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Degree

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Rodney Jin Yong Ho

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ABSTRACT

A novel target-sensitive immunoliposome system was designed, characterized and demonstrated to be both an efficient site-specific drug delivery system and a basic immune detection tool. As the name implies, these liposomes exposing immunoglobulin G (IgG) antibody on their surface, could selectively attach to target antigen, self-destabilize, and release aqueous contents in a site-specific manner. This unique characteristic of the target sensitive design was derived from the ability of membrane acylated IgG to stabilize phosphatidylethanolamine (PE) in a bilayer. Antibody-stabilized PE immunoliposomes were rapidly destabilized upon binding to multivalent antigen expressing targets while monovalent, soluble antigen failed to cause liposome destabilization, indicating the necessity of multivalent binding for destabilization. In addition to the requirement of multivalent antigen on targets, kinetic and ultrastructural studies of interactions between liposomes and herpes simplex virus (HSV) virions indicated that HSV-induced destabilization of PE-immunoliposomes involved liposome-virus fusion intermediates which collapsed after increasing in size to finally allow PE to assume its original non-bilayer form, hexagonal H_{II} phase. Freeze-fracture electron microscopy further demonstrated the reversion of PE to a H_{II} phase. Using target-sensitive immunoliposomes bearing anti-HSV-glycoprotein D, with peroxidase as an encapsulated enzyme marker, we quantitated HSV in test samples by detecting peroxidase enzymatic activity expression due to HSV-induced liposome destabilization. Evaluation of the HSV target-sensitive immunoliposome-based

immunoassay (ILA) on clinical samples showed that the ILA was equally sensitive to the currently employed virus plaque assay. In comparison, a commercial enzyme-linked-immunosorbent assay for HSV antigen was less sensitive. Site-specific release of liposome contents from target-sensitive immunoliposomes was implemented for use as an anti-viral drug-delivery system to suppress HSV replication. Results showed that encapsulation of nucleoside analogs in target-sensitive immunoliposomes not only decreased the nonspecific cytotoxicity, but also enhanced the potency of the antiviral drug. This target-sensitive immunoliposome design should be applicable in site-specific drug delivery, to improve existing immune detection systems, and as an approach to treat the foci of infections or neoplasms.

PREFACE

The goal of this project is to engineer a new design of liposome that would not only recognize a target cell or virion, but also have a built-in sensor that would cause self-destruction upon the specific interactions of the liposome with the target of interest. The basic design was made possible by the recent progress in the understanding of the polymorphic phase behavior of the lipids at the molecular level. From biophysical studies it was well known that a particular sub group of naturally occurring lipids assume a non-bilayer or hexagonal phase under physiological conditions. Phosphatidylethanolamine (PE) is one class of phospholipids that favor the hexagonal phase in the absence of a membrane stabilizer. It is now well-established that PE bilayers may be stabilized by a wide variety of competent stabilizers of membrane bound molecules. Using either the membrane-bound ligand or receptor as PE membrane stabilizing molecules, a selective removal of the stabilizers to the membrane contact point by a multivalent binding with the counterpart (either ligand or receptor molecule) causes the destabilization of these liposomes. Based on this concept, the project of designing the target-sensitive liposome was initiated and the results are recorded here.

In order to appreciate the significance of this piece of work, a brief introduction of the use of liposomes in biology and medicine is described in the first chapter. Since the application of liposomes in the area of immunoassay is still relatively new, the second chapter focuses on reviewing immunoliposome assays and their current status.

Chapters III to V describe the basic designs of target-sensitive liposomes and demonstrate their capabilities. In an effort to understand the molecular mechanism of interactions of this type of liposomes with targets, kinetic and ultrastructural studies were done and are discussed in the sixth chapter. This is followed by the evaluation of the potential use of target-sensitive immunoliposomes as reagents for immunoassay detecting herpes simplex virus in clinical samples (Chapter VII). In addition, the effective use of target-sensitive liposomes as an anti-viral drug carrier was demonstrated and the mechanism of action is proposed in the eighth chapter. Finally the current understanding of the target-sensitive liposome designs is summarized and additional experiments are proposed to further extend our knowledge in the hope that it eventually will be adapted for clinical anti-tumor and anti-viral applications.

Rodney J. Y. Ho

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CHAPTER I

INTRODUCTION

Since the first discovery of the liposomes or enclosed lipid vesicles in the early 1960's by Bangham and Saundlers (reviewed by Bangham, 1980), many refinements at the molecular level have been made as we have begun to understand more about the basic biophysical and biochemical properties of liposomes. In fact, liposomes which can easily be made merely by hydration of a dried lipid film that can encapsulate or incorporate biologically useful molecules (i.e. drugs), have been the focus of a large number of literature articles for the past 25 years. A traditional view was that liposomes, mainly composed of phosphatidylcholine adsorbed on the cell surface subsequently may fuse with the cell membrane in delivering liposomal contents (Pagano & Weinstein, 1978) to the interior of the cell.

It has been well-documented that only an infinitesimal fraction of cell-attached liposomes can fuse so that most of the liposomal contents never reach the cytosol, the intended site of action. Although the incorporation of targeting molecules on the liposome membrane has increased the selectivity and the quantity of liposome attachment to target cells, it still does not enable the proper delivery of liposome-entrapped molecules to the site of biological action. Instead, most of the liposomes bound to the target cells were internalized via an endocytic pathway and destined for final lysosomal degradation (Post and Papahadjopoulos, 1978). Although some types of liposome-entrapped drug molecules can survive in the lysosome and escape

the lysosome, most drugs and biologically active molecules can not avoid lysosomal destruction (Rustum et al., 1981).

Therefore, alternative methods are sought to bypass lysosomal delivery of the liposomes. One approach is to fuse the liposomes to intact cells with the aid of fusogens such as Sendai virus (Okada, 1962; Nakanishi et al., 1985), synexin (Hong et al., 1979), and poly-histidine (Wang & Huang, 1984), polyethylene glycol (Boni et al., 1984) or pulsed electric field (Vienken & Zimmerman, 1985). Unfortunately, most of these methods produce various detrimental effects including toxicity to the target cells.

Accordingly, pH-sensitive immunoliposomes which exploit the cellular endocytosis pathway, were invented (Huang & Liu, 1984; Duzgunes et al., 1985a; Nayar & Schroit, 1985; Ellens et al., 1984; Connor et al., 1984; Straubinger et al., 1985). When the pH-sensitive immunoliposomes are internalized, endosomal acidification (pH 5.0-6.5) activates these liposomes to fuse with endosomal membranes in delivering the liposome-entrapped drugs. This approach has been demonstrated to be very effective in the cytosolic delivery of antitumor drugs (Connor & Huang, 1986), DNA (Wang et al., 1987; Duzgunes et al., 1985b) and some cytotoxins (Collins & Huang, 1987).

An endocytosis-dependent drug delivery system may not be applicable to target cells with low endocytotic activity. This is particularly relevant in cells infected by viruses whereby the infection process shuts down cellular metabolic activity (Ben-Porat & Kaplan, 1973), including endocytotic activity (Widnell et al., 1984; Wilcox et

al.,1982). Heat-sensitive immunoliposomes were developed to overcome this constraint (Sullivan & Huang, 1985). These liposomes which became permeable at the gel-to-liquid crystalline phase transition temperature of the lipid, can deliver liposomal contents to the cell surface. This type of cell delivery system depends on the rapid uptake of the drug, released from briefly heated cell-bound liposomes. Enhanced uptake of the nucleoside uridine, encapsulated in heat-sensitive liposomes composed of dipalmitoylphosphatidylcholine was shown recently (Sullivan & Huang, 1986). Additionally, the liposome-mediated enhanced cellular uridine uptake in this model system was shown to be dependent on the functional cellular nucleoside transporter. However, the localized heating of cells required by heat sensitive liposomes may not be feasible for many applications, especially those in vivo.

We have taken the initiative to design a new type of immunoliposome which is not only target-specific, but also contains a built-in sensor for self-destruction upon reaching its destination. This design is based on the observation that unsaturated phosphatidylethanolamine (PE), which by itself does not form stable bilayer under physiologic conditions (Gruner et al., 1985; Cullis & DeKruijff, 1979), can be made to do so by mixing with another type of lipid or lipid derivatives (Ho & Huang, 1985; Nayar & Schroit, 1985; Cullis & DeKruijff, 1979, Tsao & Huang, 1986), with fatty acids and derivatives (Duzgunes et al., 1985; Huang & Liu, 1985; Ellens et al., 1984; Connor et al., 1984), with proteins such as glycophorin A, or with acylated immunoglobulin G (IgG) (Taraschi et al., 1982; Ho & Huang, 1985; Ho et al., 1986). Maintenance of stable PE bilayers, thus, depends on the random

distribution of the stabilizers within the lipid bilayer; consequently, a lateral phase separation of stabilizer molecules may result in destabilization of the PE bilayer and formation of the hexagonal (H_{II}) phase. This phenomenon was initially demonstrated using the glycophorin A-stabilized PE liposomes. When lectins which bind multivalently to the glycophorin A (thereby laterally phase separating transmembrane protein glycophorin A on the PE bilayer) were added, destabilization and H_{II} phase formation was observed (Taraschi et al., 1982). However, due to the nonspecific binding of lectins to the ubiquitous sugar residues, glycophorin A-lectin model is not suitable for use in liposome targeting.

Consequently, either membrane bound antigen or acylated IgG was used as a stabilizer for an unsaturated PE bilayer in our model system. This is, in fact, the first time that an immunoglobulin has been used as a stabilizer for PE bilayer. Since these liposomes can release encapsulated contents upon binding to the target, we proposed to call them **target-sensitive liposomes**. Use of the target-sensitive liposome is not limited to the delivery of biologically active molecules to target cells. Specific destabilization of these liposomes by multiple antigen-antibody bindings indicates that this technology is also useful in the application of immunoliposome assays. This dissertation will address both aspects in detail.

