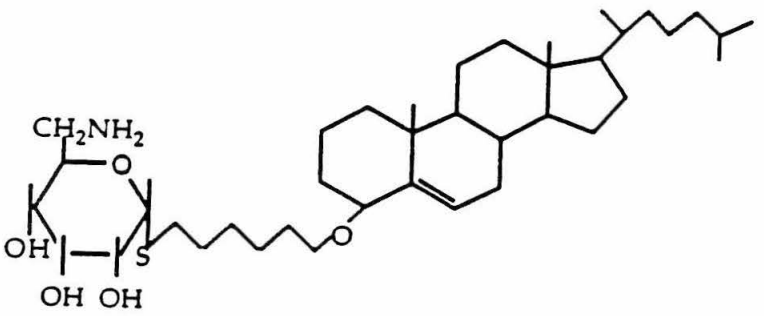


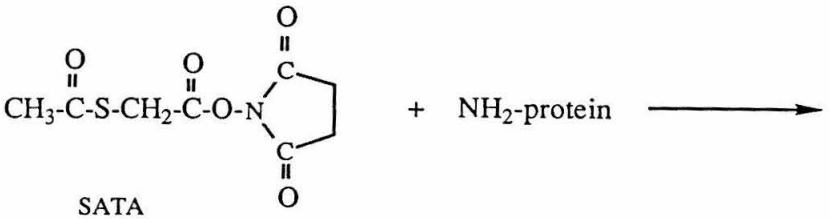
HUMAN GAMMA GLOBULIN



AMINOMANNOSE

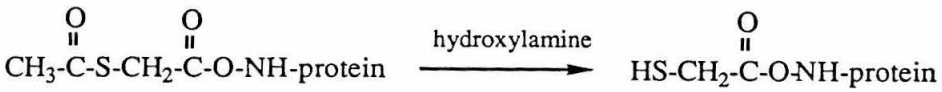
Figure 1: Structures of the human gamma globulin (HGG) and aminomannose (Am) ligands.

1.

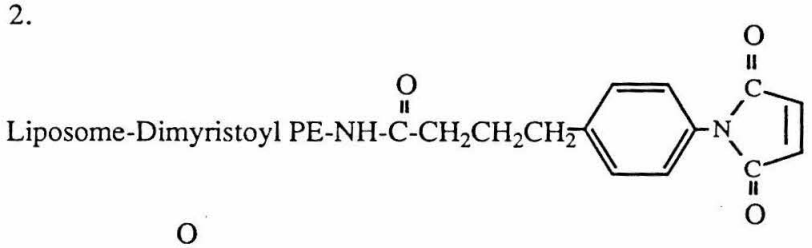


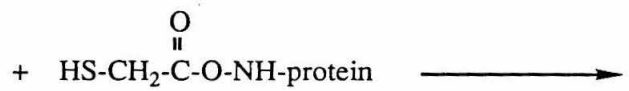
SATA

(N-hydroxysuccinimide)



2.





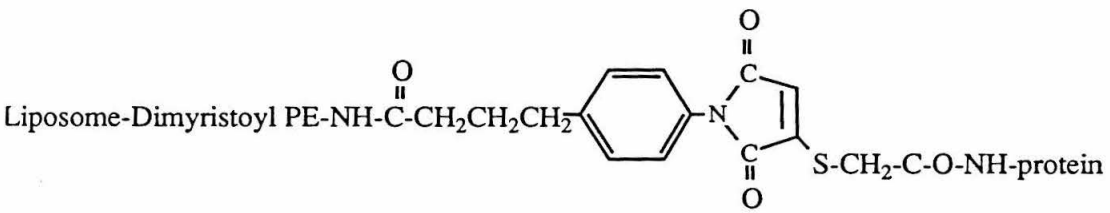


Figure 2: Synthesis scheme for the attachment of HGG and Tf to the liposomes. (PE = phosphatidylethanolamine)

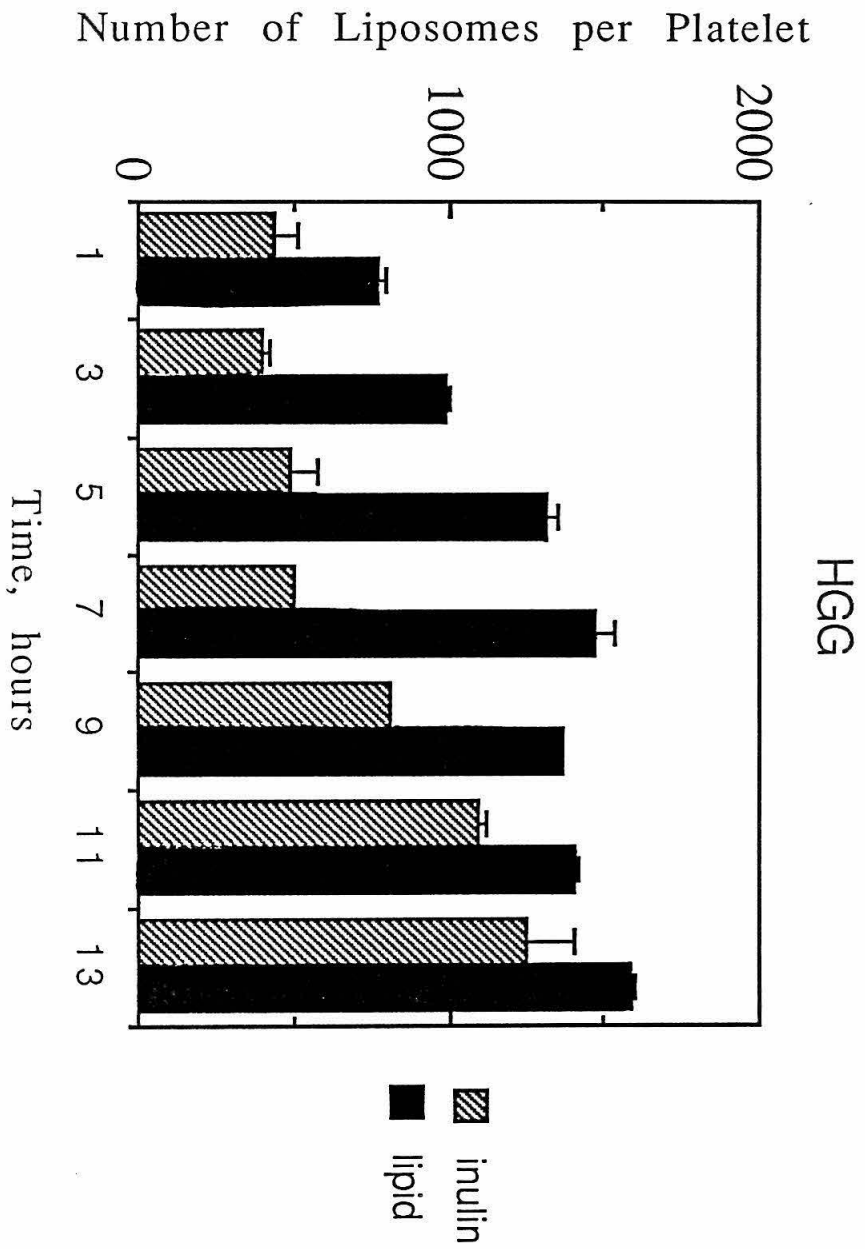

Number of Liposomes per Platelet
0
1000
2000
HGG
1
3
5
7
9
11
13
Time, hours
inulin
lipid

Figure 3: Platelet uptake of ^{3}H-cholesterylhexadecyl ether (■) (n=6) and ^{3}H-inulin (❑) (n=3) HGG liposomes versus time. 3 x 10^{8} platelets were incubated with 7.1 x 10^{11} liposomes at 37°C for varying amounts of time.

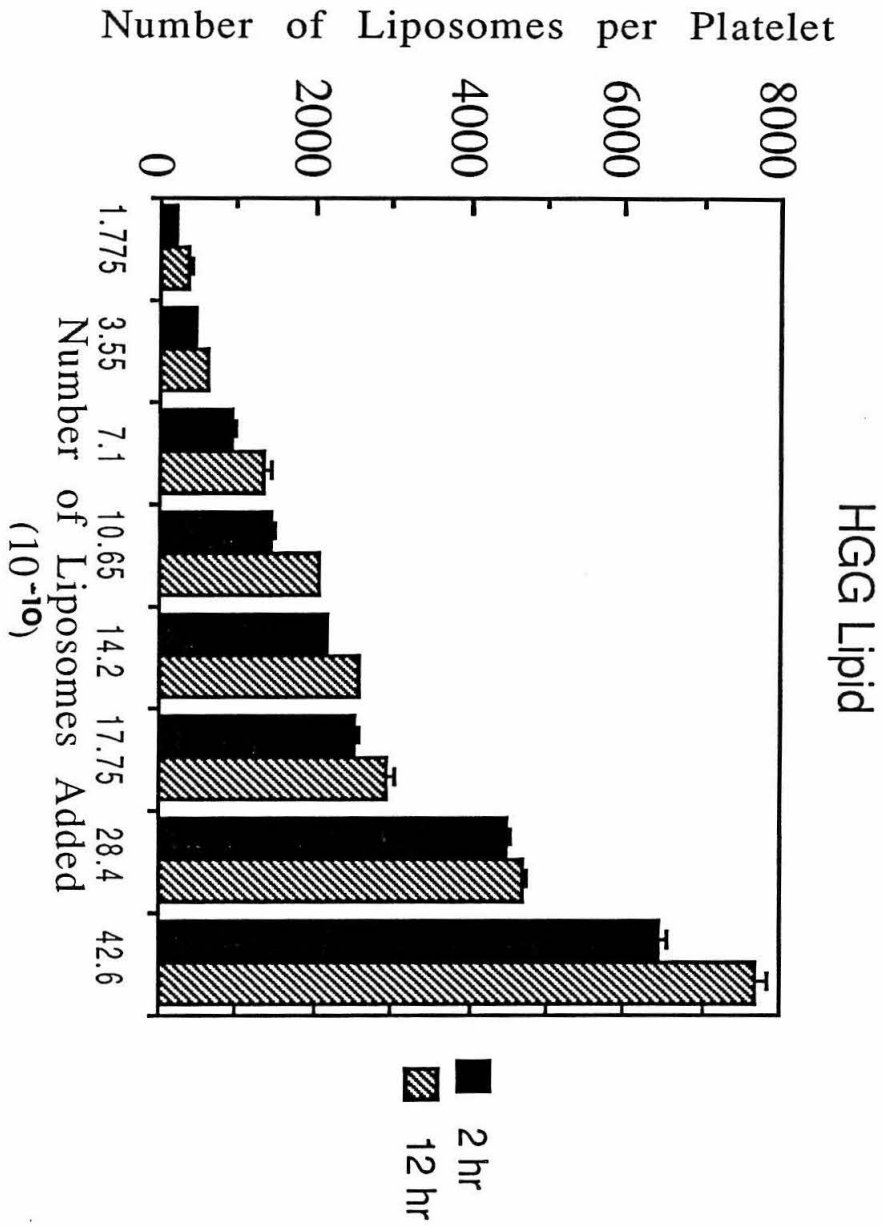

Number of Liposomes per Platelet
8000
6000
4000
2000
0
HGG Lipid
2 hr
12 hr
1.775
3.55
7.1
10.65
14.2
17.75
28.4
42.6
Number of Liposomes Added
(10-10)

Figure 4: Platelet uptake of ^{3}H-cholesterylhexadecyl ether HGG liposomes versus liposome concentration. 3 x 10^{8} platelets were incubated with varying numbers of liposomes at 37°C for 2 (■) and 12 hours (❑) (n=4).

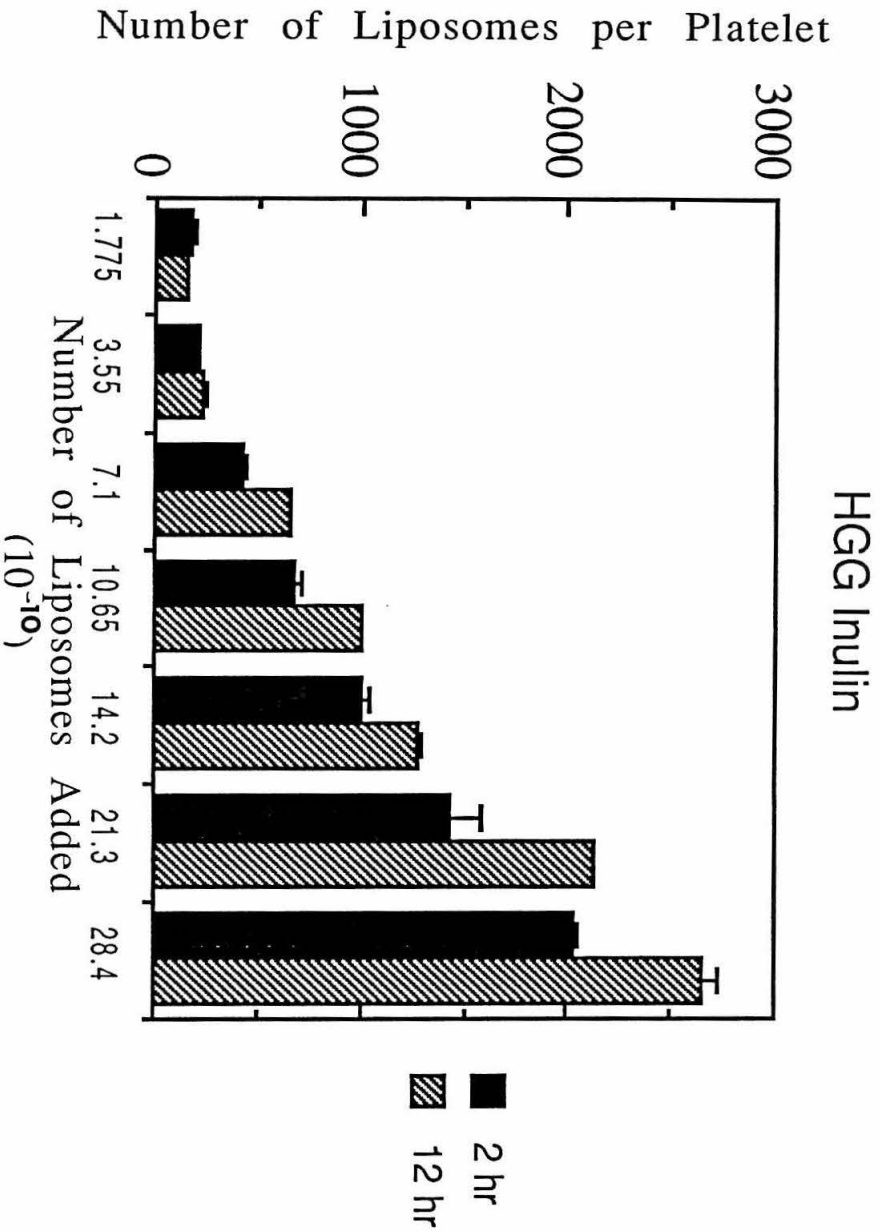

Number of Liposomes per Platelet
3000
2000
1000
0
HGG Inulin
1.775
3.55
7.1
10.65
14.2
21.3
28.4
Number of Liposomes Added
(10^{-10})
2 hr
12 hr

Figure 5: Platelet uptake of ^{3}H-inulin HGG liposomes versus liposome concentration. 3 x 10^{8} platelets were incubated with varying numbers of liposomes at 37°C for 2 (■) and 12 hours (❑) (n=4).