CHAPTER II

IMMUNOLIPOSOME ASSAYS: PERSPECTIVES, PROGRESS AND POTENTIAL

Introduction

In the past few years, a renewed interest has been placed on liposome-based immunoassays for detecting immune-related compounds or analytes of interest. This current trend is attributed to the intrinsic advantages of using immunoliposome assays (ILA) over other methods of immune detection such as radio-immunoassays (RIA) or enzyme linked immunosorbent assays (ELISA). For example, the aqueous space of liposomes can be used to carry a wide variety of reporter molecules in bypassing the use of radioactive molecules while maintaining, or even improving the sensitivity of the immunoassay. Although the improvement of using enzymes in the ELISA type assay avoids the use of radioisotopes, the intrinsic problems of elaborate time control requirements and extensive washing procedures remain as major draw backs. In addition, well-trained technicians and relatively expensive instruments are required to achieve maximum efficiency of detection. Our current understanding of membrane properties and their interaction with a wide variety of biological substances enables us to design ILA that are easily adaptable from one application to another. Another advantage of ILA is its rapidity, with most assays being completed within minutes. Accordingly, ILA fits the growing demand for a simple immunoassay method that neither requires a skilled technician nor expensive instrumentation. Thus, it is applicable for home, physician's office or

field applications. All the liposome-based immunoassays reviewed in this article will be referred to as immunoliposome assays or ILA.

A wide variety of ILA have been developed with different approaches and sensitivities. However, for the purpose of discussion, we will divide them in terms of heterogeneous ILA which require separation or washing procedures, and homogeneous ILA which do not require separation or washing steps.

Heterogeneous Immunoliposome Assays

A heterogeneous ILA is an immunoassay which requires removal of potentially interfering components prior to the detection of the appropriate signal.

Immune Precipitation of Liposomes

Since Alving and Kinsky (1971) first reported the agglutination of antigen sensitized liposomes by rabbit antiserum, only a few applications employing such an approach have been documented. A basic strategy of this method is to prepare surface antigen or analyte bearing liposomes which also carry a reporter molecule. When these liposomes are exposed to the antibody, a specific binding of the primary antibody can be followed with binding of a secondary antibody against the first antibody. This promotes extensive crosslinking which results in a precipitable liposome-immune aggregate. After separating the precipitate from the unreacted liposome suspension either by filtration (Fishman et al., 1979) or centrifugation (Fry et al.,1976; Mansbach et al., 1985), liposome-immune complex can be quantitated. Since the

addition of a free soluble antigen in the assay mixture can inhibit the precipitation of antigen-bearing liposomes, an inhibition curve of an standard analyte can be generated. This standard curve is then subsequently used to estimate the amount of the analyte in unknown samples.

A wide variety of markers have been used. Lipid soluble markers such as [^{3}H]Cholesterol (Fry et al., 1976) or FITC-phosphatidylserine (Axelsson et al.,1981), and aqueous fluorescent markers such as sulforhodamine B (Mansbach et al., 1985) were used to demonstrate that this inhibition type of ILA has a comparable sensitivity to RIA. Although the earlier design used a radiolabelled marker (Fry et al., 1976), the later assays with chromophore markers are shown to retain a sensitivity similar to that for RIA (Axelsson et al, 1981; Mansbach et al., 1985). Despite the requirement for the separation step, this assay avoids the use of radioisotopes without compromising sensitivity.

Complement-induced Lysis of Liposomes

Slovick et al. (1980) reported this type of assay by employing ^{86}Rubidium encapsulated liposomes bearing galactocerebroside. When these liposomes were exposed to anti-galactocerebroside antibody and complement, liposome lysis occurred. The immune-complex-dependent complement mediated lysis of liposomes triggers the release of liposome encapsulated ^{86}Rb. ^{86}Rb released was separated by filtration from intact liposomes. Hence, the degree of lysis can be determined by detecting the ^{86}Rb radioactivity found in the filtrate.

Cell-mediated Lysis of Liposomes

Henney (1980) described this type of assay with ^{86}Rb encapsulated DNP sensitized liposomes. These liposomes immobilized on anti-DNP-antisera coated microtitre wells were used as a target for assaying the cytolytic K(killer) cell. After incubation of the immobilized liposomes with cytolytic cells, the cells were separated by centrifugation, and the released ^{86}Rb was quantitated by counting the radioactivity in the supernatant.

Liposome as A Signal Carrier

This method is the most simple and basic application of liposome-based immunoassay. Basically, the design involves the use of a receptor or ligand that recognizes the analyte of interest which has been immobilized on solid supports such as a test tube wall or a dipstick. In the liposome, a reporter molecule such as a fluorescent dye in the case of fluorometric assay, a colored dye in the case of spectrophotometric assay, or an enzyme in the case of the enzymatic-spectrophotometric assay, is encapsulated. To detect the analyte in the test sample, a direct assay or inhibition assay can be employed. In the direct assay, the solid support is first coated with the analyte in the test sample; after removing the unadsorbed analyte by washing, the signal carrying liposome which bears the cognant receptor is introduced. Again unbound liposomes were removed by washing and finally, the signal molecule is detected with an appropriate method as mentioned above. In the competition assay , a soluble analyte can be used to inhibit the binding of liposomes to the solid support employing a

similar procedure as mentioned above. Although the basic principle of this method is very similar to the conventional ELISA method, the advantage of this ILA design is to increase the sensitivity up to several hundred fold over ELISA. In ELISA, only a few enzyme molecules are coupled to each antibody molecule, mainly due to the denaturation of antibody at a higher degree of conjugation. On the contrary, one can conjugate more receptor molecules on each liposome that can carry several hundred signal molecules. For example, a 1000Å liposome would have internal volume of about $4.2 \times 10^{8} Å^{3}$ which could carry about 40,000 molecules of 23.6Kd α-chymotrypsinogen (hydrated diameter of about 42Å) (Adrian & Huang, 1979). In addition, it has been demonstrated that only three to six membrane bound receptor molecules such as antibody were required for specific binding of liposomes to their target (Babbitt & Huang, 1985). Consequently, in theory the sensitivity of this type of immunoassay is enhanced 1000 fold over that of ELISA. Indeed, this methodology was recently used by O'Connell et al. (1985) and Elliott et al. (1985).

The disadvantage of this assay design is the same as that of either ELISA or RIA, in that, extensive washing procedures are required which lead to the high cost and long assay time.

Homogeneous Immunoliposome Assays

Homogeneous ILA are assays which require no separation steps. The generation of the signal is mediated by the specific ligand-receptor or antibody-antigen interactions.

Agglutination of Liposomes

Cross-linking of Forssman antigen bearing liposomes by rabbit antiserum was first reported by Alving and Kinsky (1971). Although this type of immunoassay is not very sensitive, the simple procedure made it very attractive for on-site application. The agglutination assay can be used to detect the anti-galactocerebroside(GC) antibody in serum using GC-sensitized liposomes to form immune-liposome complexes, followed by secondary anti-human antibody to enhance the visual detection (Fry et al.,1976). The sensitivity of this assay is at least three orders of magnitude lower than that of RIA. Recently, Kung et al. (1985) used liposomes bearing antibody to human IgM to improve the latex agglutination assay for rheumatoid "factor" in the diagnosis of rheumatoid arthritis. Since rheumatoid "factor", mainly IgM, recognizes denatured human IgG, the latex beads coated with denatured IgG were first allowed to bind with IgM ("factor"); the rate and the degree of agglutination was further enhanced by the addition of multivalent liposomes which had been prepared with surface anti-human IgM. This additional liposome binding to the latex bound IgM ("factor") increased the sensitivity as well as the time efficiency of the current existing latex agglutination assay for rheumatoid "factor". The principles of these assays are potentially applicable to enhance other latex agglutination assays.

Fluorescence Protection of Fluorescein-conjugated Liposomes

It is well established that binding of anti-fluorescein antibody to fluorescein quenches the fluorescence of the fluorophore. In order

to apply this phenomenon in detecting anti-cardiolipin antibody in reagin positive human serum, Brinkly et al., (1979) coupled fluorescein to cardiolipin. Fluorescence quenching by binding of anti-fluorescein to the fluorescein-cardiolipin bearing liposomes could be quantitatively inhibited by the presence of anti-cardiolipin antibody in the test sample. This is probably due to the steric hindrance provided by the binding of anti-cardiolipin antibody that is preventing the binding of anti-fluorescein antibody. In theory, a similar approach could be extended to detect a protein analyte.

Complement-induced Lysis of Liposomes

Since Kinsky and his colleagues (Haxby et al., 1968 & 1969; Alving et al., 1969; Kinsky et al., 1969) first showed that antibody binding to hapten bearing liposomes can fix complement which could result in the lysis of liposomes by activated complement, complement-mediated lysis of liposomes has been a focal point of a large number of papers (for a review, see Alving & Richards, 1983). The complement system is a series of proteins (C1q to C9) that recognize and bind to antibody-antigen immune complexes at membrane surfaces. The binding of complement components in correct order triggers a series of proteolytic cleavages leading to the final membrane lytic event. This process is defined as the classical pathway of complement activation. The chronological actions of membrane lysis mediated by activated complement components have been described in detail by Lewis and McConnell (1978) and McConnell (1978) for the early events and by Kinsky & Nicolotti (1977) for the late events leading to membrane lysis. At least two

adjacent IgG molecules bound to the membrane antigen are required to activate the complement system (Brulet & McConnell, 1976 & 1977). Upon activation, complement components do not promote the hydrolysis of lipid (Inoue & Kinsky, 1970) although a transient membrane lesion of about 100Å in size is detectable (Iles et al., 1973; Kataoka et al., 1973; Lachmann, 1970). These events lead to the final release of liposome encapsulated signal molecules.

Based on the finding that antigen specific lysis can be inhibited by the addition of a soluble antigen to compete for the binding of antibody to the antigen on liposomes, a battery of inhibition assays have been developed. These include assays for Forssman antigen (N-Ac-Gal (α1-3) Gal (β1-3) Gal (β1-4)-Glc-1 Ceramide) (Kinsky et al., 1969; Haxby et al., 1968 & 1969; Uemura et al., 1982; Wei et al., 1975; Humphries & McConnell, 1974); globoside I (Inoue & Kinsky, 1970; Uemura et al.,1980); dinitrophenyl hapten (Six et al., 1973 & 1974; Bowden et al., 1986; Hsia & Tan, 1978; Uemura & Kinsky, 1972; Thompson & Gaber, 1985; Shiba et al., 1980); galactocerebroside (Alving et al., 1977 & 1980); gangliosides (Geiger & Smolarsky, 1977; Shiba et al., 1980; Uemura et al., 1980 & 1982 ; Endo et al., 1984); bacterial lipopolysaccharides (Kataoka et al., 1971); cardiolipin (Rosenqvist & Vistnes, 1977; Umezawa et al., 1982; Ruysschaert et al., 1977); theophyline (Haga et al., 1980 & 1981); thyroxine, T4 (Hsia & Tan, 1978; Adolfson et al., 1985); anti-human IgG (Ishimori et al., 1984); complement (Knudson et al., 1971; Bowden et al., 1986); and phenytoin & phenobarbital (Fan et al., 1985).

The sensitivity of the assays vary from one application to another and are mainly dependent on the detection strategy rather than the affinity of the antibody. Although an antibody with high binding affinity is preferred, it is not an absolute requirement since excess antibody will compensate for lower affinity. The major factor controlling the sensitivity of this assay is the design in detection of the signal released from the liposomes. An initial approach was to encapsulate glucose as a marker inside the liposomes and detect the release of glucose by a coupled enzyme assay of glucose oxidase and glucose-6-phosphate dehydrogenase with the coenzyme NADP. In the presence of glucose and the two enzymes, NADP is converted into NADPH which is detectable spectrophotometrically at λ_{max} = 340 nm (Kinsky, 1974). The sensitivity of the assay was further improved by using fluorogenic substrates, mainly umbelliferone derivatives. In this method, either 4-methylumbelliferyl-phosphate (Six et al., 1974; Uemura et al., 1982) or 4-methylumbelliferyl-β-D-glucuronide (Thompson & Gaber, 1985) was encapsulated and the fluorescent product was generated by the addition of either alkaline phosphatase or β-glucuronidase, respectively. Conversely, some enzymes such as alkaline phosphatase (Bowden et al., 1986), β-galactosidase (Adolfson et al., 1985; Fan et al., 1985) and horseradish peroxidase (Haga et al.,1980 & 1981) have been successfully encapsulated in the liposomes. The latter approach is a better method since the sensitivity of the detection system is not limited by the number of substrate molecules which can be encapsulated in the liposomes. Thus, one can increase the detection signal by increasing the time of the enzymatic reaction in an excess of added substrate.

Other types of detection systems have also been developed. One of the approaches takes advantage of spin-spin interactions between spin labels. At high concentrations (mM range) of a spin label, the electron spin resonance (ESR) spectrum is broadened due to the spin exchange interaction between neighboring spin probes. When the spin probes are diluted as they are released from the liposomal compartment, the ESR intensity is greatly enhanced (Kornberg & McConnell, 1971). Based on this principle, a high concentration of the spin label, such as tempocholine chloride, was encapsulated in liposomes: lysis of these liposomes was detected by the release of the electron spin exchange as the tempocholine chloride was diluted. By integrating the enhancement detected of the ESR spectrum, the amount of liposomal release of spin-label molecules was estimated (Hsia & Tan,1978; Humphries & McConnell, 1974; Ruysschaert et al., 1977). Although spin-label assays are quite sensitive, they require expensive instrumentation and the relatively long assay times (normally about 20 min per ESR scan). Fluorophore has also been used as a marker in the complement mediated liposome-lysis assay. In particular, a fluorescer and quencher pair, dipyridinium-p-xylene (DPX) and 1-aminonapthalene-3-6-8-trisulfonate (ANTS) have been used (Geiger & Smolarsky, 1977; Smolarsky et al., 1977). When high concentrations of DPX and ANTS are encapsulated in liposomes, the collision between the fluorescer DPX and quencher ANTS, quenches the DPX fluorescence almost totally. Upon lysis, released DPX and ANT are diluted and the DPX fluorescence is greatly enhanced. As a result, the amount of lysis can be determined from the degree of DPX fluorescence enhancement. Another fluorophore used in the complement dependent

immune lysis assay is carboxyfluorescein (CF). At high concentrations of CF, the fluorescence is self quenched. As CF is released from the liposome, CF is diluted resulting in enhancement of the fluorescence (Ishimori et al., 1984).

Some potentiometric detection methods were also demonstrated to be quite useful. Umezawa et al. (1982), had designed an electrode capable of detecting the release of glucose which consumes molecular O_2 via a coupled enzyme assay of glucose oxidase and peroxidase. The consumption of molecular O_2 due to liposomal release of glucose was detected potentiometrically by the polypropylene membrane mounted Clark type O_2 electrode. A similar strategy was to encapsulate peroxidase which in the presence of coenzyme NADH, converts molecular O_2 in the medium into H_2O, thereby depleting O_2 in the buffer (Haga et al., 1981). A slightly different approach in potentiometric detection is to encapsulate the ionic tetraphenylammonium (TPA^+) in the liposome and detect the release of TPA^+ from liposomes by detecting the change in electrical potential of the assay mixture (Shiba et al., 1980a & b).

Although complement dependent liposome lytic assays were repeatedly improved to have a comparable or even higher sensitivity than RIA or ELISA, there remains a common draw back of the strong dependency on a rather labile components of complement. Inactivation of any one of more than 12 known complement components can hinder the cascade reaction leading to reduction or total lost of lytic activity. Thus, the stability of the reagent is a major problem.

Lytic Molecule-mediated Lysis of Liposomes

Two different types of lytic molecules have been used for for homogeneous ILA. They are the water soluble cytolysin, mellitin and the lipid soluble Sendai virus protein. In the water soluble cytolysin design, a small hapten, ouabain, an analog of digoxin was conjugated to mellitin without abolishing lytic activity (Litchfield et al.,1984). When the hapten-mellitin conjugate was added to alkaline phosphatase encapsulated liposomes, lysis of liposomes was characterized by the ability of the substrate p-nitrophenylphosphate to cross the membrane barrier and develop a colored product. The lysis activity of ouabain-mellitin was blocked by binding of antibody against ouabain due to steric hindrence. Since digoxin, an analog of ouabain, was able to inhibit this process in a concentration dependent manner, this procedure was used by Litchfield et al.(1984) to show that this digoxin assay was as sensitive as a RIA. It is not clear, however, whether this type of assay can be used for protein antigens.

Another approach is to use Sendai virus which has been shown to contain hemolytic activity (Okada, 1962). When an acylated antibody against the analyte was incorporated into the Sendai viral membrane, the resulting target virus can bind and lyse liposomes bearing the analyte. Since the free soluble antibody or soluble antigen can inhibit lysis, this principle allows one to assay for either the analyte or cognate antibody. Employing either calcein or β-galactosidase as a marker, Heath et al., (1986) have shown that this strategy is promising for assaying human IgG and 4-amino-phthalate. Compared to the complement dependent ILA, the design of this assay is simpler.

However, the instability of the lytic reagent, i.e. virus, is likely to be a major drawback.

Membrane Lytic ILA that do not Require A Lytic Molecule

The designs of the ILA in this section are mainly attributed to our recent progress in understanding of the biophysical properties of naturally occurring lipids such as phosphatidylethanolamine(PE) and cardiolipin. These lipids can adapt a variety of phases in addition to the bilayer (lamellar) phase. This phenomenum is known as lipid polymorphism. For a more detailed discussion of lipid polymorphism, we recommend the recent review by Gruner et al. (1985).

Based on the observation that cardiolipin containing lipid bilayer can be induced to form a non-bilayer (inverted micelle) structure known as hexagonal (H_{II}) phase by binding of divalent cations such as Ca^{+2} or Mg^{+2} (Rand & Sengupta, 1972; DeKruijff et al., 1982), Janoff et al., (1983) had designed an ILA to detect autoantibody in the serum of systemic lupus erythematosis (SLE) patients. The basic design was to encapsulate a divalent cation sensitive dye Arsenazo III inside liposomes which contained 30 mole percent cardiolipin. When Mg^{+2} was introduced into the liposome suspension, binding of Mg^{+2} caused the cardiolipin to transform into the H_{II} phase, resulting in lysis of liposomes which permitted Mg^{+2} to penetrate into liposomes. As a result, Arsenazo III complexed with Mg^{+2}. The color change was quantitated spectrophotometrically. Since the binding of Mg^{+2} was inhibited by the binding of autoantibody (found in SLE patients) to the phosphate group of cardiolipin, the color change due to Arsenazo III-Mg^{+2} complex

was inhibited. This ILA has been applied to the diagnosis of SLE. However, the application is limited to SLE autoantibody since other autoantibodies do not bind to cardiolipin. Conjugation of antigen to cardiolipin is likely to eliminate the ability of cardiolipin to form the H_{II} phase, particularly with protein antigens.

Recently our laboratory (Ho & Huang, 1985a & b) has taken a new approach: we designed a new type of liposomes that not only specifically recognize and bind the analyte of interest, but also contain a self-destruct mechanism which causes the lysis of liposomes upon binding with the analyte. This design was made possible by recent progress in understanding of lipid polymorphism. It is well-known that unsaturated PE such as dioleoylphosphatidylethanolamine (DOPE) does not form bilayer by itself at physiological temperature and neutral pH (Reiss-Husson, 1967). Instead, PE forms inverted micelles which give rise to bundles of tubular lipidic structures easily detectable by X-ray diffraction, ^{31}P-NMR, or freeze-fracture electron microscopy. When these unsaturated PE with an average geometry of an inverted cone are complemented by another lipid which has an average dynamic geometry of a cone, such as lyso phosphatidylcholine (lyso PC), the bilayer structure of PE is stabilized (Rand et al., 1971; Cullis & DeKruijff, 1979; Gruner et al., 1985). Based on this unique polymorphic behavior of PE, we have attempted to use a haptenated lipid or a transmembrane protein which exhibits a similar dynamic geometry as lysoPC, as a bilayer stabilizer of PE. As predicted, when the haptenated lipid, dinitrophenyl-caproyl-PE (DNP-cap-PE) or glycophorin A (a transmembrane protein of human red cell) was added to DOPE, stable liposomes which

encapsulated a fluorescent dye, calcein,resulted. However, when the hapten or glycophorin stabilized liposomes were allowed to bind with an appropriate multivalent antibody (IgG antibody immobilized on a glass surface), lysis of the liposomes was detected by the enhancement of calcein fluorescence. As schematically shown in figure 1A, lysis of the PE membrane probably involved a multivalent antibody induced phase separation (contact capping) of the stabilizing antigen in the liposome bilayer. As a result of the reduction in the local concentration of the stabilizer molecule in the PE bilayer, liposomes were destabilized, which resulted in the release of calcein. Liposome lysis was antigen specific in that it could be inhibited by preincubation of the immobilized antibody with a free antigen prior to the addition of liposomes. Free bivalent antibody could not lyse the liposomes, but it inhibited the subsequent lysis by immobilized antibody. Thus, we have demonstrated a new type of homogeneous, solid phase immunoliposome assay for either antigen or antibody. The assay is sensitive to a few pmoles of antigen and a few ng antibody (Ho & Huang, 1985a & b).