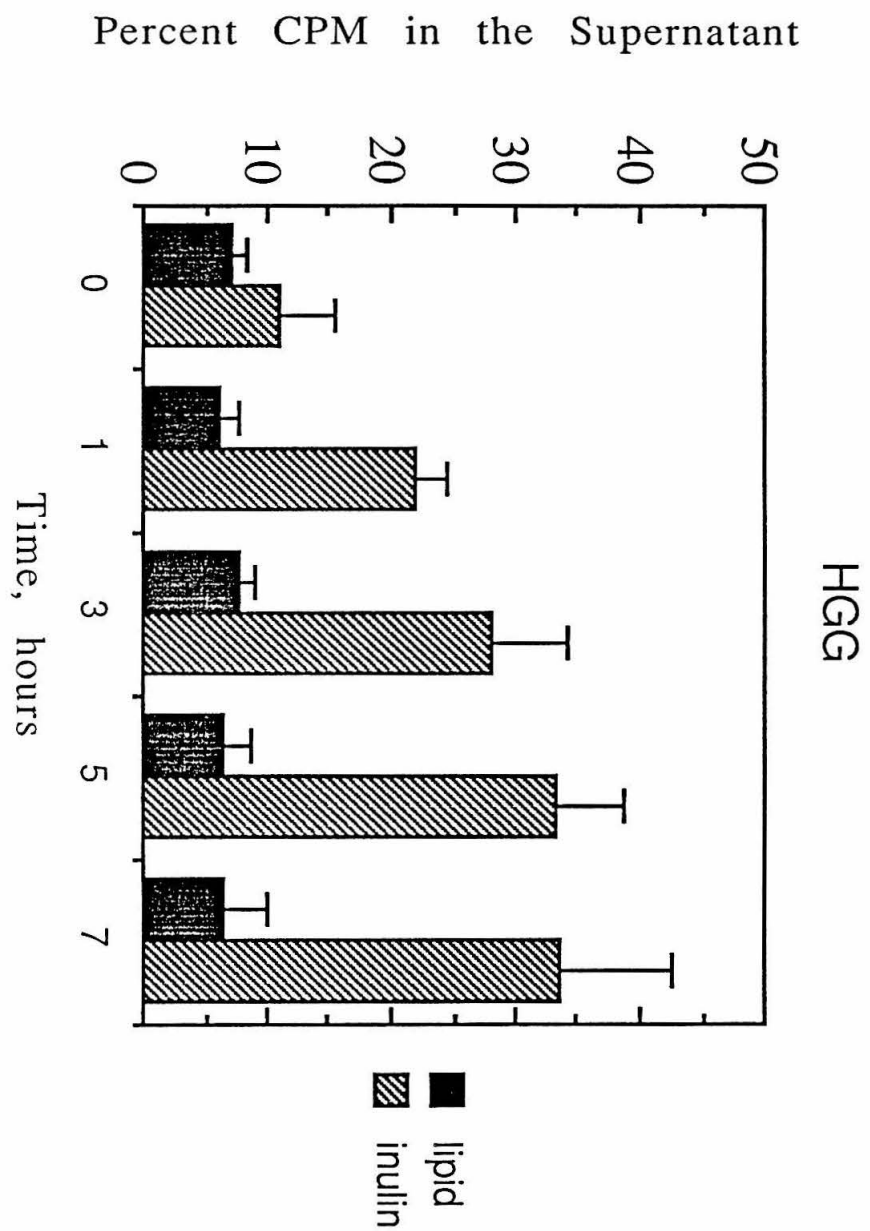

Percent CPM in the Supernatant
0
10
20
30
40
50
HGG
0
1
3
5
7
Time, hours
lipid
inulin

Figure 6: Release studies of lipid and aqueous probes. 3×10^8 platelets were incubated with 14×10^{11} ^{3}H-cholesterylhexadecyl ether (■) or ^{3}H-inulin (❑) HGG liposomes at 37°C for 3 hours. Cells were pelleted, washed twice with buffer, resuspended in buffer, and incubated for various incubation times at 37°C. Percentages of ^{3}H-cholesterylhexadecyl ether and ^{3}H-inulin in the supernatants were determined (n=4).

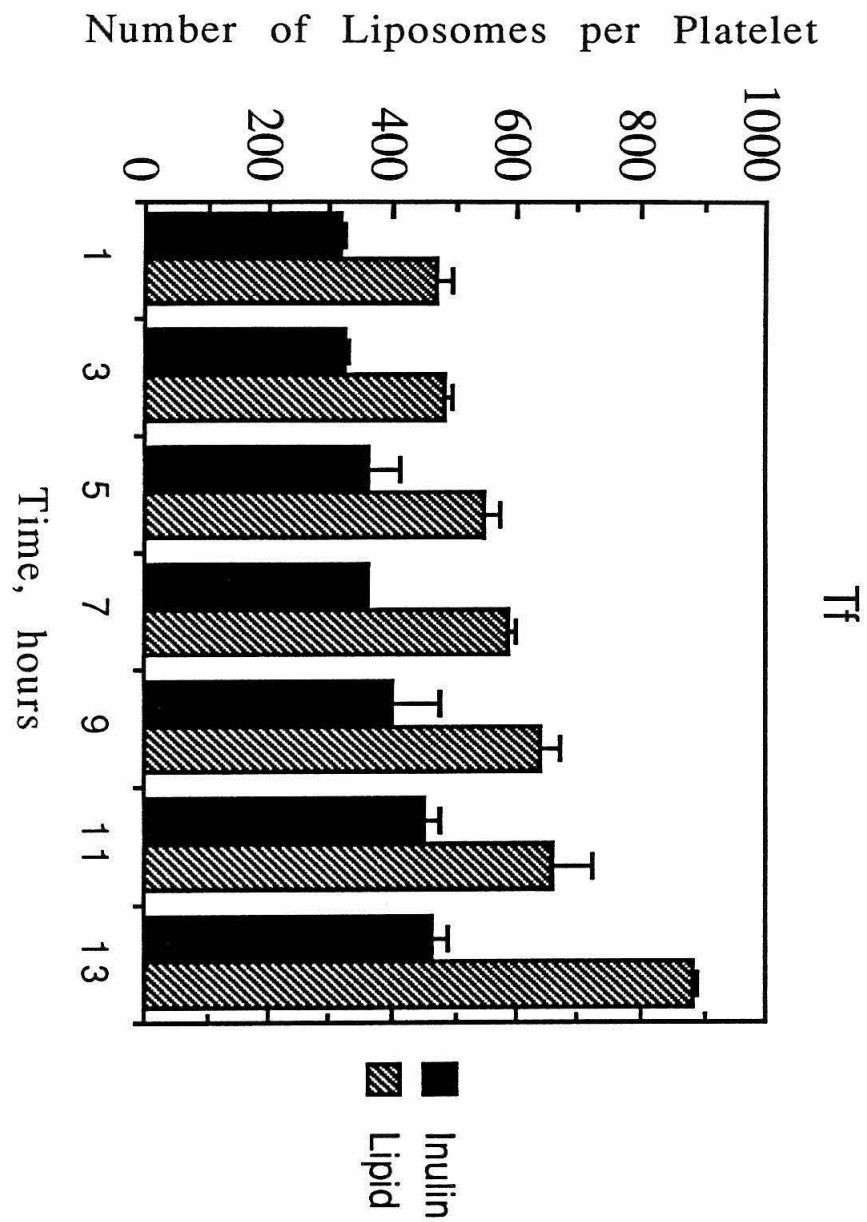

Number of Liposomes per Platelet
0
200
400
600
800
1000
Tf
1
3
5
7
9
11
13
Time, hours
Inulin
Lipid

Figure 7: Platelet uptake of ^{3}H-cholesterylhexadecyl ether (■) (n=4) and ^{3}H-inulin (❑) (n=3) Tf liposomes versus time. 3 x 10^{8} platelets were incubated with 7.1 x 10^{11} liposomes at 37°C for varying amounts of time.

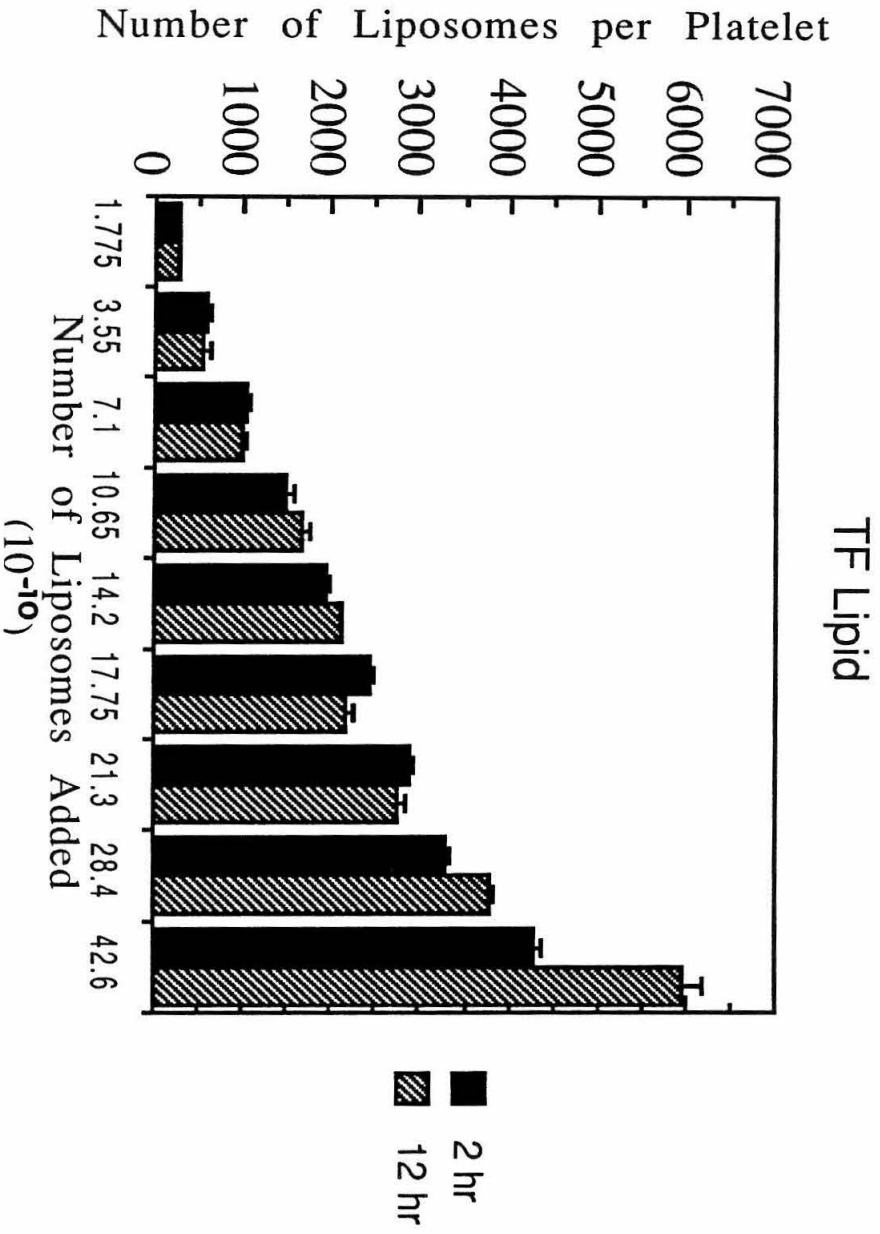

Number of Liposomes per Platelet
7000
6000
5000
4000
3000
2000
1000
0
TF Lipid
2 hr
12 hr
1.775
3.55
7.1
10.65
14.2
17.75
21.3
28.4
42.6
Number of Liposomes Added
(10-10)

Figure 8: Platelet uptake of ^{3}H-cholesterylhexadecyl ether Tf liposomes versus liposome concentration. 3 x 10^{8} platelets were incubated with varying numbers of liposomes at 37°C for 2 (■) and 12 (❑) hours (n=3).

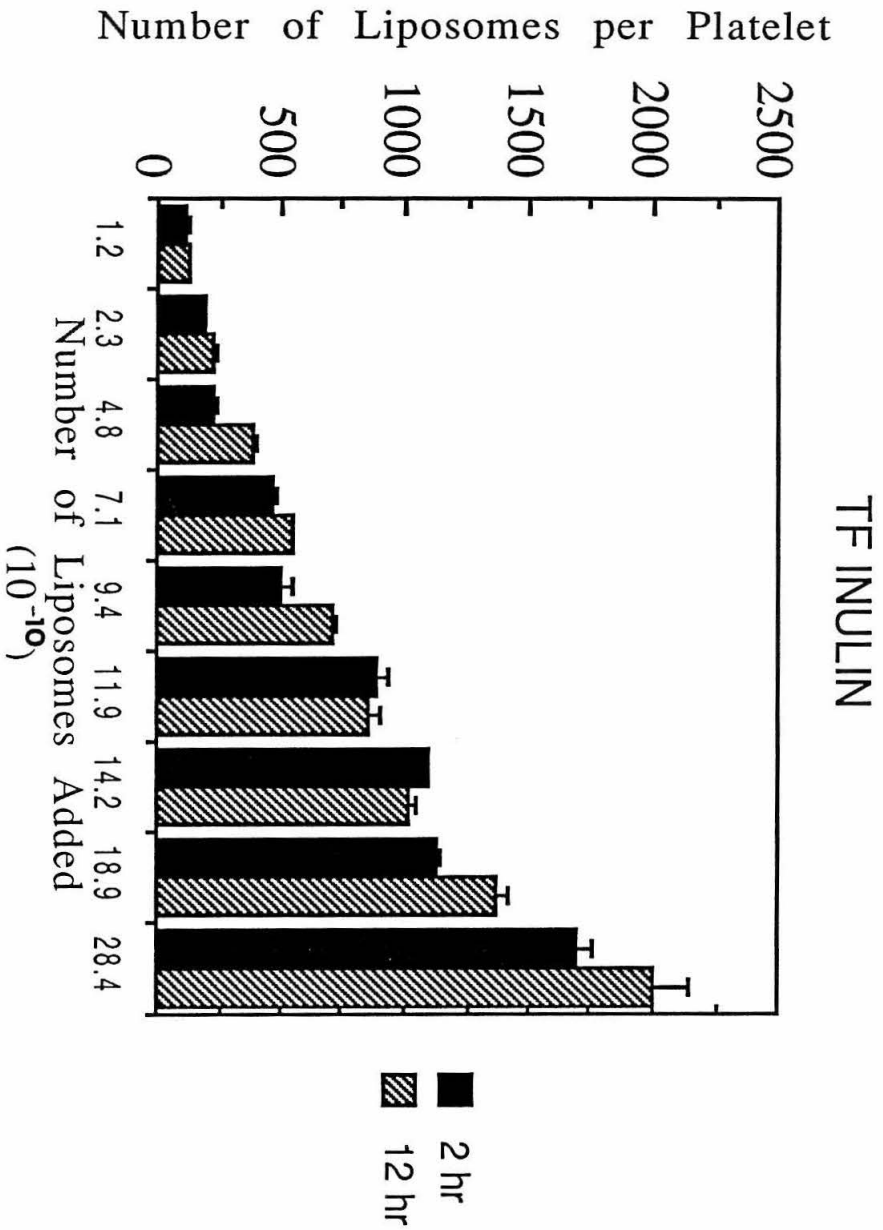

Number of Liposomes per Platelet
2500
2000
1500
1000
500
0
TF INULIN
2 hr
12 hr
1.2
2.3
4.8
7.1
9.4
11.9
14.2
18.9
28.4
Number of Liposomes Added
(10^{-10})

Figure 9: Platelet uptake of ^{3}H-inulin Tf liposomes versus liposome concentration. 3 x 10^{8} platelets were incubated with varying numbers of liposomes at 37°C for 2 (■) and 12 (❑) hours (n=2).