In principle, this type of assay can be designed in a reciprocal manner such that antibody is used as a stabilizer of the PE bilayer and a multivalent antigen should be able to mediate lysis of immunoliposomes (Fig. 1B). Since many antigens are naturally multivalent (eg. bacteria, viruses or cell surface antigens) or can be easily made multivalent by absorption to a solid support, no inhibition assay for the antigen will be required. Immunoliposomes would be the only reagent involved in the assay.

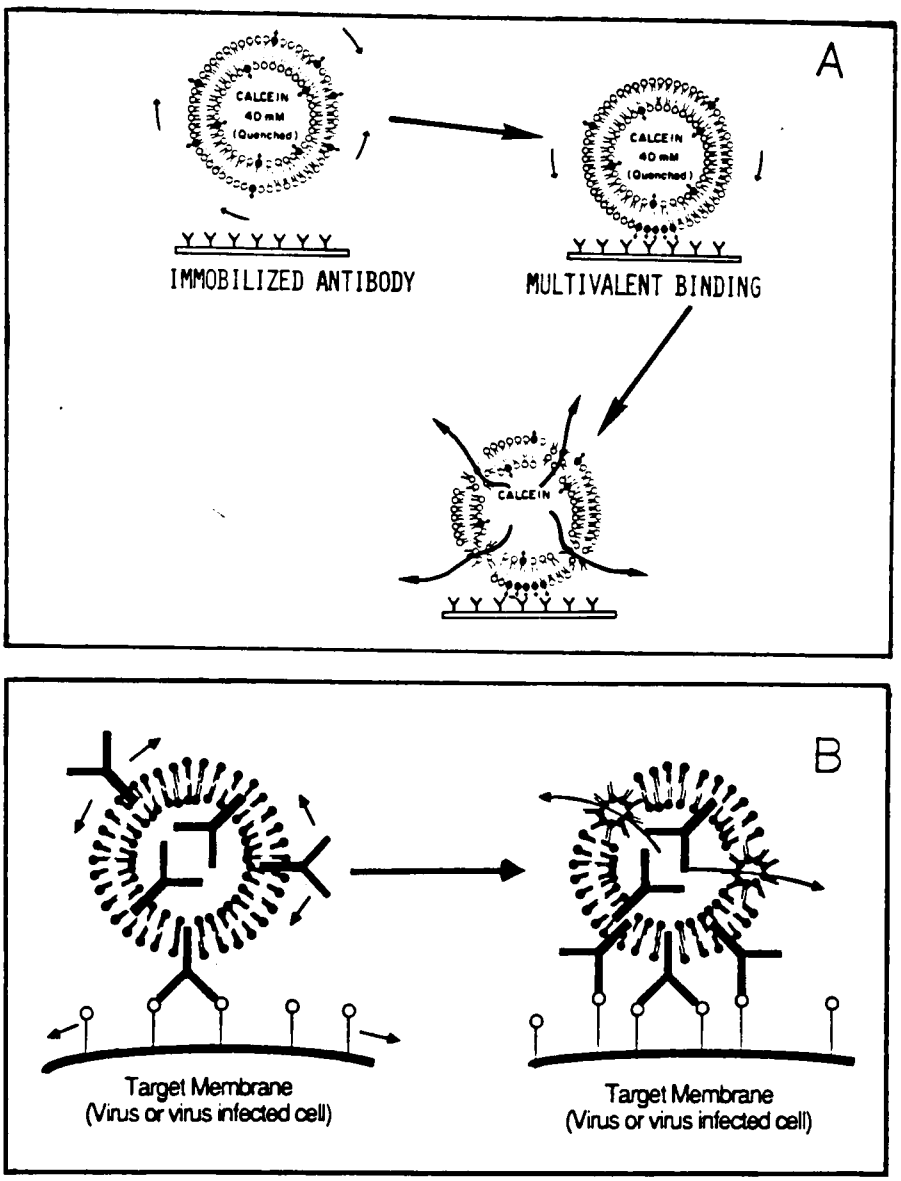

Figure 1. Schematic Representation of the Solid-phase Inhibition ILA (A), and Fluid-Phase Direct ILA (B). In antigen-stabilized liposomes (A), membrane stabilizers or antigens are freely diffusable in the plane of bilayer. When the antigen-stabilized liposomes are bound to the immobilized antibody, multiple immune complex formation (contact capping) between the stabilizer and antibody causes the phase separation of the stabilizer. This process eventually transforms the liposome bilayer to hexagonal phase leading to the lysis of liposomes. Similarly in (B), the PE immunoliposomes are stabilized by the freely diffusable membrane bound antibody. After binding with a multivalent target (i.e. virus or virus-infected cell), the formation of multiple immune complex leads to the depletion of the stabilizing antibody from the bulk PE bilayer which eventually transforms to the hexagonal phase and causes liposome lysis.

We have demonstrated this principle by employing the anti-HSV-gD, a mouse monoclonal IgG antibody against the herpes simplex virus (HSV) glycoprotein antigen gD, to stabilize the PE bilayer, and HSV as the multivalent antigen (Ho et al., 1986a-c). It is well-established that free IgG does not stably associate with membrane due to insufficient hydrophobicity of the molecule (Huang & Kennel, 1979). We have acylated the Fc portion of the antibody with palmitic acid (Ho et al., 1986a) to a stoichiometric ratio of 2 to 5 fatty acyl chains per IgG molecule. The palmitoyl IgG (pIgG) stabilized the DOPE liposomes at a lipid to pIgG ratio of $2\text{-}4X10^3$. Antibody stabilized PE immunoliposomes about 500Å in diameter could be prepared by brief sonication; almost all pIgG were incorporated into liposomes. When several different viruses were tested, only HSV could induce the rapid lysis of liposomes. HSV preincubated with free anti-HSV-gD could not. The assay is also applicable to the detection of HSV infected cells (Ho et al., 1986a-c). When HSV was used to infect mouse fibroblast L929 cells, the viral antigen gD expressed on the cell surface could induce the lysis of immunoliposomes. The cell-induced lysis was specifically blocked by free antibody. When PE was replaced with PC in the immunoliposomes, the target induced lytic activity on liposomes was lost, indicating that the lytic activity probably involved phase separation which triggered the bilayer to hexagonal (H_{II}) phase transition (Fig. 1B). Since the PE immunoliposomes are triggered to release encapsulated contents upon binding to the target (Fig 1B), we refer to them as **target-sensitive immunoliposomes**. Based on this principle, we were able to detect HSV at $5x10^4$ pfu in 7μL with calcein encapsulated liposomes and

16 pfu in 5μL with alkaline phosphatase encapsulated liposomes. The total assay time required was 20 min using calcein as a reporter molecule and 1.5 hr using alkaline phosphatase as a reporter molecule.

Although this ILA for HSV may not be as sensitive as the plaque assay using cultured cells, the time required for the latter assay is usually days (Table 1). Therefore, the plaque assay is not suitable for the situations such as detection of the neonatal HSV infection during the final stage of child delivery where rapidity of the assay is essential. A rapid assay employing target-sensitive immunoliposomes such as this one will allow detection of HSV antigen in less than two hr. In addition, the sensitivity of this ILA is within the concentration range of HSV found in most clinical samples (Arens & Swierkosz, 1986).

One of the major advantages of the assay design is the ease of adapting the basic principle to detect other analytes for which pure antibody is available. Since neither lytic molecules nor a complement is required, the assay is simpler and potentially more reproducible than other homogeneous ILA's. The design of this assay has eliminated the time consuming washing procedure and the use of expensive instrumentation, thereby providing an excellent potential for assembling it into test kits for use at home or in a physician's office.

Acknowledgment

I thank Dr. Stephen J. Kennel for a critical reading on this review.

Table 1

Comparison of Some Immunoassays

Analyte	Type	Washing required	Total time	Sensitivity range	Commercial application	Ref.
Digoxin	ILA	no	7 min	2-30 ng/mL	no	Litchfield et al., 1984
	ILA	yes	1 hr	2-4 ng/mL	no	O'Connell et al., 1985
	EIA	yes	2.5 hr	0.5-8 ng/mL	yes	Beckman Epsillon-E[a]
Tyroxine	ILA	no	1.5 hr	0.5-4.5 ng/mL	no	Hsia & Tan, 1978
	Agglutination	no	10 min	6-240 ng/mL	no	Reichberg et al., 1985
Rheumatoid factor	ILA	no	2 min	0.7 IU/mL	no	Kung et al., 1985
	Latex-agglutination	no	2 min	6.25 IU/mL	yes	Helena Lab[a]
HSV (gD)	ILA	no	1 hr	$3.2X10^3$ pfu/mL	no	Ho et al., 1986 a,b & d
	Plaque assay	yes	24-72 hrs	$5X10^2$ pfu/mL	yes	Zhao et al., 1986
CEA	RIA	yes	2 hr	0.6-60 ng/mL	yes	Abbott CEA-RIA[a]
	EIA	yes	4.5 hr	0.5-60 ng/mL	yes	Abbott CEA-EIA[a]

[a]Data from manufacturer's product inserts.

CHAPTER III

INTERACTIONS OF ANTIGEN-STABILIZED LIPOSOMES WITH IMMOBILIZED ANTIBODY: A NOVEL DESIGN OF TARGET-SENSITIVE LIPOSOME

Dioleoyl phosphatidylethanolamine (DOPE) does not form stable bilayer liposomes at room temperature and neutral pH. However, stable unilamellar liposomes could be prepared by mixing DOPE with a minimum of 12% of a haptenated lipid, N-[dinitrophenylaminocaproyl]-phosphatidylethanolamine [DNP-cap-PE]. When the liposomes bound to rabbit anti-DNP IgG that had been adsorbed on a glass surface, lysis of the liposome occurred with the release of the contents into the medium as judged by the fluorescence enhancement of an entrapped self-quenching dye, calcein. On the other hand, incubation of the same liposomes with glass surfaces coated with normal rabbit IgG had little effect. In addition, free anti-DNP IgG induced aggregation of the liposomes but did not cause any dye release. Liposomes composed of dioleoyl phosphatidylcholine (DOPC) and DNP-cap-PE did not lyse when added to the glass surfaces coated with either anti-DNP IgG or normal IgG. A likely mechanism for liposome lysis is that the DNP-cap-PE laterally diffuse to the contact area between the liposome and the glass. Binding of the haptenated lipid with the immobilized and multivalent antibody trap the haptenated lipids in the contact area. As a result of lateral phase separation, lipids may undergo the bilayer to hexagonal phase transition, leading to the leakage of the entrapped dye. Because both the free hapten and the free antibody inhibited the liposome leakage, this process could be used to assay for the free hapten or antibody. We

have shown that inhibition assays performed by using this principle can easily detect 10 pmol of free DNP-glycine in 40μL. Furthermore, by substituting human glycophorin A, a transmembrane glycoprotein, for the lipid hapten, we have demonstrated that this assay system is also applicable to detect protein antigen with a sensitivity of sub-nanogram level.

Introduction

During the past 20 yr, interactions between antibody and hapten bearing lipid in various model membrane systems have been studied extensively. Studies on liposomes containing hapten have revealed that immunogenic activity of the hapten is strongly dependent on the chain length as well as the molecular structure linking the hapten and the phosphodiester group of the lipid (Brulet & McConnell, 1977; Six et al., 1973; Dancey et al., 1979; Cooper et al., 1981; Hashimoto et al., 1982). These investigators also found that the lipid composition of the liposomes regulates the immunogenicity of the hapten. Most of the earlier experiments were focused on the interaction of hapten-sensitized liposomes and free antibody by using haptens such as the spin-labeled hapten (Parce et al., 1978; Parce et al., 1979; Smith et al., 1979). Immune complex-dependent complement fixation has also been studied (Haxby et al., 1968). In recent years, investigators in some laboratories have studied the interaction between the hapten dinitrophenyl (DNP), which bears liposomes, and the immobilized antibody, generally MOPC 315 cells, expressing surface anti-DNP IgA (Lesserman et al., 1979). It was concluded that binding of DNP-sensitized liposomes

to MOPC 315 is dependent on lipid composition, fluidity of membrane, and the hapten density (Hashimoto et al., 1983).

In all of the studies mentioned, liposomes were made of phospholipids such as phosphatidylcholine (PC), which is capable of forming stable bilayer liposomes. In the present study, we chose to use the unsaturated phosphatidylethanolamine (PE), which does not form stable bilayer at neutral pH and room temperature (Reiss-Husson, 1967; Rand et al., 1971; Cullis & DeKruijff, 1979). However, stable dioleoyl phosphatidylethanolamine (DOPE) liposomes can be obtained by the addition of either a haptenated lipid, N-(dinitrophenylaminocaproyl)-phosphatidylethanolamine (DNP-cap-PE) or a transmembrane glycoprotein human glycophorin A. The purpose of the study is to design an immunoliposome assay that is based on the binding of the antigen-sensitized liposomes with immobilized antibody. Haxby, Alving, Kinsky, et al. (Haxby et al., 1968; Alving et al., 1969; Kinsky et al., 1969) first showed that liposomes containing haptenated lipids can bind with antibody and fix complement, resulting in lysis of liposomes by activated complement components. Since then, the complement-mediated immune lysis has been the focal point of a large amount of literature (for a recent review, see Alving & Richards, 1983). Recently, other noncomplement-mediated immunoliposome assays have been developed. For example, binding of antibody to haptens conjugated to melittin, a membrane lytic protein, blocks liposome lytic activity of the melittin (Litchfield et al., 1984). Binding of antibody in lupus serum to liposomes containing cardiolipin prevents the lysis of liposomes by Mg^{2+} ions (Janoff et al., 1983). In all of the assays developed, a membrane lytic

molecule(s) or ion(s) is required. Although some of these immunoassays may be quite sensitive, they often involve many steps and are sometimes difficult to reproduce.

We describe here principles of a novel immunoliposome assay by using antigen-sensitized liposomes composed of unsaturated PE. These liposomes lyse when they come in contact with immobilized antibody in the absence of any lytic molecules, such as the complement. The principle may be widely applicable to many assays that involve the antigen-antibody interaction.

Materials and Methods

Materials

DOPE, dioleoyl phosphatidylcholine (DOPC), and DNP-cap-PE were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Calcein, N-(dinitrophenyl)-glycine (DNP-Gly), human glycophorin A, fetuin, and glycine were purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents were analytical grade.

Antibody

Anti-DNP serum was prepared from rabbits immunized with DNP-derivatized bovine serum albumin (BSA) (Eisen et al., 1967) and was a generous gift from Dr. Stephen Kennel. Monoclonal antibody against human glycoprotein A (from LICR LON/R10) (Edwards, 1980) was isolated from ascites fluid and was kindly provided by Dr. Timothy Heath. IgG fractions were purified from the serum or ascites fluid by protein A affinity column chromatography (Warr, 1982) and were stored in

phosphate-buffered saline (PBS) or NTE buffer (100 mM NaCl, 10 mM Tris-HCl, and 0.2 mM EDTA, pH 7.4), respectively, at -20°C. Antibody was adsorbed to glass surfaces by adding 40μL of IgG solution at various concentrations to a spot of 1.5 cm in diameter on a clean glass slide. After 20 min at room temperature, the slide was washed thoroughly with 6 to 8mL PBS or NTE, blotted to dryness except at the spot where antibody had been deposited, and immediately used for the subsequent experiment.

Liposome Preparation

In routine experiments, DOPE or DOPC (0.88μmol), DNP-cap-PE (0.12μmol), and a trace amount of hexadecyl [^{3}H] cholestanyl ether (final specific activity 5.7 X 10^9cpm/mol) were mixed and evaporated free of solvent with a stream of N_2 gas. The dry lipid was vacuum desiccated for at least 30 min. One hundred microliters of PBS containing 40 mM calcein, pH 7.4, were added. The mixture was sonicated for 20 min at room temperature in a bath sonicator (Laboratory Supplies, Inc., Hicksville, NY) until a uniform translucent liposome suspension was obtained. Glycophorin A-stabilized liposomes were prepared by adding concentrated glycophorin A and calcein in NTE buffer to previously dried DOPE with a trace amount of hexadecyl [^{3}H] cholestanyl ether (final specific activity 4.3 X 10^{12} cpm/mol) to obtain a final concentration of 10 mM lipid, 0.5 mol percent glycophorin A, and 100 mM calcein. The mixture was sonicated for 20 min. The liposome suspension was then chromatographed on a Biogel A-0.5m column to remove the untrapped calcein. Liposomes eluted with either PBS or NTE in the void

volume fractions were detected by counting ^{3}H-radioactivity (Pool et al., 1982). These fractions were pooled and stored at $4^{o}C$.

Liposome-antibody Interactions

Liposome suspension (0.9 to 2.9nmol lipid in 5 to 45μL) was added to the spot on the glass slide, which had been previously coated with IgG. After a 20-min incubation in a moist chamber at room temperature, the glass slide was rinsed with 2mL PBS or NTE to quantitatively transfer the liposomes into a quartz cuvette. The fluorescence was measured with a Perkin Elmer LS5 spectrofluorometer with λ_{ex} = 490nm and λ_{em}=520nm. These measurements were reproducible within ±7%.

The total calcein fluorescence in the recovered liposome preparation was measured after the addition of sodium deoxycholate to a final concentration of 0.12% for DNP hapten assay and 0.24% for glycophorin A assay. The percentage of dye release is defined as

$$\% \text{ release} = [(F - F_o)/(F_t - F_o)] \times 100$$

where F_o and F are the calcein fluorescence of the liposome sample before and after the interaction with the immobilized antibody. F_t is the total calcein fluorescence of the liposome sample before and after the interaction with the immobilized antibody. F_t is the total calcein fluorescence after release with deoxycholate.

For the inhibition of dye release, free hapten or glycoprotein in 40μL was added to the immobilized antibody on the glass slide and incubated for 20 min at room temperature before the addition of liposomes. For the inhibition by free antibody, equal volumes of liposome

and antibody were mixed and preincubated for 20 min at room temperature before addition to the glass slide. To analyze the inhibition by cell membrane-bound glycoproteins, varying number of cells in 40μL were added to the immobilized antibody on the glass slide and incubated for 20 min at room temperature, followed by washing with 6 to 8mL of NTE buffer to minimize the nonspecific binding.

Ninety-degree Light Scattering of Liposomes

To test for liposome formation, sonicated lipids were diluted 100-fold in PBS. Light scattering (at 90 degrees) was measured in a Perkin Elmer LS5 spectrofluorometer at $\lambda_{ex} = \lambda_{em} = 660$ nm with slit width = 3 nm.

Interaction of Liposomes with Free Antibody

DOPE liposomes (3.35nmol lipid in 10μL) and IgG were added into a cuvette with a final volume of 2mL PBS, pH 7.4. The amount of total antibody concentration was 0.6mg/mL with varying ratios of anti-DNP IgG to normal IgG. Aggregations caused by anti-DNP IgG was monitored by measuring 90-degree light scattering of liposomes. Release of entrapped calcein caused by liposome aggregation was determined as described above.

Electron Microscopy

Liposomes (0.75μmol/mL) were negatively stained with 0.5% aqueous uranyl acetate and viewed in a Hitachi 600 electron microscope operating at 75 KV. The size of the liposomes was determined on photographically enlarged micrographs.

Preparation of Red and White Blood Cells (RBC and WBC)

RBC from human, mouse, and sheep were prepared according to Steck et al. (1970). WBC were prepared by spinning down the RBC on a desk-top centrifuge, and the WBC in the buffy coat were collected. The contaminating RBC were removed by lysing with Tris-buffered ammonium chloride (Mishell et al., 1980). Both RBC and WBC were suspended in NTE buffer and were used immediately.

Results

Stabilization of the DOPE Liposome Bilayer by DNP-cap-PE

Formation of stable liposomes was monitored by light scattering at 660 nm. To determine the minimal amount of DNP-cap-PE that could stabilize the DOPE bilayer, various amounts of DNP-cap-PE were mixed with DOPE or DOPC, and the lipid mixtures were sonicated for 20 min before measurement of light scattering. When stable, sonicated liposomes were formed, the turbidity of the suspension was low and resulted in low light scattering. At concentrations of DNP-cap-PE above 12%, stable DOPE liposomes could be generated (Fig. 2). Between 6 and 11%, liposome suspensions were quite turbid, indicating the presence of nonuniform particles. Below 6%, however, large aggregates of lipid were seen and the light scattering was again low. Pure DOPE forms only large aggregates even after prolonged sonication. In contrast, DOPC formed stable, low light scattering liposomes regardless of the addition of DNP-cap-PE. We concluded that a minimum of 12% DNP-cap-PE was required for stable DOPE liposome formation. This composition was used for all subsequent experiments.