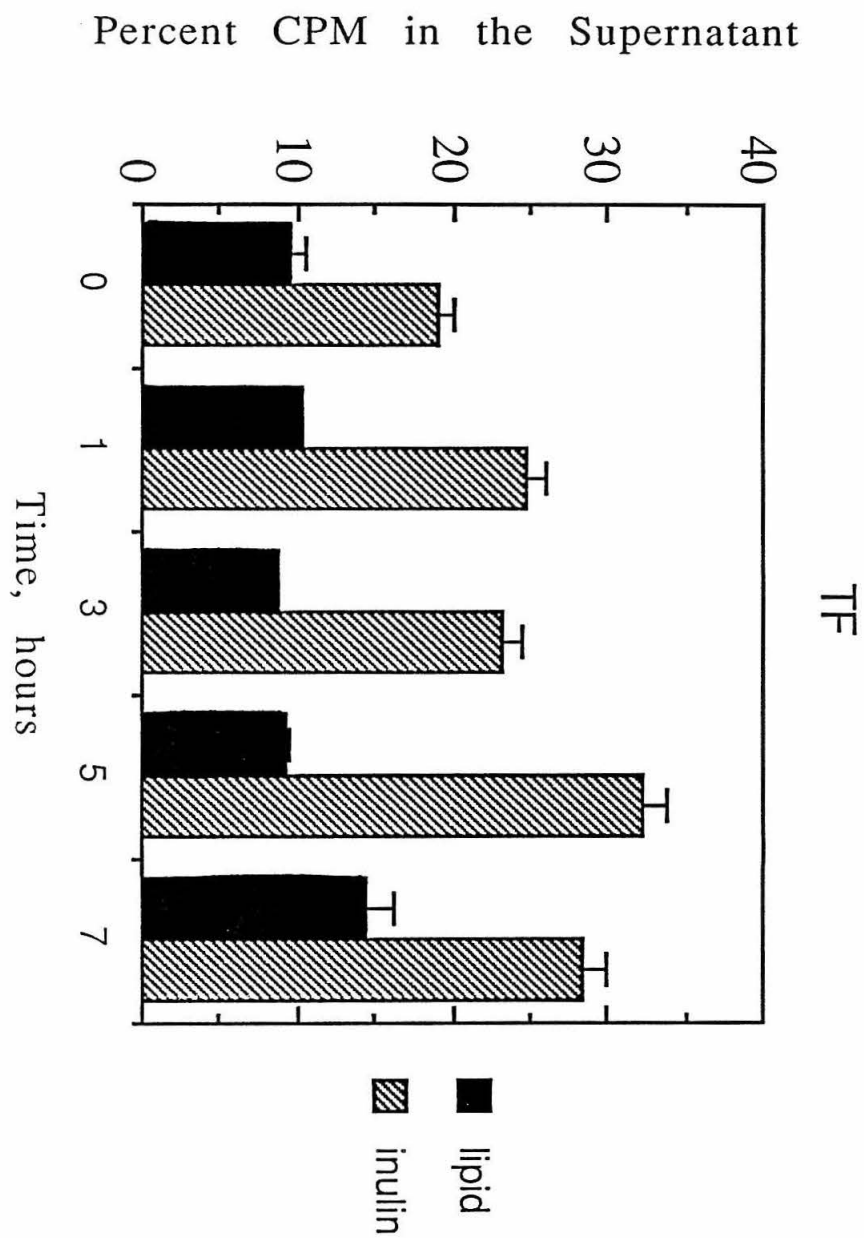

Percent CPM in the Supernatant
0
10
20
30
40
TF
0
1
3
5
7
Time, hours
lipid
inulin

Figure 10: Release studies of lipid and aqueous probes. 3×10^8 platelets were incubated with 14×10^{11} ^{3}H-cholesterylhexadecyl ether (■) or ^{3}H-inulin (❑) Tf liposomes at 37°C for 3 hours. Cells were pelleted, washed twice with buffer, resuspended in buffer, and incubated for various incubation times at 37°C. Percentages of ^{3}H-cholesterylhexadecyl ether and ^{3}H-inulin in the supernatants were determined (n=3).

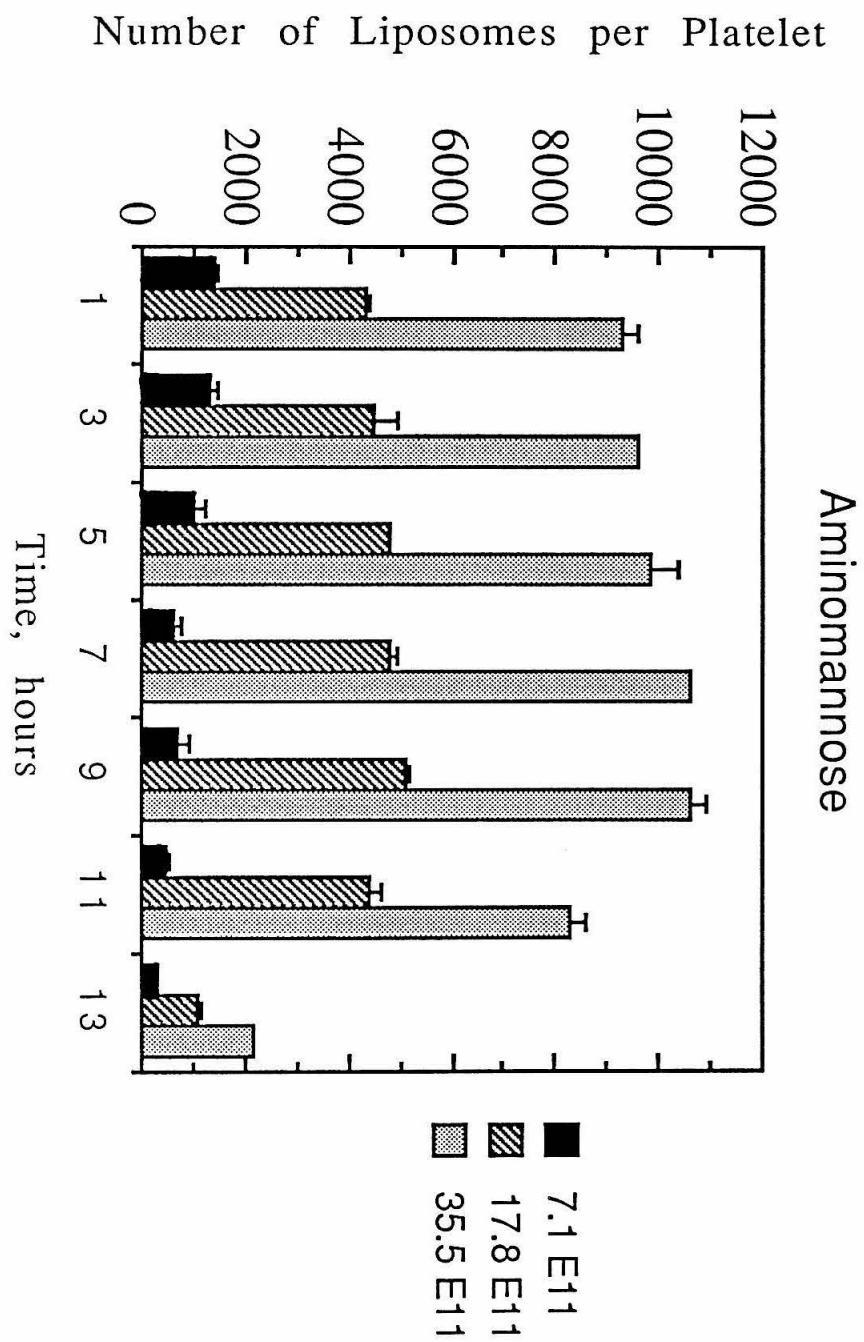

Number of Liposomes per Platelet
12000
10000
8000
6000
4000
2000
0
1
3
5
7
9
11
13
Time, hours
Aminomannose
7.1 E11
17.8 E11
35.5 E11

Figure 11: Platelet uptake of ^{3}H-cholesterylhexadecyl ether Am liposomes versus time. 3×10^{8} platelets were incubated with 7.1×10^{11} (■) (n=6), 17.8×10^{11} (❑) and 35.5×10^{11} (❑) (n=2) liposomes at 37°C for varying amounts of time.

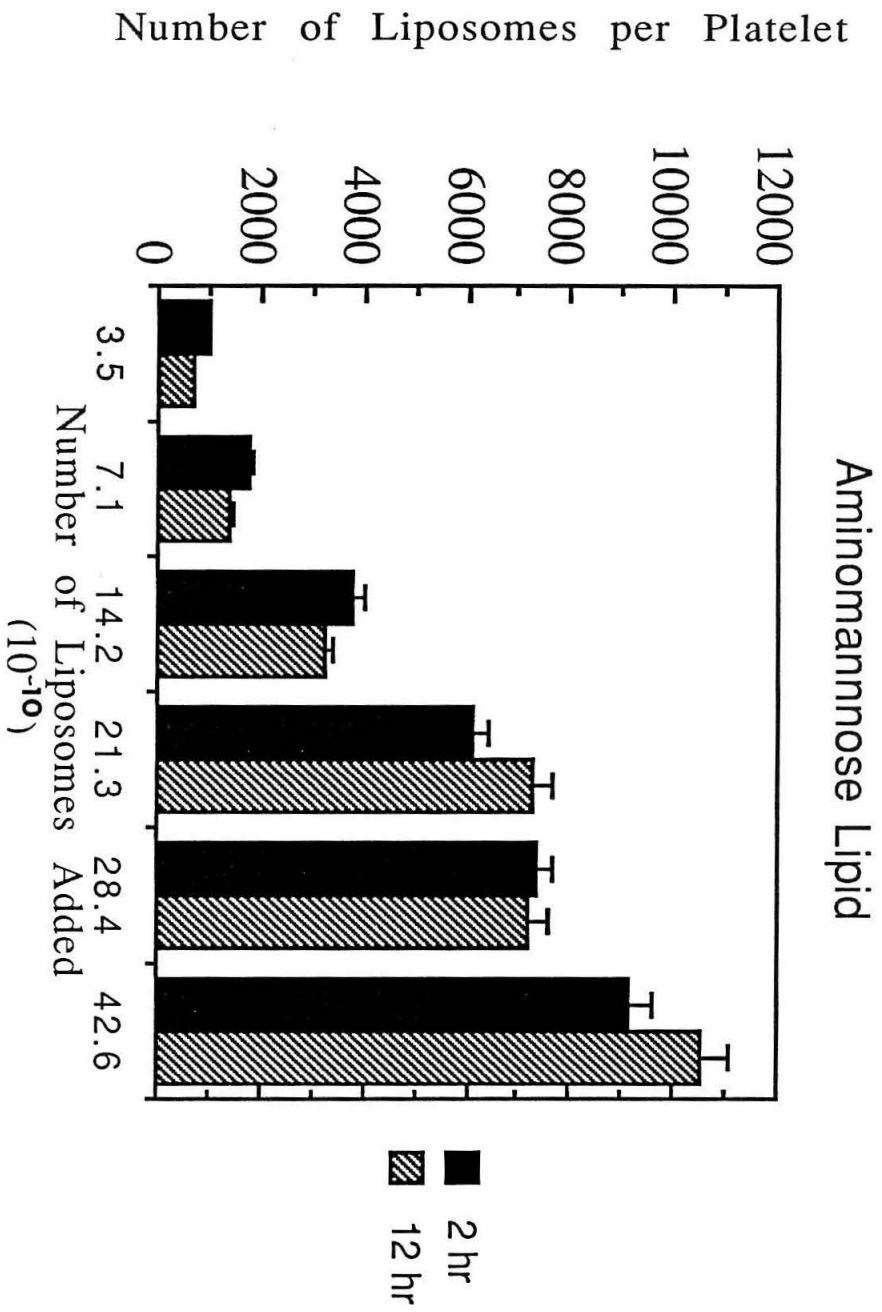

Number of Liposomes per Platelet
12000
10000
8000
6000
4000
2000
0
Aminomannnose Lipid
2 hr
12 hr
3.5
7.1
14.2
21.3
28.4
42.6
Number of Liposomes Added
(10^{-10})

Figure 12: Platelet uptake of ^{3}H-cholesterylhexadecyl ether Am liposomes versus liposome concentration. 3 x 10^{8} platelets were incubated with varying numbers of liposomes at 37°C for 2 (■) and 12 (❑) hours (n=4).

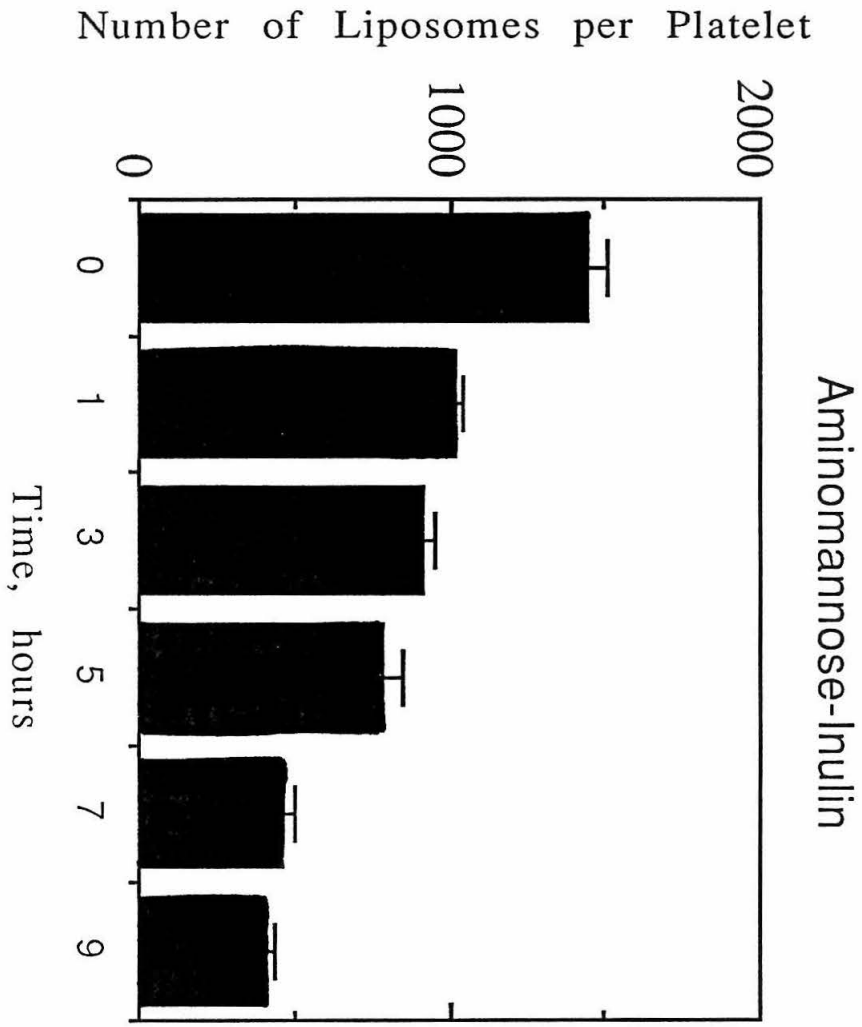

Number of Liposomes per Platelet
2000
1000
0
0
1
3
5
7
9
Time, hours
Aminomannose-Inulin

Figure 13: Platelet uptake of ^{3}H-inulin Am liposomes versus time. 3×10^{8} platelets were incubated with 7×10^{11} liposomes at 37°C for varying amounts of time (n=4).