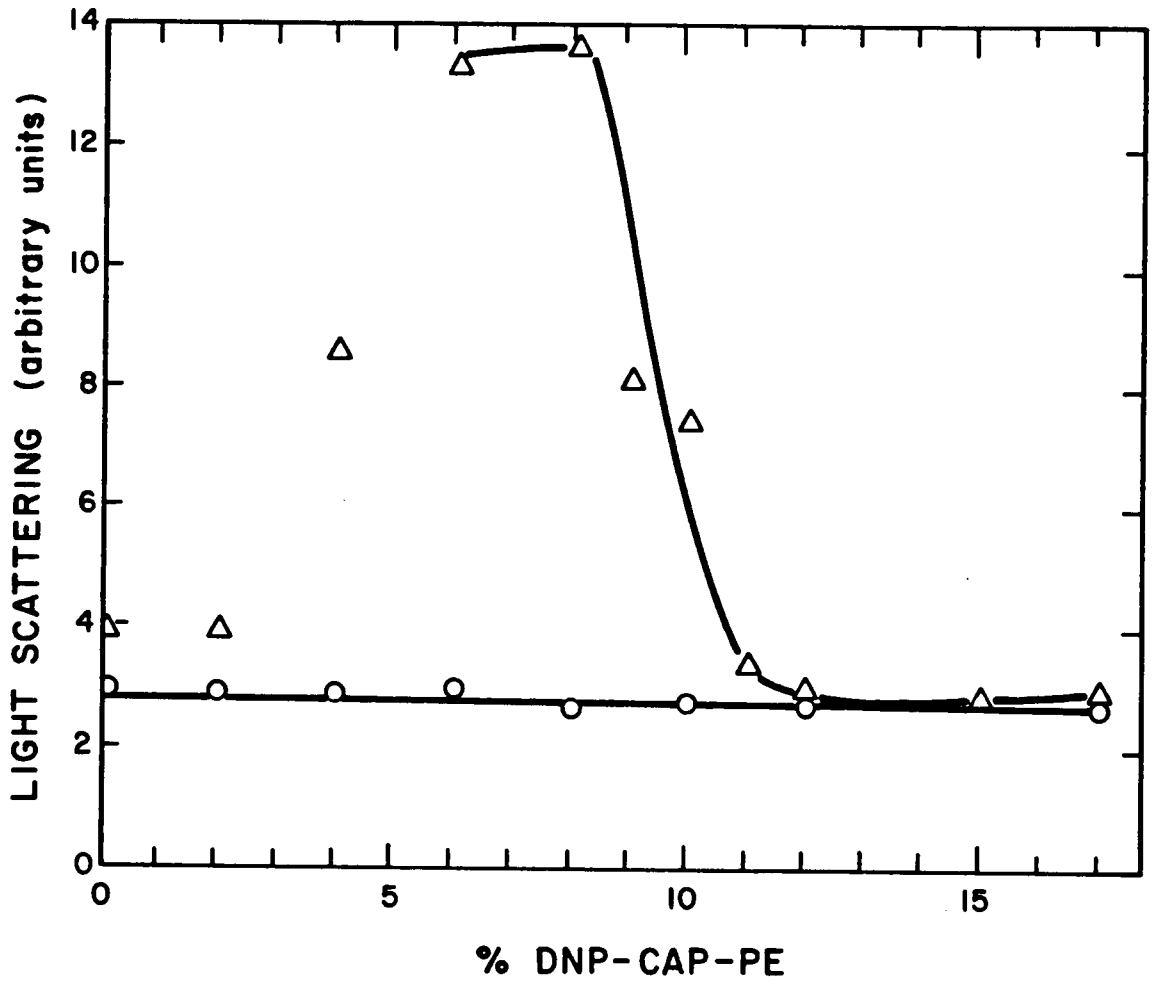

Figure 2. Stabilization of DOPE and DOPC Liposomes with DNP-cap-PE. 90° light scattering of the sonicated lipid were measured for DOPE (△) and DOPC (◯) liposomes.

Size of Liposomes

Sonicated liposomes composed of DOPE:DNP-cap-PE (88:12) (hereafter called DOPE liposomes) were unilamellar and relatively homogeneous in size as determined by negative stain electron microscopy. The average diameter of the liposomes was 908Å with a standard deviation of 135Å.

Trapped Volume of Liposomes

From the average diameter of the DOPE liposomes, the trapped volume can be calculated according to Enoch and Strittmatter (1979) to be 2.66μL/μmol lipid. The trapped volume was also directly estimated by measuring the amount of calcein trapped in the liposomes after the untrapped calcein was removed by gel filtration. The amount of calcein trapped was determined from a standard curve of fluorescence intensity vs. calcein concentration (0 to 0.5μM). The lipid concentration was determined from the amount of [^{3}H] lipid marker in the preparation (Pool et al., 1982). Assuming that the calcein concentration inside the liposomes was 40 mM (the concentration used to prepare liposomes), the trapped volume was determined to be 2.08 μL/μmole lipid. This is in remarkably good agreement with the value calculated from the size of the liposomes. The trapped volume of DOPC:DNP-cap-PE (88:12) liposomes (hereafter called DOPC liposomes) was 0.54 μL/μmol, indicating that these liposomes were much smaller in size. DOPE or DOPC liposomes containing calcein could be stably stored at 4°C for at least 2 weeks without significant dye leakage.

Dye Release upon Liposome-antibody Interaction

To measure liposome lysis, we trapped calcein (40 mM) inside the liposomes. At this concentration, calcein fluorescence is self-quenched to greater than 90%. Fluorescence is greatly enhanced when the dye is diluted by leakage from the liposomes (Allen & Cleland, 1980; Connor et al., 1984). Using glass surfaces coated with various types of proteins, we tested their ability to induce the calcein release from the liposomes. Bare glass surface and glass surface coated with BSA could not induce DOPE liposome leakage (Table 2); however, contact with a glass surface coated with anti-DNP IgG induced release of all the entrapped calcein. A glass surface coated with normal IgG induced the release of only about 40% of the contents. Dye release could be blocked by a pretreatment of the glass surface with free hapten, DNP-Gly, or by a preincubation of the liposome with free anti-DNP IgG, but not with free normal IgG or BSA. These results strongly indicate that the dye release from DOPE liposomes is a direct result of the antibody-hapten binding. DOPC liposomes were very stable; as evidenced by the fact that none of the glass surface types tested induced significant dye release (Table 2).

Effect of Immobilized Antibody Concentration on Dye Release

We varied the concentrations of the IgG solution used in the coating of the glass surface. Figure 3 shows that the dye release was dependent on antibody concentration applied to the glass surface. Nearly total release was observed for glass surfaces coated with anti-DNP IgG solutions greater than 1μg/mL. Below this concentration,

Table 2

Interaction Between Calcein-entrapped Liposomes and Immobilized Antibody

Protein[a] Coated on Glass Slide	Pretreatment		Percent of Dye Released from Liposomes[b]	
	Glass slide	Liposome	DOPE	DOPC
None	None	Deoxycholate[d]	100	100
None	None	None	2	3
Anti-DNP IgG	None	None	102	4
Anti-DNP IgG	DNP-Gly[c]	None	8	-1
Anti-DNP IgG	None	Free anti-DNP IgG[e]	-11	1
Anti-DNP IgG	None	Free normal IgG[f]	95	-3
Anti-DNP IgG	None	BSA[g]	95	1
Normal IgG	None	None	38	-3
Normal IgG	DNP-Gly[c]	None	25	3
Normal IgG	None	Free anti-DNP IgG[e]	3	1
Normal IgG	None	Free normal IgG[f]	39	-1
Normal IgG	None	BSA[g]	29	5
BSA	None	None	-6	2

[a]Four micrograms of antibody were used to apply on the glass slide, and excess antibody was washed with PBS. For BSA, 160 µg were used to coat the glass slide.

[b]Amount of lipid used for each assay: DOPE = 0.87 nmol, DOPC = 1.92 nmol.

[c]We used 0.4 µmol DNP-Gly in 40 µL.

[d]Final deoxylcholate concentration was 0.12%.

[e]We used 0.5 µg anti-DNP IgG in 5 µL.

[f]We used 0.5 µg normal IgG in 5 µL.

[g]We used 20 µg of BSA in 5 µL.

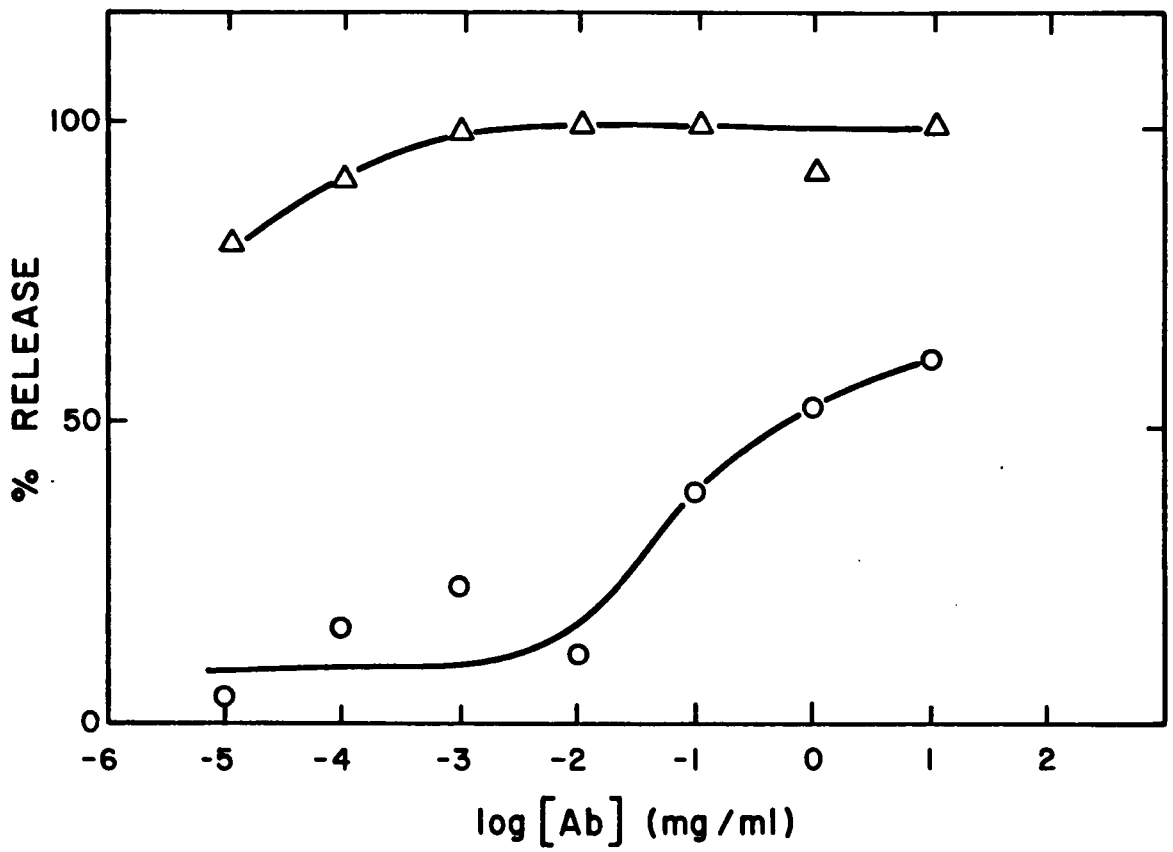

Figure 3. Effect of Adsorbed Antibody Concentration on DOPE Liposome Lysis. Anti-DNP IgG (△) or normal IgG (○) at indicated concentrations was used to coat the glass slide, and the DOPE liposome lysis was measured by calcein release.