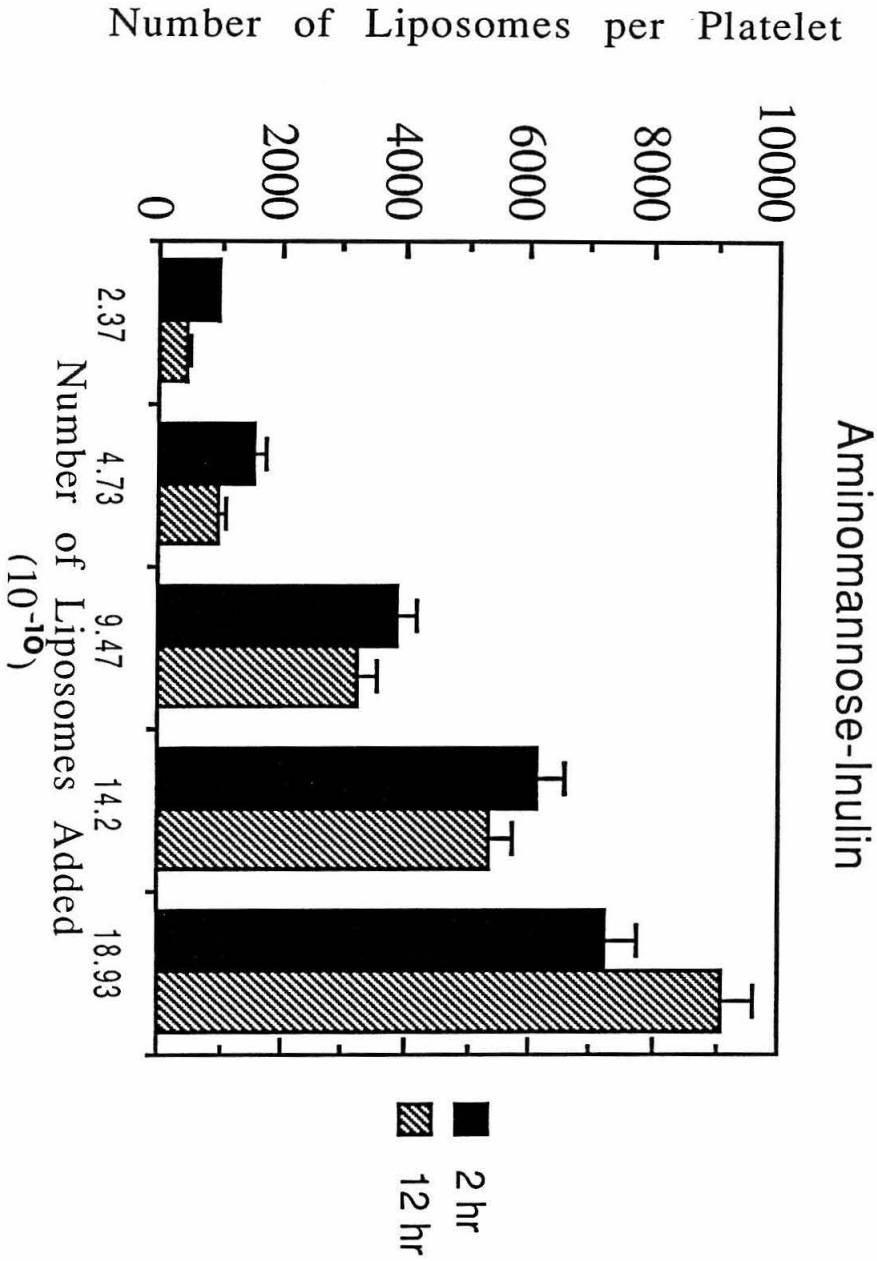

Number of Liposomes per Platelet
10000
8000
6000
4000
2000
0
Aminomannose-Inulin
2 hr
12 hr
2.37
4.73
9.47
14.2
18.93
Number of Liposomes Added
(10^{-10})

Figure 14: Platelet uptake of ^{3}H-inulin Am liposomes versus liposome concentration. 3 x 10^{8} platelets were incubated with varying numbers of liposomes at 37°C for 2 (■) and 12 hours (❑) (n=4).

CHAPTER SIX

CHARACTERIZATION OF THE INTERACTION OF PLATELETS WITH SENDAI VIRUS

Roxanne Male, Wilton E. Vannier, Qizhi He and John D. Baldeschwieler

ABSTRACT

Sendai virus particles have been shown to infect cells via fusion of the viral envelopes with the cell plasma membranes. In addition it has been demonstrated that platelets are capable of phagocytosing particles including latex beads and small neutral unilamellar vesicles. Both possible mechanisms of Sendai virus interaction with human platelets were considered and fusion was found to be the predominant process.

Incubation of platelets with ^{125}I labeled Sendai virus particles (SV) and ^{125}I labeled reconstituted Sendai virus envelopes (RSVE) resulted in uptake of both the virus particles and RSVE. Uptake of the ^{125}I labeled virus particles was proportional to the incubation time and amount of virus added. Incubation of 3×10^8 platelets with either 9.9×10^{10} ^{125}I labeled intact or reconstituted virus particles at 37°C for twelve hours resulted in approximately 125 RSVE per platelet and 50 virus particles per platelet. Uptake measurements of ^{125}I labeled SV or RSVE do not distinguish between fusion and binding mechanisms.

The octadecylrhodamine B (R_{18}) fluorescence assay was used to demonstrate and characterize the fusion of virus particles with platelets. Fusion was found to be maximal at 37°C and pH 7.4. The maximum number of Sendai virus fusion sites per platelet was determined to be approximately 55 virus particles per platelet.

INTRODUCTION

Although the interaction of viruses, including Newcastle Disease Virus and Sendai virus (paramyxovirus), Influenza virus (myxovirus) and Human Immunodeficiency Virus Type 1 (retrovirus), with platelets

have been studied previously, little is known about the mechanisms of the interactions [1-4]. Thrombocytopenia is a prominent feature of many virus infections and it has been proposed that platelets may play an important role in the scavenging of virus particles from the circulation [5].

Removal of sialic acid residues from rabbit platelets, as a result of incubation with Newcastle Disease Virus (NDV), has been shown to result in rapid removal of these cells from circulation [6]. In addition, incubation of human platelets with Sendai virus (SV) *in vitro* has been shown to result in platelet aggregation and secretion, which usually promotes reticuloendothelial uptake.

Influenza virus particles were observed by electron microscopy within the platelet open channel system (OCS) by Danon *et al.* as early as 1959 [7], but the mechanism of interaction between Influenza virus particles and platelets is still uncertain. Jerushalmy *et al.* hypothesized that Influenza virus and Newcastle Disease virus particles are incorporated inside platelets since neuraminidase treatment after virus incubation with the cells did not result in complete elution of either virus from the cells [2]. Using the neuraminidase elution technique, Terada *et al.* concluded that Influenza virus particles were absorbed to the platelet surface and do not undergo endocytosis [3].

Human Immunodeficiency Virus Type 1 (HIV-1) particles have also been observed within platelet vacuoles by electron microscopy [4].

We chose Sendai virus, a murine paramyxovirus, as a model virus to determine the mechanism and kinetics of virus interactions with platelets. Interactions of this virus with a variety of cell lines have been studied extensively by ourselves as well as others and are well

characterized [8-10]. Sendai virus particles are capable of fusing with cell membranes via the function of two glycoproteins [11-13]. The hemagglutinin / neuraminadase (HN) protein mediates binding of the virus particles to the cell surface receptors and the fusion (F) protein is essential for promotion of virus-cell fusion.

In addition to viral fusion, we tested for possible phagocytosis of the Sendai virus particles by the platelets. Platelets are known to phagocytose a variety of particles, including liposomes and latex particles [14,15]. Although both processes can result in viral uptake, the characteristics of viral fusion and platelet phagocytosis are very different. Phagocytosis is energy dependent and is arrested when both oxidative phosphorylation and glycolysis are inhibited [16]. Phagocytosis is also calcium dependent [17]. Fusion is temperature and pH dependent [18] and requires the presence of functional F and HN proteins.

MATERIALS AND METHODS

Purification of Platelets. Platelets were purchased from the American Blood Institute (Los Angeles) and used within 10 hours of collection. This time was necessary for HIV and Hepatitis testing. The platelets, which were stored in citrate phosphate dextrose, were centrifuged at 1,000 g for 15 minutes in a IEC Centra-8 centrifuge. The pellet was resuspended in Tyrodes buffer (0.1% dextrose, 0.1% $NaHCO_3$, 0.02% KCl, 0.8% NaCl and 0.005% Na_2HPO_4 w/w in 1 liter water, pH=7.4) and spun at 110 g for five minutes to remove all red blood cells. The supernatant was then centrifuged at 450 g for 10 minutes; the platelet pellet was resuspended in

Hank's Balanced Salt Solution (HBSS) or modified Tyrodes buffer (Tyrodes buffer plus 1.26 mM $CaCl_2$).

The final purity of the platelet preparation was greater than 95% platelets as assessed by a Coulter S+4 cell counter; the main contaminants were red blood cells.

Determination of the Number of Platelets per mg of Protein. Cell counts were performed with the Coulter S+4 Cell Counter to determine the number of platelets. The Peterson modification of the Lowry protein assay [19] with a bovine serum albumin standard and without TCA precipitation was used to determine the amounts of protein in the same samples. A milligram of protein was found to correspond to 3×10^8 platelets. Meyers *et al.* reported a value of 5.8×10^8 platelets/mg protein [20]. However, they isolated the platelet in an excess of human serum albumin, which might not have been completely removed prior to the protein assay, and did not report the protein standard used. The assay is highly dependent on the molecular weight and aromatic amino acid content of the protein standard.

Preparation of Sendai Virus. Ten day old chicken eggs were each injected with 200 microliters of a 1/500 dilution of Z strain Sendai virus (~1.3×10^{12} virus particles/ml) in PBS (0.90% NaCl, 0.12% $Na_2HPO_4 \cdot 7H_2O$, 0.013% $NaH_2PO_4 \cdot H_2O$ w/w in water, pH 7.3) containing 1% penicillin and 1% streptomycin. Prior to injection the solution was filtered through an 800 nm millipore filter. The inoculated eggs were incubated at 37°C for three days. The eggs were then frozen at -20°C for two hours to reduce bleeding during harvesting. The virus particles were harvested according to the protocols of Hoekstra *et al.* [18] and Maeda *et al.* [21]. The amounts

of protein in the preparations were determined using the Peterson modification of the Lowry protein assay. The virus fusion activities, as assessed by the R_{18} fusion assay, for the various preparations used varied. All data displayed in the same figures were taken from experiments which used the same virus preparations.

^{125}I labeling of the Virus. Approximately 4 x 10^{12} SV or RSVE particles were mixed with 0.5 mCi of Na^{125}I (Amersham, Corp.) in 0.5 mL of HBSS. Forty microliters of a 10 mg/ml solution of chloramine T were added and mixed gently for one minute. Subsequently two hundred microliters of a 4 mg/ml solution of sodium metabisulfite were added and mixed gently for five minutes. Both solutions were made immediately before use. The virus particles were washed twice by centrifugation at 84,000 g in a L 5-65 Beckman Ultracentrifuge using a SW 50.1 rotor for forty minutes to remove excess ^{125}I and resuspended in HBSS. Labeling by this method did not appear to interfere with the virus particles' activities, as assessed by the R_{18} fusion assay. The specific proteins labeled and the activity per mg protein have been previously determined by Markwell *et al.* [22].

Preparation of RSVE. Reconstituted virus particles were prepared using a modification of the procedure of Vainstein *et al.* [23]. 3.9 x 10^{12} SV (2.6 x 10^{12} particles/ml solution) were pelleted at 84,000 g for 40 minutes at 4°C. The pellet was resuspended in 100 microliters of a solution A (100 mM NaCl, 10% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) and 50 mM Tris HCl buffered to pH 7.4) and gently shaken for one hour at room temperature. The mixture was centrifuged under the same conditions as above; the supernatant was removed and shaken with 50 mg of SM-2 beads (Bio-Rad, Inc.) for 3-4 hours to remove

excess detergent. An additional 50 mg of beads and 100 microliters of solution B (100 mM NaCl, 50 mM Tris HCl and 5 mM EDTA buffered to pH 7.4) were then added and gently shaken for 12-14 hours. The supernatant was removed from the beads using a syringe and centrifuged at 84,000 g for 40 minutes at 4°C to obtain the reconstituted virus envelopes which were resuspended in solution B.

Preparation of ^{3}H-inulin Labeled RSVE. ^{3}H-inulin labeled reconstituted virus particles were prepared as above except 50 μCi of ^{3}H-inulin (5,200 molecular weight) (Amersham, Corp.) was added to solution A.

Incubation Procedure. The virus particles and platelets were incubated in 1.5 ml polypropylene centrifuge tubes for the desired incubation times and at the appropriate temperatures with occasional inversion, after which the mixtures were centrifuged at 5700 g for 5 minutes in a Beckman Microfuge 11. The pellets were washed twice in Tyrodes buffer and finally resuspended in 0.5 ml of 0.5 M NaOH/0.5% SDS and incubated overnight at room temperature. Solutions containing ^{125}I were gamma counted with a Beckman Biogamma II; solutions containing ^{3}H-inulin were mixed with 10 ml Safety Solve (Research Products, Inc.), allowed to sit for at least two hours and scintillation counted with a Beckman LS 6000. For each experiment, all samples were done in duplicate; the number of independent experiments (n) is noted in the figure legends or in the text. Bars on the graphs figures represent the range of the data.

Exocytosis Studies. Platelets and ^{125}I labeled or ^{3}H-inulin labeled reconstituted virus particles were incubated as above for 12.0 hours. The mixtures were centrifuged and the platelets washed by centrifugation twice

in HBSS or modified Tyrodes buffer to remove excess RSVE and further incubated at 37°C for varying amounts of time. Both the pellets and supernatants were collected by centrifugation and solubilized in NaOH/SDS solution overnight.

Octadecylrhodamine B (R_{18}) Fluorescent Assay. The fluorescent probe R_{18} has been used by a number of other workers to investigate viral fusion (for a general review see 8). The probe inserts spontaneously into the viral membrane and rapidly equilibrates throughout the target membrane when fusion occurs. The probe self-dequenches by resonance energy transfer and subsequent trivial decay mechanisms due to the overlap of the emission and excitation fluorescence bands. In dilute concentrations (<10% of the lipid content), the fluorescent dequenching is linearly proportional to the concentration of the probe in the membrane [24]. Upon fusion of the virus with a cell, the probe is diluted in the target membrane and is dequenched.