progressively lower release was seen. At concentrations above 10μg/mL, normal IgG also induced dye release, although the magnitude was much lower than that caused by anti-DNP IgG. This is in agreement with the observation of naturally occurring anti-DNP activity in normal rabbit IgG (Farah, 1973). However, because DNP-Gly could only partially inhibit the dye release (Table 2) caused by the immobilized normal rabbit IgG, some additional interfering factor(s) may be present in the normal rabbit IgG.

Effect of Free Antibody on Liposome Aggregation without Dye Release

We have studied the interactions of antibody with DOPE liposomes. The total IgG concentration was kept constant in this experiment, but the mole fraction of anti-DNP IgG was varied to test the haptenated liposome aggregation caused by free antibody. Figure 4 shows that aggregation of DOPE liposome was indeed dependent on the anti-DNP IgG concentration. In addition, the aggregation did not cause the release of the entrapped dye. No dye release was observed even with a high antibody concentration of 10mg/mL. The aggregation occurred instantaneously, and even after a prolonged period (up to 3 to 4 hr) of incubation at room temperature, there was no detectable amount of increase in either aggregation or leakage of the entrapped dye. Also note that antibody-induced aggregation was not reversible by the addition of free hapten, DNP-Gly up to 100μM. DOPC liposomes were similarly aggregated by the free anti-DNP IgG without the release of dye (data not shown).

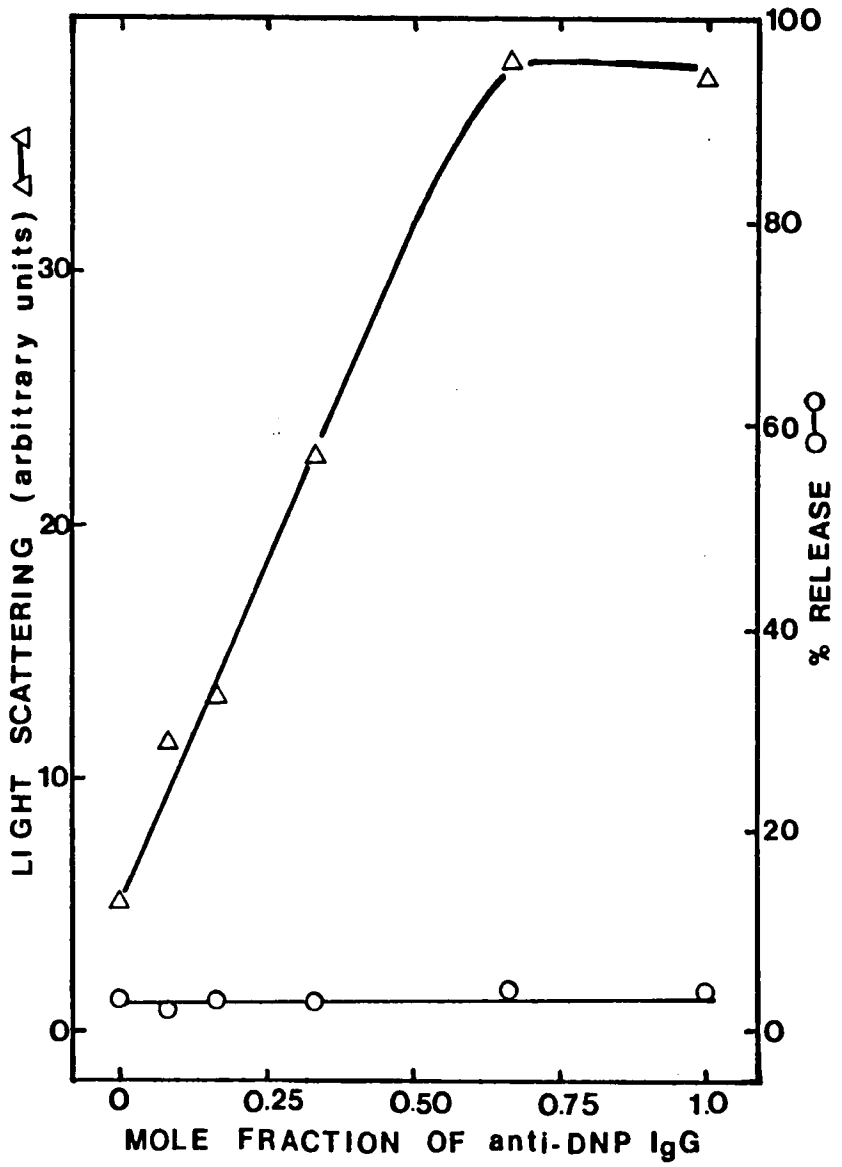

Figure 4. Effect of Free Anti-DNP IgG on DOPE Liposomes. Immune complex (DOPE liposome and antibody complex) formation is measured by 90-degree light scattering (△), and the DOPE liposome lysis is measured by entrapped calcein release (○).

Inhibition of Dye Release by Free Hapten

We examined the ability of free hapten to inhibit liposome lysis. As can be seen in figure 5, DNP-Gly could effectively inhibit dye release from the DOPE liposomes. The concentration of DNP-Gly that caused 50% inhibition was calculated to be 0.35μM, which is equal to 14pmol in 40μL of the preincubation medium. Incubation with a non-hapten analog, Gly, had no effect on dye release even at 1 mM concentration.

Inhibition of Dye Release by Free Antibody

Although free (fluid phase) anti-DNP IgG did not cause dye release, preincubation of the DOPE liposomes with free anti-DNP IgG, but not normal IgG, caused inhibition of the dye release by the immobilized antibody (Fig. 6). Fifty percent inhibition took place at free antibody concentration of 0.5mg/mL, which is equal to 2.5μg in a 5-μL preincubation volume. Thus, assays for antibodies based on this type of inhibition are likely to be less sensitive than those for hapten.

Immunoliposome Assay for Glycophorin A

To further demonstrate the general applicability of this solid-phase immunoliposome assay, we used 0.5 mol percent human glycophorin A to stabilize the DOPE liposomes. Taraschi et al. (1982) had shown that at this molar ratio, all of the DOPE were in bilayer form, and increasing fraction of DOPE in the Hexagonal (H_{II}) phase was detected with lower amounts of glycophorin A incorporated.

To measure liposome lysis, we trapped calcein (100 nM) inside these liposomes and followed the enhancement of calcein fluorescence

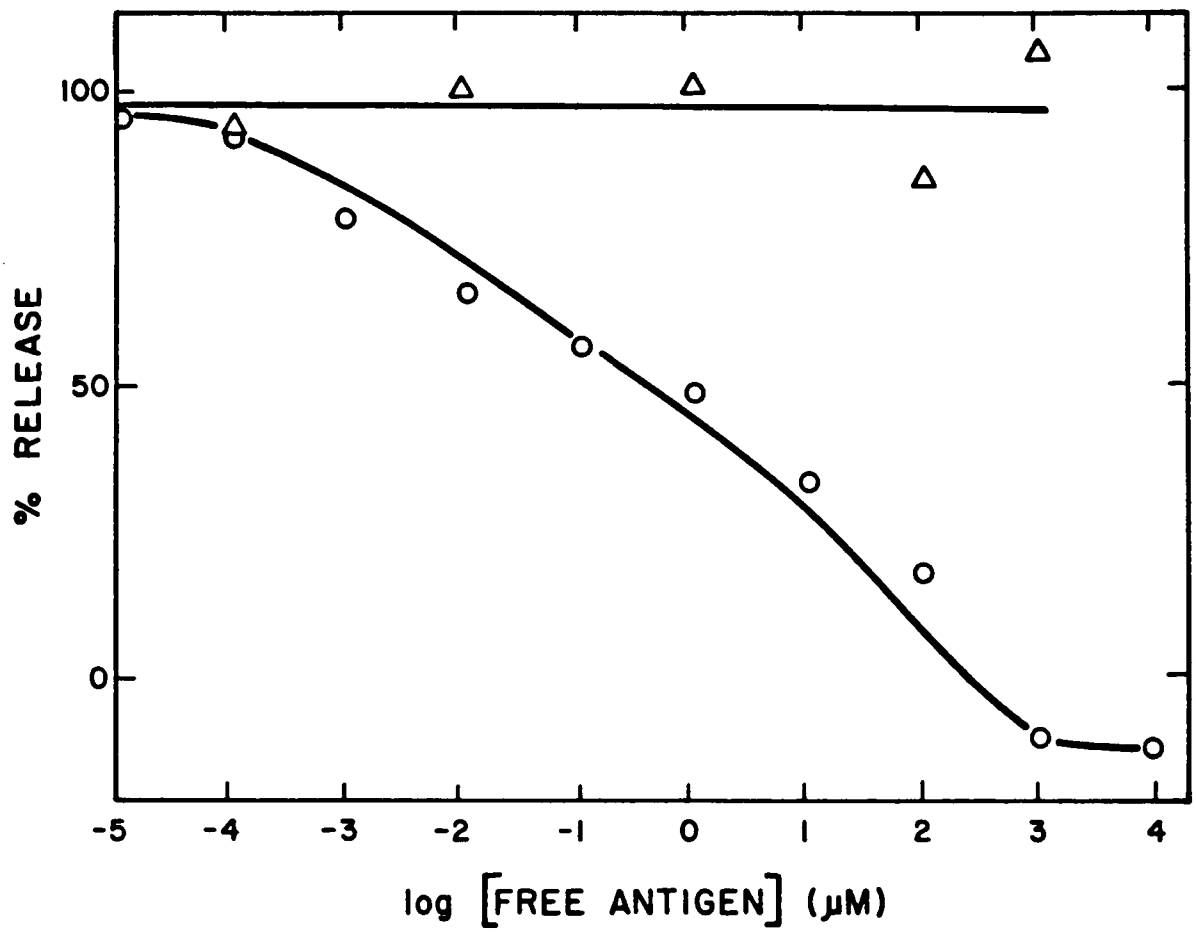

Figure 5. Inhibition of DOPE Liposome Lysis by Free Hapten. DNP-Gly (○) or Gly (△) of indicated concentration was added to adsorbed anti-DNP IgG on the glass slide before liposome addition.