A volume of ten microliters of a 1 mM solution of R_{18} (Molecular Bioprobes, Inc.) in ethanol was added to a one milliliter solution containing approximately 2.6×10^{12} SV (1 mg of protein = 1.3×10^{12} SV [25]) and incubated for one hour at room temperature. Excess R_{18} was removed by centrifugation at 84,000 g for 40 minutes at 4°C. The pellet was resuspended in HBSS or Tyrodes buffer.

Fluorescence measurements were done with a SLM 4800 fluorimeter with a stirred, temperature controlled cuvette by monitoring the emission at 590 nm with an excitation wavelength of 560 nm. The temperature and pH dependences of fusion were determined by performing kinetic experiments of thirty minute duration. R_{18} labeled Sendai virus were

preincubated at the appropriate temperature in the cuvette with 2 ml of HBSS or Tyrodes buffer. Data collection was initiated and after a baseline had been established the platelets were added. At the conclusion of the experiments 1% Triton X-100 was added to determine the maximum fluorescence intensity.

To determine the viral and platelet concentration dependences of fusion, R18 labeled Sendai virus particles were incubated with platelets at 37oC for thirty minutes. Prior to and after incubation, fluorescence measurements were performed. One percent Triton X-100 was added to the samples at the conclusion of each experiment to determine the maximum fluorescence intensity.

HPTS Labeled RSVE. HPTS, 1-hydropyrene-3,6,8-trisulfonic acid (Molecular Probes), is a pH-dependent fluorescent dye which exhibits two major fluorescence maxima (403 and 460 nm) which have a complementary pH dependence in the range 5-9; the peak at 403 is maximal at low pH while the peak at 460 nm is maximal at high pH values. The fluorescence values at 413 and 447 nm are relatively pH-independent and can be used to standardize the concentration of dye associated with the cells. The fraction of dye taken up by phagocytosis and delivered to an acidic compartment is calculated using the 460/413 nm ratio (or 460/447 nm ratio) and the equation:

Fraction phagocytosed = $(ratio_{pH7.4}-ratio_{meas.})/(ratio_{pH7.4}-ratio_{pH6.0})$ where $ratio_{meas.}$ is the 460/413 ratio of the virus treated cells and $ratio_{pH\ 7.4}$ and $ratio_{pH\ 6.0}$ are the 460/413 ratios of virus in buffer and acidified buffer respectively. In the original paper by Daleke *et al.* [26], the second

peak occurred at 450 nm; however, according to our data the peak maximum is at 460 nm.

HPTS labeled RSVE were prepared according to the procedure for RSVE using a modification of solution A (75 mM NaCl, 35 mM HPTS, 10% Triton X-100, 0.1 mM PMSF and 50 mM Tris HCl buffered to pH 7.4). After incubation with HPTS labeled reconstituted virus envelopes for various times, the platelets were washed twice with buffer. Fluorescence excitation spectra (λ_{ex} 395-465 nm, 4 nm bandwidth) were measured at 510 nm emission (4 nm bandwidth) using a SLM 4800 spectrofluorimeter (SLM Instruments, Inc.) with a stirred, temperature controlled cuvette (20°C). The 447 nm fluorescence values were used to standardize the data since degradation of the probe resulted in a peak at 432 nm which interfered with the readings at 413 nm used for data standardization.

Control experiments were performed to assess the leakage of HPTS from the RSVE. No significant leakage occurred within 12 hours.

Thin Section Electron Microscopy. Samples of 3×10^8 platelets were incubated with 1×10^{11} virus for 6 hours at 37°C and washed twice by centrifugation in Tyrodes buffer. The samples were incubated in 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 15 minutes and then centrifuged at 800 g. Postfixing in 2% osmium tetroxide was done in the dark at 4oC for 0.5 to 2 hours followed by centrifugation at 800 g and washing in the buffer. Dehydration was accomplished by immersing the sample in a series of solutions 50% through 100% in ethanol for 5 minutes each. At this point the sample was transferred to a gelatin capsule and centrifuged. LR white, the embedding agent, was added and crosslinked and dried at 4oC for 2-3 days under an ultraviolet lamp. Sliced sections

were stained with 2% uranyl acetate for 5 minutes, then rinsed 50 times and blotted dry. An 8 mM solution of acidic lead citrate was then added and rinsed thoroughly.

In addition, platelets suspended in HBSS or modified Tyrodes buffer incubated with or without virus particles for 1 hour at 37°C and prepared as above were studied to determine changes in platelet morphology under the conditions used for studying the virus particle interaction.

***In vitro* Studies to Assess Platelet Function.** *Serotonin Release Assay.* The serotonin release assay was performed with 3 x 10^8 platelets (0.5 ml final volume), suspended in HBSS or modified Tyrodes buffer and incubated with or without 9.9 x 10^{10} Sendai virus particles for one to seven hours as described [27]. ^{14}C-serotonin loaded platelets were prepared by preincubation in Tyrodes buffer containing 1 mM ^{14}C-serotonin for 0.5 hours, centrifugation to remove excess ^{14}C-serotonin and resuspension in modified Tyrodes buffer containing 2 mM imipramine to prevent reuptake of the probe.

Microaggregation Assay. Platelets suspended in HBSS or modified Tyrodes buffer were incubated at 37°C, with or without Sendai virus particles, for one to nine hours and then fixed with 1% glutaraldehyde. The percentages of aggregated platelets were estimated by examining the cells in a hemocytometer and using the equation, % aggregation = number of cells in aggregates/ total number of cells.

RESULTS

^{125}I-labeled Virus Uptake Studies. To begin characterizing the uptake of Sendai virus by platelets, ^{125}I-labeled virus particles were incubated

with platelets. The incubation time, concentration of the incubated virus and incubation temperature dependences were studied. The values reported for the uptake as determined by the use of ^{125}I do not differentiate between binding, fusion and phagocytosis.

Platelet uptake of the ^{125}I-labeled virus particles increased with increasing incubation times over the 18 hour range studied. Uptake occurred rapidly during the first 3 hours, but then began to saturate (Figure 1). After incubation of 3×10^8 platelets with 9.9×10^{10} virus particles for 12 hours at 37°C, approximately 40 virus particles were associated with each platelet. Viral uptake increased with increasing numbers of incubated virus. Two incubation times are shown in Figure 2. Incubation for 17.5 hours resulted in only slightly greater uptake values than the 12.5 hour incubation which suggests the onset of saturation during these incubation times. Uptake at 37°C, following incubation of 3×10^8 platelets with varying numbers of virus particles for 12 hours, was greater than uptake at 4°C. At both temperatures the uptake increased with increasing numbers of incubated virus (Figure 3). The ratios of platelet associated virus particles to platelets were approximately 50 virus particles/platelet for 37°C and 25 virus particles/platelet at 4°C.

Release Studies of the Lipid and Aqueous Compartments of the RSVE. To study the possible exocytosis of the reconstituted virus particles, the amounts of ^{125}I and ^{3}H-inulin released from platelets after 12.0 hours of incubation with reconstituted virus particles, two centrifugation washes and resuspension in buffer were examined (Figure 4). After 12 hours neither the membrane protein associated probe nor the aqueous phase probe was released.

Competition Assay. A competition assay was performed to study the saturation of viral uptake (data not shown, n=4). Preincubation of 3×10^8 platelets with 0 to 2.0×10^{11} non-radiolabeled virus particles prior to incubation with 9.9×10^{10} ^{125}I-labeled virus particles for preincubation times of 0.5 and 6 hours and incubation times of 2 and 6 hours resulted in decreased uptake with increasing amounts of non-radiolabeled virus particles added. In addition, increased preincubation times resulted in less uptake of the labeled virus particles.

Fusion of Virus With Platelets. The R^{18} fluorescence assay was employed to study fusion of Sendai virus particles with platelets and to characterize the temperature, pH and viral and platelet concentration dependences. Plots of the fluorescence versus time showed biphasic behavior for the fusion of virus particles with platelets. Initially the fluorescence increased rapidly for approximately two minutes and then continued at a slower rate of change until saturation. Fusion was maximal at 37°C and pH 7.4. The ranges of temperature and pH studied were 10°C to 50°C and 5 to 8 respectively (n=4).

In addition, the percent fusion decreased with increasing numbers of virus particles added. The percent fusion is defined as: % Fusion = (F(t) - F(I))/(F(T) - F(I)) where F(t) is the fluorescence at time t, F(I) is the initial fluorescence and F(T) is the total fluorescence. Incubation of 4.5×10^7 platelets (1 mg of protein = 3×10^8 platelets [14]) with 6.5×10^9 to 3.9×10^{10} virus particles (1 mg of protein = 1.3×10^{12} Sendai virus particles [25]) at 37°C for 30 minutes resulted in a decrease in the percent fusion from 10% to 4% while the percent fusion remained constant at approximately 4% at 4°C over the same range (Figure 5a). The percent fusion increased from

4% to 12% when 6.5 x 10^9 virus particles were incubated with 1.5 x 10^7 to 6.0 x 10^7 platelets for 30 minutes at 37°C and decreased from 5% to 2% at 4°C (Figure 5b).

By replotting the data of figure 5 as a reciprocal plot we determined the maximum number of fusion sites per platelet from the values for the y-intercept, the number of platelets per fused virus at infinite dilution. A reciprocal plot graphs the number of platelets per fused virus versus the number of platelets per virus added. Approximately 55 virus particles are capable of fusing with a platelet at 37°C.

Inhibition of Viral Uptake. Fusion and platelet phagocytosis are two very different processes which can each be inhibited using different agents. For all experiments 3 x 10^8 platelet (final volume 0.5 ml) were incubated with 1.2 x 10^{10} to 40 x 10^{10} virus particles at 37°C for 12 hours. The amounts of inhibitors used were estimated from the following publications and the exact amounts determined by uptake/inhibitor concentration dependence studies: gangliosides [28], trypsin and trypsin inhibitor [18], EDTA [17], cytochalsin B [29] and dinitrophenol/iodoacetate [16]. Preincubation of the virus particles with 50 μg/ml of either GD1a or GT1b (Sigma Chemical Co.) at 37°C for 20 minutes decreased uptake by 45% and 78% respectively (n=6). Trypsinization by preincubation with 40 mg/ml of trypsin at 37°C for 20 minutes followed by the addition of 80 mg/ml soybean trypsin inhibitor inhibited uptake by 70% (n=4). Incubation in the presence of 1 mM EDTA, 4 mg/ml cytochalasin B or 2.5 x 10^{-4} M 2,4-dinitrophenol and 5 x 10^{-5} M iodoacetate did not inhibit uptake (n=2).

Gangliosides are known receptors for the HN protein of Sendai virus [28]. The addition of suitable gangliosides blocks the binding proteins on the virus. Trypsinization of the virus particles cleaves the reactive sequence of peptides of the fusion protein that is essential for fusion [18]. EDTA chelates divalent cations whose presence are essential for platelet phagocytosis [17]. Cytochalasin B alters the plasticity of platelet membranes and decreases OCS uptake [29]. The combination of 2,4-dinitrophenol/iodoacetate inhibits glycolysis and oxidative phosphorylation which are necessary to produce energy for phagocytosis [16].

HPTS Labeled RSVE Uptake. In order to determine whether Sendai virus particles were phagocytosed by platelets and subsequently delivered to an acidic compartment, the time dependence of the uptake of fluorescently labeled (HPTS) reconstituted virus particles was measured by fluorimetry. No change in the pH-dependent fluorescence was noted; therefore there was no significant RSVE accumulation in acid-containing vesicles, such as lysosomes or prelysosomal vesicles.

Uptake of 125I-labeled RSVE. To study the uptake of RSVE by platelets, varying amounts of 125I-labeled reconstituted virus particles were incubated with platelets . Assuming that the HN and F proteins are 25% of the total virus protein [30] and are the only proteins present in RSVE, 1 mg of protein should correspond to 5.2×10^9 reconstituted virus particles. Using this conversion factor, more RSVE than virus were taken up by the platelets (Figure 6). Incubation of 3×10^8 platelets with either 9.9×10^{10} virus particles or 9.9×10^{10} reconstituted virus particles at 37°C for 12

hours, resulted in approximately 125 RSVE per platelet and 50 virus particles per platelet.

Electron Microscopy. Electron microscopy was performed to study the effects of HBSS and modified Tyrodes buffer on platelet morphology and to investigate the location of the virus particles with respect to the platelets. Platelets maintained a discoid shape when suspended in modified Tyrodes buffer. However, platelets suspended in HBSS tended to have irregular shapes and approximately half had pseudopodia. Therefore, even though the amounts of uptake by platelets suspended in both buffer are identical, we believe that incubation in modified Tyrodes buffer is superior. A number of virus particles were located within the open channel system (OCS). Examination of a large number of sections (n=30) showed an average of 8 ± 2 virus particles bound to the surface and within the OCS of each cell (Figure 7).

***In vitro* Assays to Assess Platelet Function.** Platelets secreted 15% at 0.17 hr., 62% at 1 hr., 64% at 3 hr, 66% at 5 hr., and 65% at 7 hr. of the radiolabeled serotonin when incubated with Sendai virus particles and 18% at 0.16 hr., 19% at 1hr., 19% at 3 hr., 20% at 5 hr., and 28% at 7 hr. when incubated without Sendai virus particles (n=2). Incubation with Sendai virus also resulted in platelet microaggregates (<15 cells per aggregate): 18% at 0.17 hr., 16% at 1 hr., 16% at 3 hr., 15% at 5 hr., and 12% at 7 hr. as compared to 1% at 0.16 hr, 2% at 1 hr., 2.5% at 3 hr., 3% at 5 hr., and 8% at 7 hr. in the absence of virus particles (n=2). It is interesting to note that microaggregation occurred without significant secretion during the first several minutes of incubation. Secretion and aggregation in the absence of virus particles may have been due to the unfavorable

conditions that exist when cells are kept in small polypropylene tubes for extended periods of time at 37°C without stirring. In addition secretion may have been caused by the presence of imipramine which can induce small amounts of leakage.

DISCUSSION

Two possible mechanisms of platelet/Sendai virus interaction: viral fusion and platelet phagocytosis, were examined. Sendai virus fusion is a receptor mediated process in which the virus envelope fuses with the cell membrane and the envelope's contents are deposited into the cell's cytoplasm. Platelet phagocytosis of particles begins with particle sequestration within the OCS and is followed by localization and degradation of the particles within acid-containing vacuoles and eventual exocytosis of the aqueous components of the particle. Note, platelet phagocytosis, as defined in this paper, is not receptor mediated and should not be confused with receptor-mediated endocytosis. Fusion was determined to be the dominant process. However, binding of the virus particles to the platelets was also significant, as shown by the uptake data taken at 4°C.

Initially we examined the interactions of ^{125}I labeled virus or reconstituted virus particles with platelets. Uptake values using ^{125}I labeled SV or RSVE experiments do not differentiate between fusion and phagocytosis and the data reported reflect all processes of uptake including binding. Uptake of ^{125}I labeled virus particles was time dependent and showed saturation at longer incubation periods. Uptake was also dependent on the number of virus particles incubated with the platelets

and increased with increasing virus concentration until saturation at high concentrations. Platelet uptake of virus particles at 37°C was approximately twice that at 4°C. The majority of Sendai virus associated with the cells at 4°C were probably bound to the platelets' surfaces since fusion and phagocytosis are negligible at this temperature. Uptake values for 125I labeled reconstituted virus particles exceeded those for ^{125}I labeled intact virus. Although there is no clear explanation for this difference, Harmsen *et al.* [31] suggested that a disturbance of the membrane lipid asymmetry may affect the functional properties of the reconstituted proteins. In addition, Lee *et al.* [32] suggested a possible correlation between the fusion capacity of Sendai virus particles and the rotational mobility of the F protein; the fusion activity increases with increasing mobility. In the native membrane the rotational freedom of the F protein may be more restricted than in the RSVE due to the rigidity of the intact virus membrane.

Virus localization within the OCS, as shown by electron microscopy, suggested that platelet phagocytosis might be occurring. However, there was no accumulation of virus in acid-containing vesicles as detected by HPTS, a pH-sensitive probe. Retention of both the aqueous components and the protein of the virus is also inconsistent with results expected for platelet phagocytosis, as previously shown in studies of platelet phagocytosis of liposomes [14]. In addition, preincubation of platelets with non-radiolabeled virus resulted in decreased uptake of radiolabeled virus. For experiments with similar amounts of liposomes and platelets, where uptake by the platelets is believed to occur via phagocytosis, uptake is not saturable [14]. Since fusion is receptor mediated, we believe that blockage

of the receptors by unlabeled virus particles or free gangliosides reduced the availability of the receptors to the labeled particles and decreased fusion and uptake.

Fusion was monitored using the R_{18} fluorescence assay. The fusion plots of Sendai virus particles with platelets are similar to those seen for other cells [8-10]. Fusion showed biphasic kinetics; initially fusion increases rapidly over two minutes and then proceeded more slowly until the fusion was saturated. The percent fusion decreases with increasing amounts of incubated virus and increased with increasing amounts of platelets added. The low percent fusion seen at 4°C may represent fusion-independent transfer of the R_{18} probe. As expected, fusion was maximal at 37°C and pH 7.4.

To further support the hypothesis that fusion is the predominant process, the platelets and/or virus particles were incubated with agents known to inhibit fusion or phagocytosis. The addition of the gangliosides GD1a and GT1b to the virus particles prior to incubation with the platelets inhibited uptake. Trypsinization of the virus particles prior to incubation also decreased uptake. However, uptake was not inhibited by incubation in EDTA, preincubation of the cells with cytochalasin B or the addition of 2,4-dinitrophenol and iodoacetate to the platelet solution. Therefore, we conclude that the predominant interaction between platelets and Sendai virus particles is fusion.

Reconstituted Sendai virus envelopes have been previously used in vitro to deliver drugs to cells (for a general review, 33). Various lipophilic and aqueous substances can be introduced during reconstitution which can be delivered to various cells via fusion. The results of our platelet

function assays and those of Chernesky *et al.* [1] indicate that incubation with Sendai virus particles adversely affects platelet function. Therfore, the use of RSVE for drug delivery to platelets may not be feasible unless the concentration of RSVE can be kept below that required to induce aggregation and secretion. As of now, the concentration dependence of platelet secretion and aggregation has not been studied.

ACKNOWLEDGMENTS

We are indebted to Chris Di Simone for his advice about Sendai virus. We would also like to thank Alex Wein for his help in preparing the reconstituted virus. Funding for this work was provided by ARO grant #DAAL-03-87-K-0044, and the Caltech Consortium in Chemistry and Chemical Engineering; Founding Members: E.I. du Pont de Nemours and Company, Inc., Eastman Kodak Company, Minnesota Mining and Manufacturing Company, Shell Oil Company Foundation.

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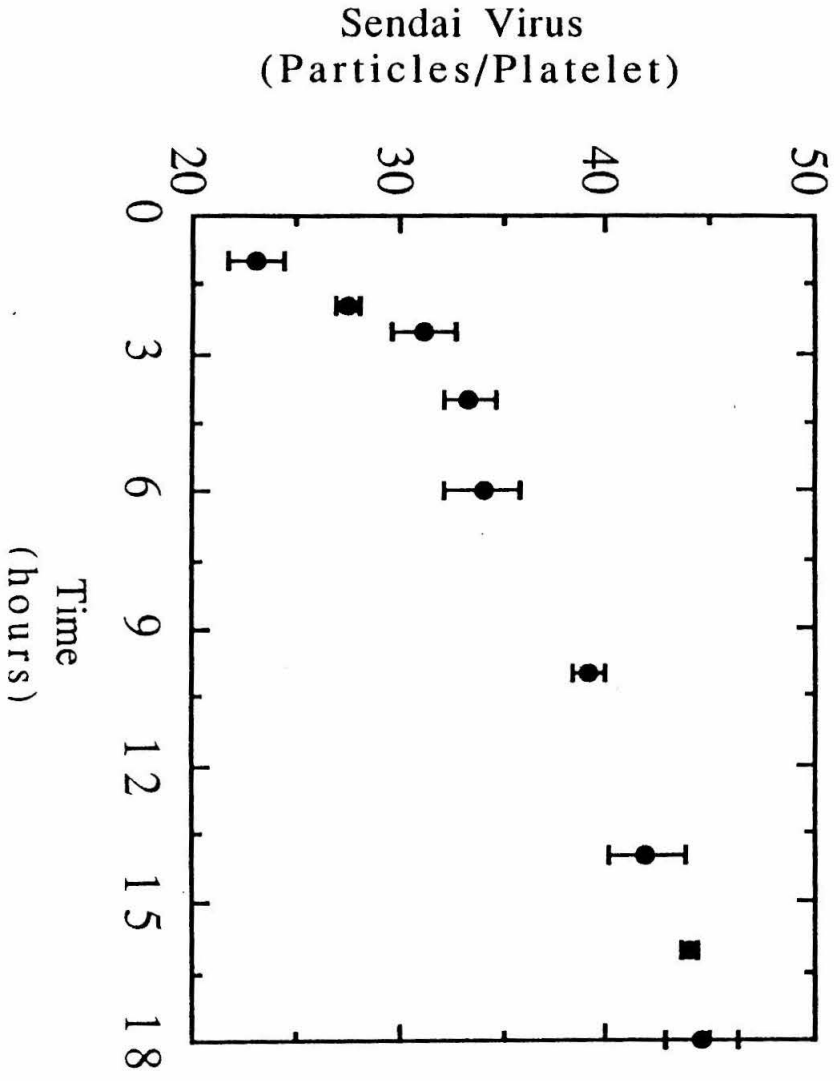

Sendai Virus
(Particles/Platelet)
20
30
40
50
0
3
6
9
12
15
18
Time
(hours)

Figure 1: Incubation time dependence of platelet uptake of ^{125}I labeled Sendai virus. 3×10^8 platelets were incubated with 9.9×10^{10} virus at 37°C for varying incubation times (n=2).

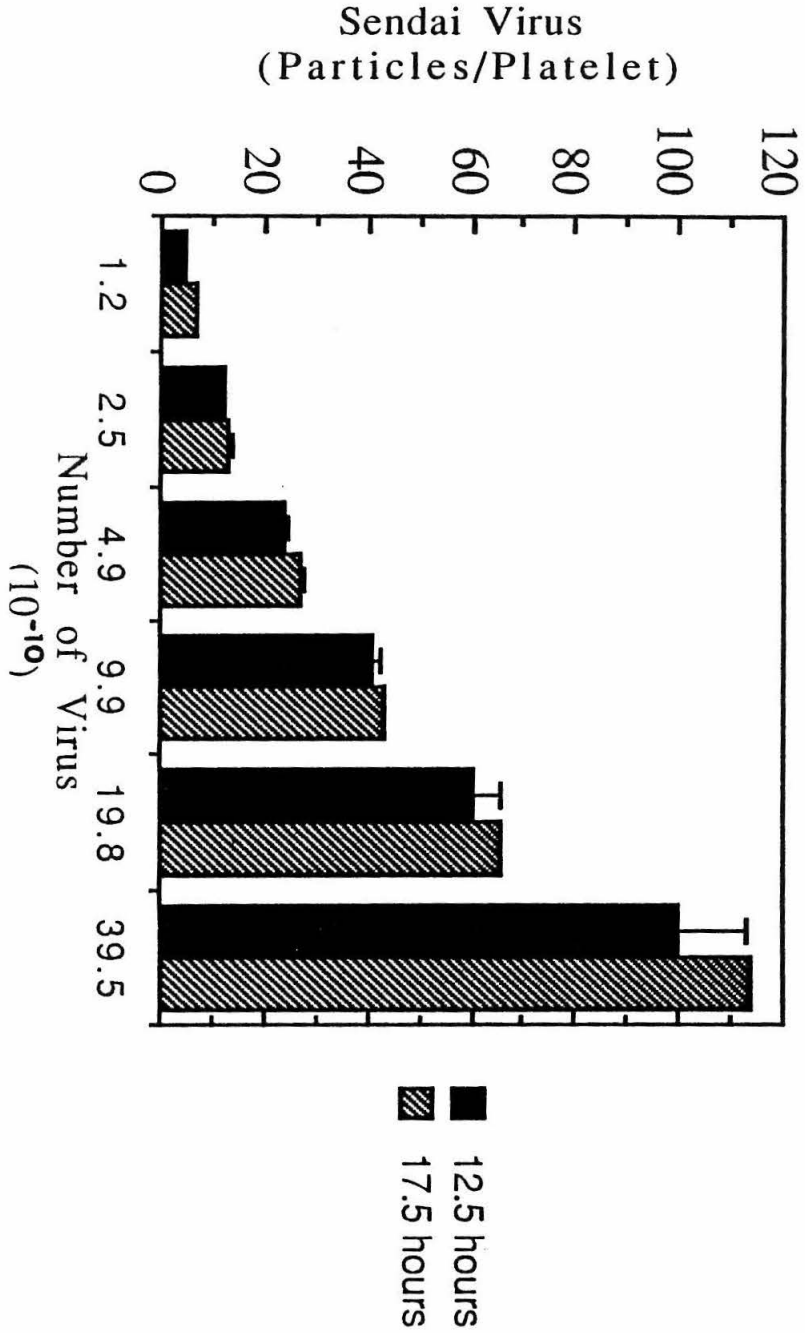

Sendai Virus
(Particles/Platelet)
0
20
40
60
80
100
120
1.2
2.5
4.9
9.9
19.8
39.5
Number of Virus
(10^{-10})
12.5 hours
17.5 hours

Figure 2: Sendai virus concentration dependence of platelet uptake of ^{125}I labeled virus. 3×10^8 platelets were incubated with varying amounts of ^{125}I labeled virus at 37°C for 12.5 hours (■) or 17.5 hours (❑) (n=2).

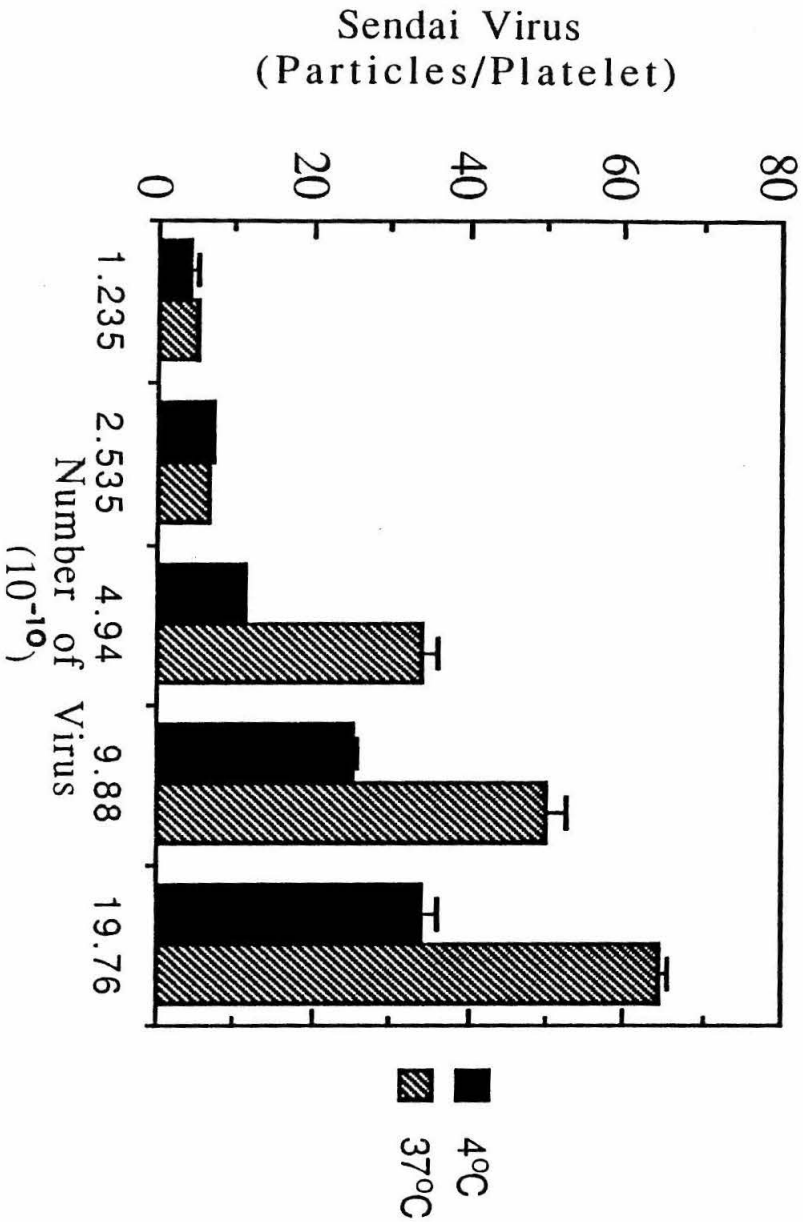

Sendai Virus
(Particles/Platelet)
0
20
40
60
80
1.235
2.535
4.94
9.88
19.76
Number of Virus
(10^{-10})
4°C
37°C

Figure 3: Temperature dependence of platelet uptake of ^{125}I labeled virus. 3×10^8 platelets were incubated with varying amounts of ^{125}I labeled virus at 37°C (❑) and 4°C (■) for 12.0 hours (n=2).

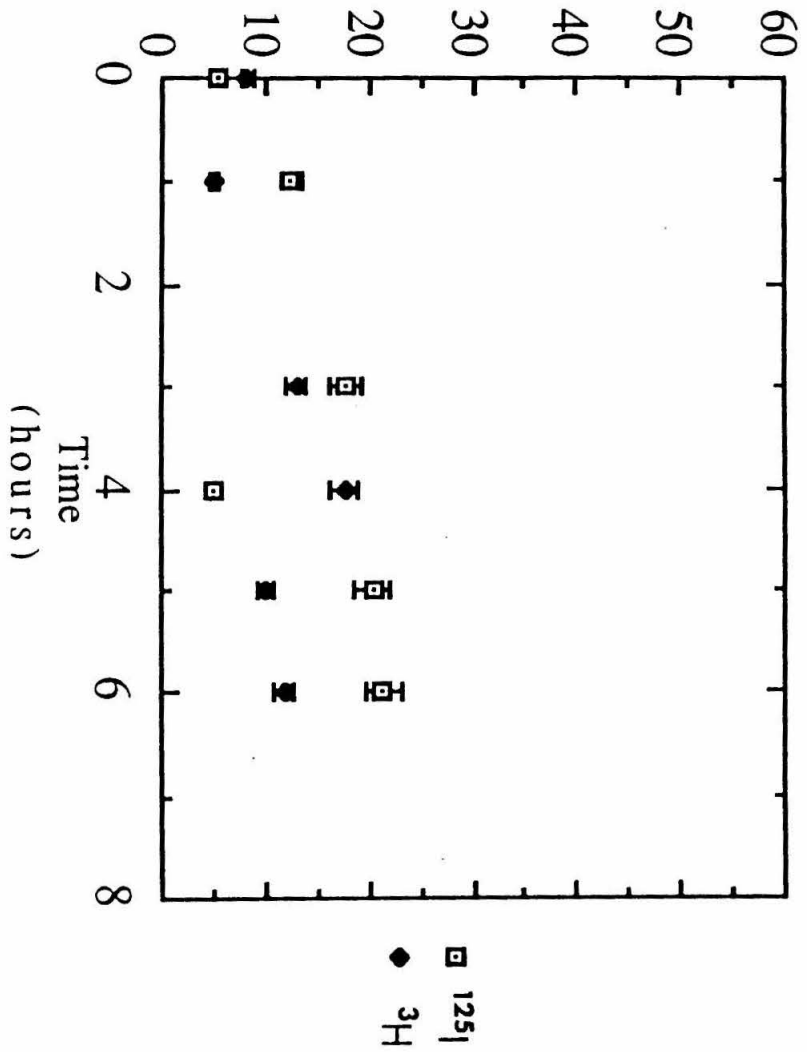
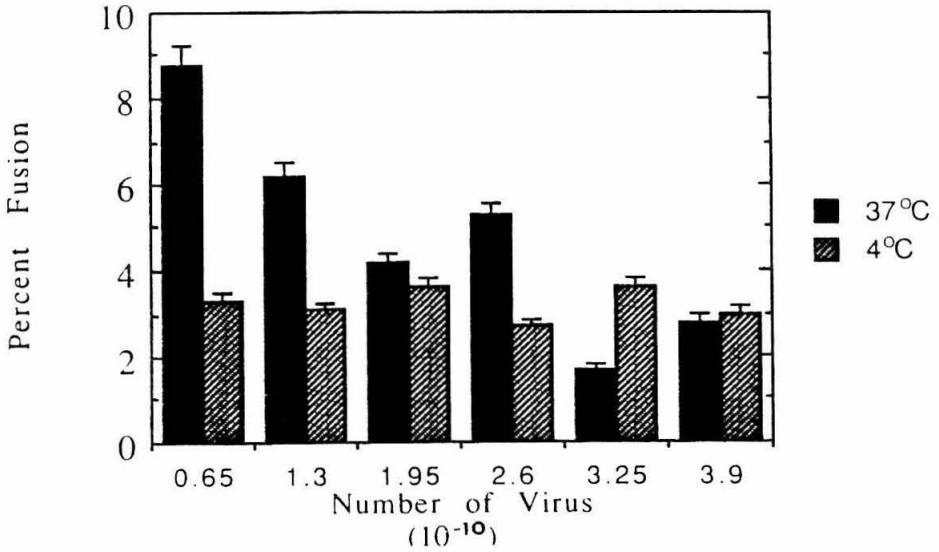

Percent CPM in the Supernatant
0
10
20
30
40
50
60
0
2
4
6
8
Time
(hours)
125I
3H

Figure 4: Release studies of the protein associated and aqueous probes. 3 x 10^8 platelets were preincubated with 9.9 x 10^{10} ^{3}H-inulin labeled RSVE or 9.9 x 10^{10} ^{125}I labeled virus at 37°C for 12.0 hours. The cells were then pelleted, washed twice in buffer and incubated for various incubation times at 37°C. The percentages of the ^{3}H-inulin (●) and the ^{125}I (○) labels in the supernatants were determined (n=2).


Percent Fusion
10
8
6
4
2
0
0.65
1.3
1.95
2.6
3.25
3.9
Number of Virus
(10-10)
37°C
4°C

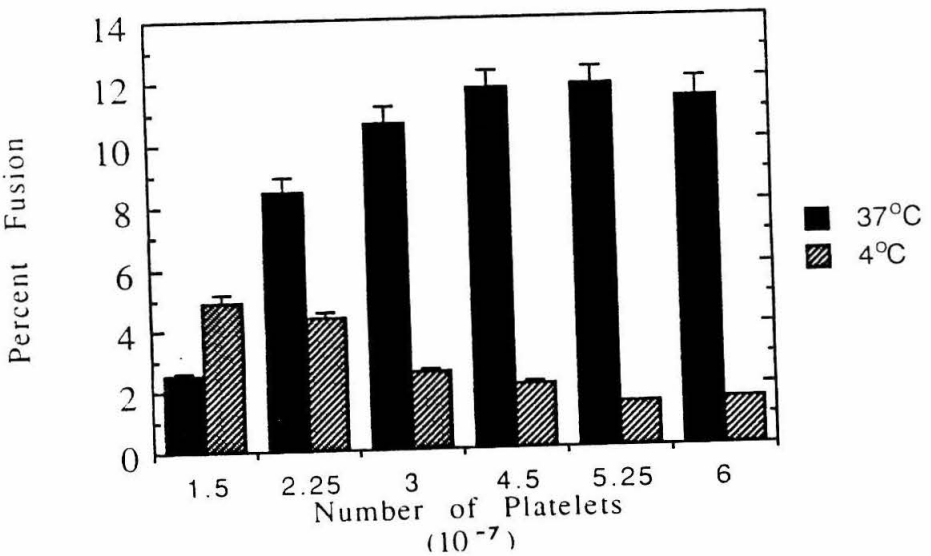

Percent Fusion
14
12
10
8
6
4
2
0
1.5
2.25
3
4.5
5.25
6
Number of Platelets
(10-7)
37°C
4°C

Figure 5: Concentration dependence of fusion. (A) 4.5 x 10^7 platelets were incubated with varying amounts of R_{18} labeled virus at 37°C (■) and 4°C (❑) for 30 minutes (n=2). (B) 6.5 x 10^9 R_{18} labeled Sendai virus particles were incubated with varying amounts of platelets at 37°C (■) and 4°C (❑) for 30 minutes (n=2).

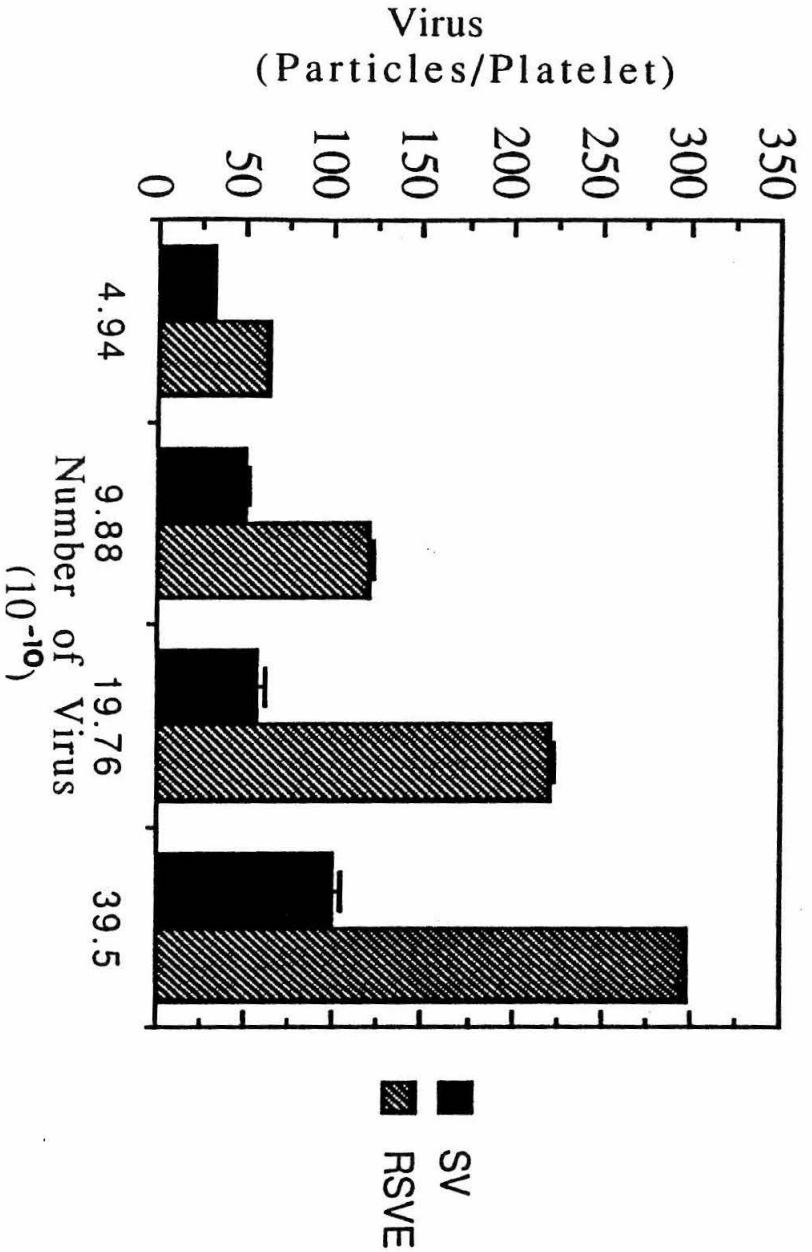

Virus
(Particles/Platelet)
0
50
100
150
200
250
300
350
4.94
9.88
19.76
39.5
Number of Virus
(10^{-10})
SV
RSVE

Figure 6: Reconstituted virus concentration dependence of platelet uptake of ^{125}I-labeled reconstituted virus. 3 x 10^8 platelets were incubated with varying amounts of ^{125}I-labeled reconstituted virus (❑) and ^{125}I-labeled virus (■) at 37°C for 12.0 hours (n=2).

Figure 7: Electron micrograph of a platelet containing virus. 3×10^8 platelets were incubated with 1×10^{11} virus for 6.0 hours at 37°C. Note the virus within the open channel system (n=2). Bar, 0.1 mm.

APPENDIX A

ASSESSMENT OF THE THROMBOLYTIC EFFICACY OF LIPOSOME ENCAPSULATED STREPTOKINASE

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ABSTRACT

The thrombolytic efficacy of streptokinase encapsulated in 1-palmitoyl 2-oleoyl phosphatidylcholine large unilamellar vesicles (LUV) in close-chested dogs 1 hour post-artery occlusion and 15 minutes and 1 hour post-injection was studied. No evidence was found that liposome encapsulation enhances the thrombolytic efficacy of streptokinase.

INTRODUCTION

The formation of an occlusive thrombus (clot) within an atherosclerotic artery is the principal event which initiates acute myocardial infarction (heart attack) (1-3). The vast majority of deaths following myocardial infarction occur within hours of the initial occlusive event (4). Canine studies have shown that 30-40% of the infarcted area is lost at 40 minutes, and 80-90% is lost at 3 hours (5). Though one must be cautious in applying these results to humans, clinical studies in humans do support the contention that myocardium loss is rapid and significant before 3-6 hours (6-8).

Recent therapies for the rapid restoration of vessel patency, along with decreased mortality and improved ventricular function, have included the use of fibrinolytic agents, especially plasminogen activators. Fibrinolytic agents convert plasminogen to plasmin which initiates lysis of the thrombus. Plasminogen activators such as streptokinase (SK), urokinase, tissue plasminogen activator (t-PA) and genetically engineered one- and two-chain versions of t-PA and urokinase have been administered effectively by intravenous infusion. However, the risk of hemorrhage, as a result of treatment with any of these thrombolytic

agents, is significant. For patients treated with SK or urokinase, the risk of bleeding complications is between 23 and 47% (9).

Although SK can induce immunological side-effects (10) and cause hemorrhaging (9), it is currently the least expensive and most widely used plasminogen activator. In order to decrease unwanted side-effects, prevent serum protein inactivation and enhance myocardium uptake, several investigators have encapsulated SK in liposomes (11,12). Encapsulation of SK in 1-palmitoyl 2-oleoyl phosphatidylcholine large unilamellar vesicles (LUV) has been shown to decrease the *in vitro* clot dissolving time by 24% in the presence of plasma proteins, as compared to free SK (11). Caride *et al.* reported liposome accumulation in canine ischemic myocardium 24 hours following coronary occlusion in dogs (13). Accumulation was dependent on the net charge of the liposome membrane. Positively charged egg phosphatidylcholine:cholesterol: stearylamine (7:1:2 mole ratio) multilamellar vesicles (MLV) accumulated within the infarcted myocardium; negatively charged egg phosphatidylcholine:cholesterol: dicetylphosphate (8:1:1 mole ratio) MLV did not accumulate. Cole *et al.* found no evidence of accumulation of phosphatidylcholine: cholesterol:stearylamine (8:1:1 mole ratio) small unilamellar vesicles (SUV) within canine infarcted myocardium 5 hours post occlusion and 1 hour post injection (5). Nguyen *et al.* reported that injection of SK containing 1-palmitoyl-2-oleoyl phosphatidylcholine LUV into dogs decreased the reperfusion time (time needed to restore blood flow) by 50%, as compared to free SK (12).

Accumulation of liposomes in infaracted myocardium at 24 hours, as studied by Caride *et al.* (13) is interesting, but of little clinical use.

Accumulation at shorter times is questionable, since there are conflicting data from studies which used vastly different protocols. The present study was designed to establish definitively whether liposome encapsulated SK (LESK) is an effective treatment for occlusive thrombi during shorter time periods. The average reperfusion time for LESK treatment reported by Nguyen *et al.* was 32 ± 28 minutes. Therefore we chose to examine the efficacy of 1-palmitoyl-2-oleoyl phosphatidylcholine LUV encapsulated SK treatment in dogs within 15 and 60 minutes post injection.

MATERIALS AND METHODS

Liposome Encapsulated SK (LESK) Preparation. Sixty-five mg of 1-palmitoyl-2-oleoyl phosphatidyl-choline (POPC) (Avanti Polar Lipids) in chloroform was taken to dryness in a 100 ml round bottom flask with a Büchler "Rotovap" apparatus and was dried under vacuum overnight. The lipid film was resuspended in 4 ml of Tyrodes buffer containing 10^6 units of SK (KabiVitrum, Sweden), Vortex mixed and sonicated for 15 minutes with a Heat Systems-Ultrasonics sonicator with a microprobe. During sonication the tube was emersed in ice water to prevent solution heating. Unencapsulated SK was removed by centrifugation at 110,000 g for 30 minutes using a ficoll gradient. The ficoll gradient consisted of three layers (from bottom to top): (1) 1 ml of 30% ficoll mixed with 0.5 ml liposomes was layered on the bottom of the tube, (2) 2.5 ml of 10% ficoll and (3) 0.75 ml Tyrodes buffer. Liposomes were stored at 4°C and warmed to room temperature prior to use. The average liposome diameter was 100 ± 20 nm, as assessed by photon correlation spectroscopy.

Streptokinase Encapsulation Efficiency. The Peterson modification of the Lowry protein assay (14) with a bovine serum albumin standard and without trichloroacetic acid precipitation was used to determine the amounts of protein added and encapsulated. The encapsulation efficiencies ranged from 25 to 30%.

Streptokinase Activity Assay. A chromogenic substrate assay (Kabi Diagnostica, Franklin, Oh.) was performed to determine the activity of the liposome encapsulated SK. S-2251, a chromogenic substrate is hydrolyzed by plasmin, thus liberating the chromophoric group p-nitroaniline. Standards and samples were prediluted to 1000 units/ml and then further to 1:50, 1:100 and 1:200. One hundred and twenty five μg of S-2251 and 125 units of plasminogen were mixed with the standards or liposomes, pre-dissolved with 10% triton X-100, and allowed to sit at room temperature for 15 minutes. A volume of 50 μl of citrate buffer pH 3.0 was added to stop hydrolysis. The absorbances at 405 nm, which are directly proportional to the streptokinase activity, were determined.

***In vitro* Activity of free SK.** Samples with 50 units of thrombin and 4 x 10^{-5} moles of $CaCl_2$ were added to 1 ml of plasma and incubated for 2 hours at room temperature. The resulting clot was removed, rinsed in isotonic saline and weighed. The clot was then resuspended in plasma with 1,000 units/ml free or encapsulated streptokinase.

***In vivo* Experimental Preparation.** The study was performed in closed-chested mongrel dogs, 16 to 28 kg in body weight, anesthetized with 30 mg/kg sodium pentobarbitol and additional doses as needed. The dogs were intubated and artificially ventilated with room air with use of a Harvard respirator. An electrocardiographic lead was continually

monitored. Both the jugular and femoral veins were exposed and a 25 mm long copper coil of slightly conical shape, 2 mm in outside diameter, was inserted into each vein. The coils were weighed before insertion. One hour after implantation the coils with the thrombi were removed, weighed again to determine the initial thrombus weight, and then inserted into the dissected carotid and femoral arteries of the same animal. Shortly before insertion of the coils, the exposed femoral arteries and all their branches had been ligated. After insertion the main femoral artery proximal to the coil and one side branch distal to the coil were released to permit blood flow around the thrombus, which was evidenced in all experiments by the presence of distinctly papable pulsations in the distal side branch. All other branches remained ligated during the study (Figure 1). Ten thousand units per kg weight of dog of free SK and LESK were injected *i.v.*. The coils were removed 15 minutes or 1 hour post-injection and weighed; the percent weight decreases were determined.

RESULTS

***In vitro* lysis.** The average size of the clot formed was 490 ± 40 mg. Incubation with 2000 units SK or LESK for 1 hour reduced the clot weight by 76 ± 6.0% (n=4) and 48 ± 6.6%, respectively (n=4).

***In vivo* activity.** In all experiments the weight of the thrombus decreased upon addition of free SK. The average percent lysis, defined as $\%\ \text{lysis} = \frac{\text{weight clot}_{\text{initial}} - \text{weight clot}_{\text{final}}}{\text{weight clot}_{\text{initial}}}$, was 26.7 ± 13.2. The average percent lysis 15 and 60 minutes post-injection of LESK were -4.3 ± 19.2 and -9.6 ± 7.9, respectively. The results of these studies are summarized in Table 1.

DISCUSSION

From this study, we conclude that liposome encapsulated SK does not significantly decrease thrombus weight 1 hour post-occlusion and 15 minutes or 1 hour post-injection. Our results are contrary to data reported by Nguyen *et al.* (12), which showed significant decreases in reperfusion times for LESK, as compared to SK. According to Nguyen *et al.* , the reperfusion times and thrombus weight remaining were 78 ± 43 minutes and 28.6 ± 31.8 mg and 32 ± 28 minutes and 3.4 ± 4.3 mg for free and liposome encapsulated SK, respectively. Some differences in the protocols of the two experiments should be noted that may be responsible for the discepencies. Nguyen *et al.* prepared their liposomes by the addition of an n-octyl-β-D-glucopyranoside (OG)/SK mixture and dialysis. OG is a detergent, and it is uncertain whether it has any thrombolytic activity; the residual amount of OG was not reported. The size and encapsulation efficiencies of the liposomes and estimated percent SK residing within the interior, as compared to that bound to the surface, were comparable to those measured in this study. Nguyen *et al.* prepared their clots by the repeated injection of thrombin. Decreases in reperfusion times were reported as evidence of thrombolysis. However, the initial weights of the thrombi were not measured and the thrombi were not held in place. Therefore it is possible that the decreased reperfusion times were due to fragmentation of the thrombus rather than lysis.

For effective thrombolysis using LESK, the liposomes must accumulate at the site of the occlusion and the streptokinase must be released. The half-life of intact POPC liposomes in plasma at 37°C is on the

order of a few minutes; therefore the liposomes must accumulate within this time period. Otherwise the efficacy of LESK should be similar to or less than that for free SK, since liposomes also tend to rapidly accumulate within the reticuloendothelial system (RES). Future studies should examine the biodistributions of POPC liposomes as well as the SK. The addition of antibodies or other ligands to the surface of the liposomes to enhance thrombus uptake and/or decrease RES uptake might be worth pursuing.

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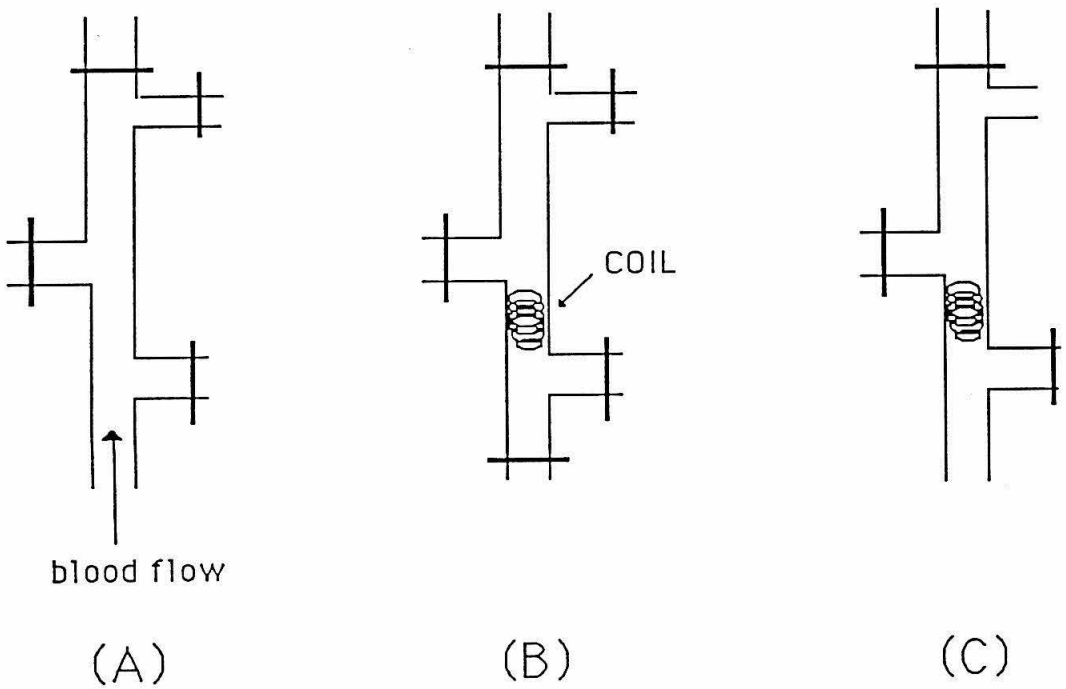

(A) (B) (C)

Figure 1: Ligation of the artery and side branches prior to (A), during (B) and after (C) insertion of the coppper coil.

DOG	SK	LESK	ave. % Lysis	number of clots
1	X		34.4 ± 12.5	4
2	X		16.4 ± 2.5	4
3		X	5.8 ± 7.6	2
4		X	- 6.5 ± 19.9	4
5		X	- 7.1 ± 24.7	4
6		X	- 9.6 ± 7.9	3

Table 1: Free and liposome encapsulated SK were injected *i. v.* . The percent lysis was measured 15 minutes (Dogs 1-5) and 60 minutes (Dog 6) post-injection. % lysis = $\frac{\text{weight clot}_{\text{initial}} - \text{weight clot}_{\text{final}}}{\text{weight clot}_{\text{initial}}}$.

APPENDIX B

USEFUL ASSAYS FOR THE JDB GROUP

Böttcher Modification of the Bartlett Assay for Phosphate Determination

Reagents:

70% $HClO_4$

Molybdate reagent

2.2 g $(NH_4)_6Mo_7O_{24} \cdot H_2O$

14.3 ml conc. H_2SO_4

dilute to 1 liter with deionized H_2O

10% w/w ascorbic acid (stable 2-3 weeks in refrigerator)

Standard:

712 mg $Na_2HPO4 \cdot 2H_2O$ in 100 ml deionized H_2O (store in refrigerator)

Dilute 1 ml of stock to 100 ml (0.4 nmol/ul) for use in the assay

Procedure:

1. Pipet $Na_2HPO_4 \cdot 2H_2O$ standards of 0, 20, 40, 60, 80, 100 nmol and samples in duplicate
2. Add 0.4 ml of 70% $HClO_4$ to samples and standards
3. Heat 30 minutes at 180-190°C in a heating block
4. Leat cool 15 minutes
5. Add 4 ml molybdate reagent
6. Add 0.5 ml ascorbic acid solution
7. Vortex and let clear 10 minutes in a boiling water bath
8. Measure $A_{812\ nm}$

Peterson Modification of the Lowry Protein Assay

Reagents:

CTC solution

20% Na_2CO_3

add equal volume of a 0.2% $CuSO_4$. $5H_2O$/ 0.4% sodium tartate solution (stable > 2 months at room temperature)

10% Sodium dodecylsulfate (SDS)

0.8 N NaOH

2 N Folin-Ciocalteu phenol reagent

BSA, fraction V, 1 mg/ml BSA with 1 mg/ml NaN_3 (keep in freezer)

Procedure:

1. Pipet BSA standards of 10, 20, 40 mg and samples in duplicate

2. Add equal volumes of CTC, SDS, NaOH solutions and water (solution A)
3. Add 0.5 ml deionized H_2O and 0.5 ml solution A to standards and samples
4. Let sit 10 minutes
5. Prepare a 1:5 Folin-Ciocalteu soultion:water solution
6. Add 0.25 ml Folin-Ciocalteu phenol solution; vortex
7. Let sit 30 minutes
8. Measure $A_{750\ nm}$

Sendai Virus Preparation

Reagents:

Phosphate Buffered Salt (PBS): 0.90% NaCl: 0.12% $Na_2HPO_4 \cdot 7H_2O$: 0.013% $NaH_2PO_4 \cdot H_2O$ w/w in water, pH 7.3

Procedure:

1. Inject 10 day old chicken eggs with 2 μλ of a 2μg/ml virus solution containing 1% penicillin and 1% streptomycin (per egg); make sure the eggs are fertilized
2. Incubate at 37°C
3. Freeze the 13 day old eggs at -20°C for 1 hr to prevent bleeding
4. Remove the top of the shell and carefully pipet out the fluid
5. Centrifuge the fluid in 250 ml centrifuge bottles at 3500 rpm (GSA rotor) for 30 minutes
6. Discard pellet; centrifuge the supernatant in 25 ml tubes at 25,000 rpm (SS34 rotor) for 45 minutes
7. Discard the supernatant; resuspend the pellet with 1.5-2 ml of PBS using a 15 gauge syringe
8. If there are still red blood cells present repeat steps 6 and 7
9. Store the virus at -70°C.

Red Blood Cell Ghost Preparation

Reagents:

Prelysis Buffer
150 mM NaCl
5 mM Na_2HPO_4
pH 7.4

Lysis Buffer
5 mM Na_2HPO_4
pH 8.0

KNP Buffer
120 mM KCl
30 mM NaCl
10 mM Na_2HPO_4
pH = 7.4

Procedure:

1. Add 15 ml RBC to an Erlemyer flask emersed in ice
2. Add the 20 ml of prelysis buffer
3. Stir for 15 minutes
4. Centrifuge in a SS34 rotor at 5500 rpm for 12 minutes at 10°C
5. Remove the supernatant
6. Add 100 ml of lysis buffer to the pellet; suck the solution into a pipet 10-15 times
7. Place in an Erlemyer flask in ice and stir for 30 minutes
8. Centrifuge in a SS34 rotor at 15,000 rpm for 15 minutes at 10°C
9. Repeat steps 6-8 two more times
10. Remove the supernatant
11. Add 35 ml of the lysis buffer to the pellet; suck the solution into a pipet 10-15 times
12. Centrifuge in a SS34 rotor at 12,000 rpm for 20 minutes
13. Remove the supernatant
14. Add 10 ml of KNP solution; 0.01 ml of a 1.0 M solution of $MgSO_4$
15. Incubate at 37°C for 45 minutes
16. Store the ghosts on ice or in the refrigerator

10% SDS Gel Electrophoresis

Reagents:

Stock solution
40 g acrylamide
1.06 g methylene-bis-acrylamide
diluted in 100 ml distilled water

Separating gel
5 ml stock solution
5 ml of 1.5 M tris·HCl pH 8.8
10 ml H_2O
200 μλ of 10% SDS
100 μλ of a 10% solution of ammonium persulfate solution (freshly prepared)
7.5 μλ tetramethylenediamine, TEMED (add immediately before use)

Concentrating gel
- 1 ml stock solution
- 3 ml of 0.5 M Tris·HCl pH6.8
- 4 ml H_2O
- 80 μλ of 10% SDS
- 100 μλ of a 10% solution of ammonium persulfate solution (freshly prepared)
- 5.0 μλ tetramethylenediamine, TEMED (add immediately before use)

Sample Buffer
- 5% SDS
- 5% β-mercaptoethanol
- 8 M urea
- 62.5 mM Tris·HCl pH 6.8
- 0.01% bromophenol blue

Running Buffer
- 144 g glycine
- 10 g SDS
- 30 g Tris·HCl
- dilute in 1 liter water, final pH=8.8

Staining solution
- 0.2% Coomassie blue
- 25% methanol
- 10% glacial acetic acid

Destaining Solution
- 25% methanol
- 10% glacial acetic acid

Procedure:

1. Clean glass plates with soap and water followed by ethanol
2. Apply vaseline to the edges; place plastic tabs; put plates together and hold together with clamps
3. Prepare the separating gel (usually double the recipe); stir 30 times with a spatula
4. Tip the plate and add the separating gel; add 5 ml butanol saturated with water to break the bubbles
5. Cover with Saran wrap; let sit 2-2.5 hours
6. Remove the butanol
7. Prepare the concentrating gel; stir 30 times with a spatula
8. Tip the plate and add the concentrating gel
9. Add the comb at an angle; press gently to remove air bubbles
10. Cover with Saran wrap; let sit for 2 hours
11. Remove the comb carefully

12. Remove clips from the bottom of the plates
13. Clip the plates to the rack
14. Dilute the running buffer (1:10) correct pH to pH 8.8
15. Add the running buffer
16. Remove air bubbles
17. PREPARE SAMPLE

    Add 75-100 µl of a 72% solution of trichloroacetic acid to 0.5-1.0 ml sample; mix

    Place on ice for 1 hour

    Centrifuge in an Eppendorf centrifuge at 9000 rpm for 10 minutes at 10°C

    Remove the supernatant

    Add 10 µl of 1 M NaOH; add 50-60 µl of sample buffer to the pellet

    Boil 3 minutes in a water bath

18. Pipet ~60 µl/ lane of standard or sample
19. Plugs in leads
20. In concentrating gel run at 100 mV; lower current for separating gel
21. Remove plugs
22. Remove the gel
23. Stain; destain