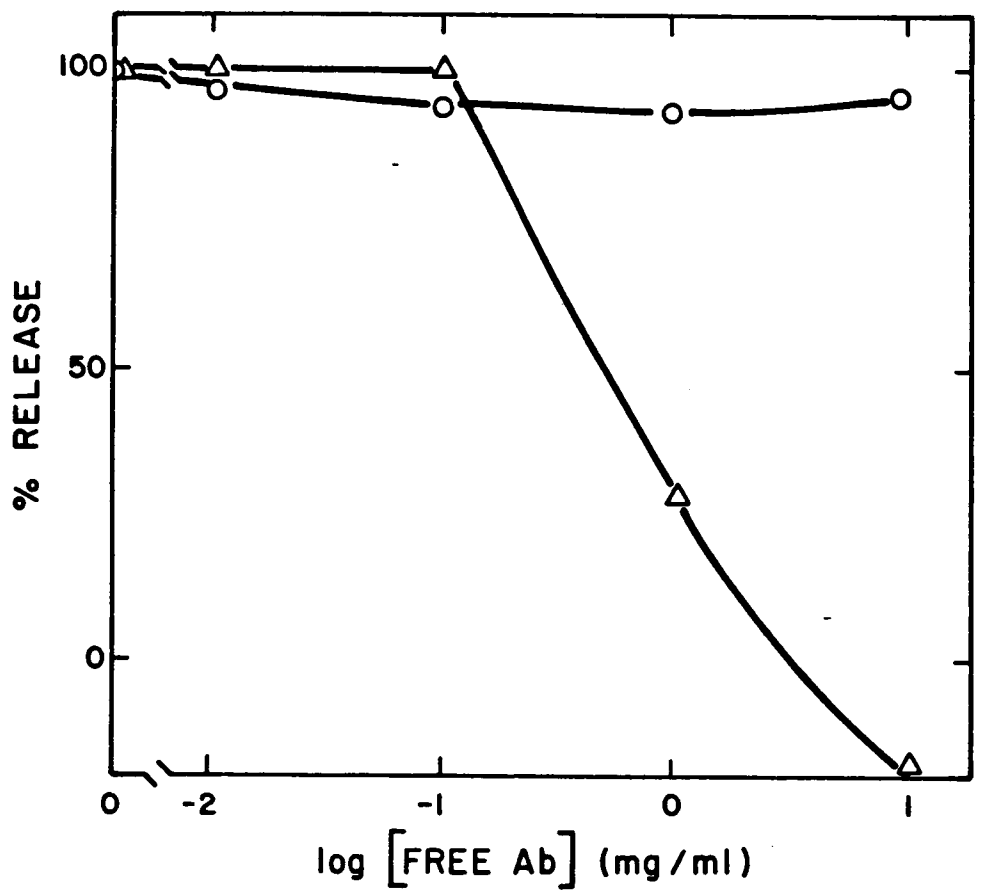

Figure 6. Inhibition of DOPE Liposome Lysis by Free Antibody. Liposomes were preincubated with free anti-DNP IgG (△) or normal IgG (○) at indicated concentrations before adding to the adsorbed antibody on the glass slide.

upon the release of dye into the medium. Using the immobilized anti-human-glycophorin A IgG, we were able to use as little as 0.1μg/mL of antibody to coat the glass surface. On the other hand, neither anti-DNP IgG nor anti-HSV-gB IgG (a monoclonal antibody against the gB glycoprotein of the herpes simplex virus type 1) was able to induce the dye leakage. Therefore, 1μg/mL of antibody was used for all of the subsequent experiments to assure a complete effect. Figure 7A shows the inhibition of the dye release by free human glycophorin A. Because no other glycoproteins tested (fetuin or BSA) could significantly inhibit the dye release form the liposomes, this assay was specific for glycophorin A. Fifty percent inhibition took place at the free glycophorin A concentration of 12ng/mL or 0.48ng in a 40-μL assay volume. The specificity of this monoclonal IgG toward human glycophorin A has been reported elsewhere (Edwards, 1980; Bragman et al., 1983). Figure 7B shows the inhibition of dye release by free antibody. Again, the inhibition was only observed with anti-human-glycophorin A IgG, but not with anti-HSV-gB IgG. The concentration of the free antibody that caused 50% inhibition was calculated to be 0.2μg/mL, or 1ng in 5μL preincubation medium.

Finally, figure 7C shows the inhibition assay for membrane-bound glycoprotein. Only RBC that contain the glycophorin A can inhibit the lysis of DOPE liposomes and only insignificant amount of inhibition was observed with the WBC. This small inhibitory activity might be due to the residual red cells in the WBC preparation. Although there was also some cross-reactivity with the mouse and sheep RBC, the inhibition took place at a cell concentration one to three orders of magnitude

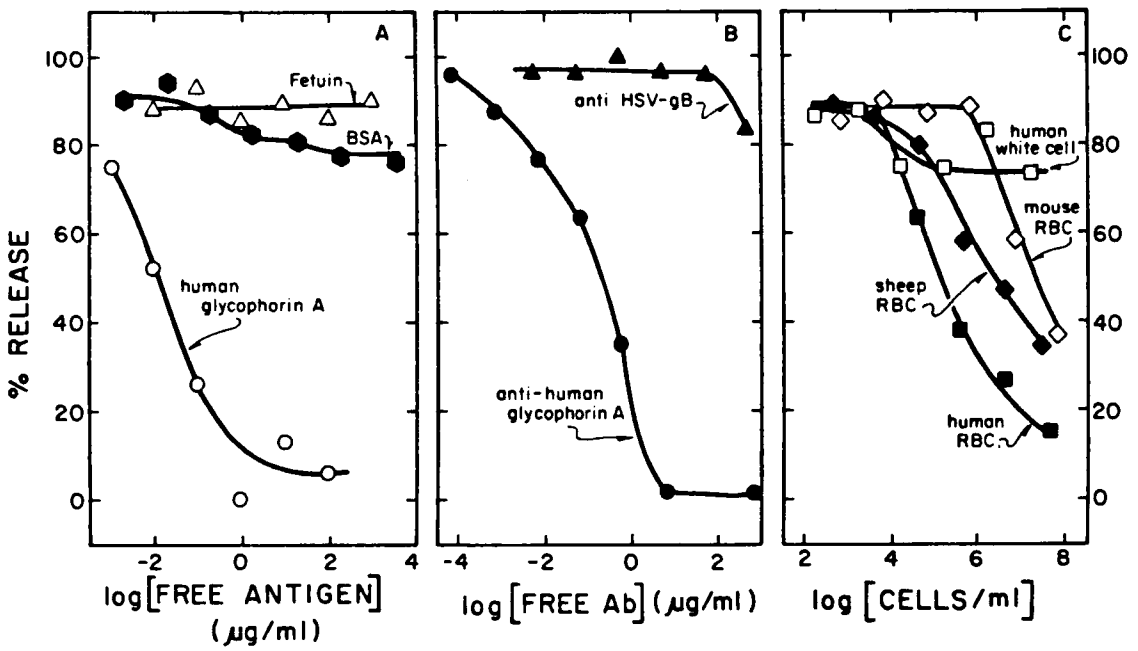

Figure 7. Effect of Glycoproteins (A), Free Antibodies (B), and Blood Cells (C) on Glycophorin A-DOPE Liposome Lysis. Glass surfaces coated with 40μL of 1μg/mL anti-human-glycophorin A IgG and liposome suspensions containing 2.9nmol of lipids in 5μL were used for all of the experiments. A, antibody coated glass slides were preincubated with free glycophorin A (○), fetuin (△), or BSA (⬢) before the addition of liposomes. B, Liposomes were preincubated with anti-human-glycophorin A IgG (●) or with anti-HSV-gB IgG (▲) before being added to the immobilized antibody. C, Antibody-coated glass slides were preincubated with human RBC (■), human WBC (□), sheep RBC (◆) or mouse RBC (◇) as described in Materials and Methods before the addition of liposomes.

higher than that of human RBC. Parallel inhibition was also observed with RBC ghosts (data not shown).

Discussion

The design of the assay is based on the inability of unsaturated PE to form stable bilayer liposomes at physiologic conditions (Reiss-Husson, 1967; Rand et al., 1971; Cullis & DeKruijff, 1979). We used the minimal amount of DNP-cap-PE or glycophorin A required to stabilize DOPE bilayer liposomes such that a small perturbation of lipid distribution, leading to a decrease in the effective concentration of the haptenated lipid or glycophorin A in the bilayer, would cause destabilization. We chose the perturbation factor to be the binding of the haptenated lipid or glycophorin A with the immobilized, multivalent antibody.

The simplest explanation for antibody-induced liposome lysis involves a lateral phase separation of the phospholipids. As shown in figure 8, we envision that the haptenated lipids or glycophorin A diffuse rapidly in the plane of the fluid lipid bilayer with a diffusion coefficient about 1×10^{-8} to 1×10^{-9} cm^2/sec (Peters, 1981). This means that only a fraction of a second is necessary for the haptenated lipid or glycophorin A to diffuse to the area of contact between liposomes and the glass surface. Formation of immune complexes prevents the random diffusion of antigen by trapping them in the contact area. Such lateral phase separation effectively decreases the haptenated lipid or protein concentration in the bulk bilayer and leads to the destabilization of liposomes. We have estimated that the entire

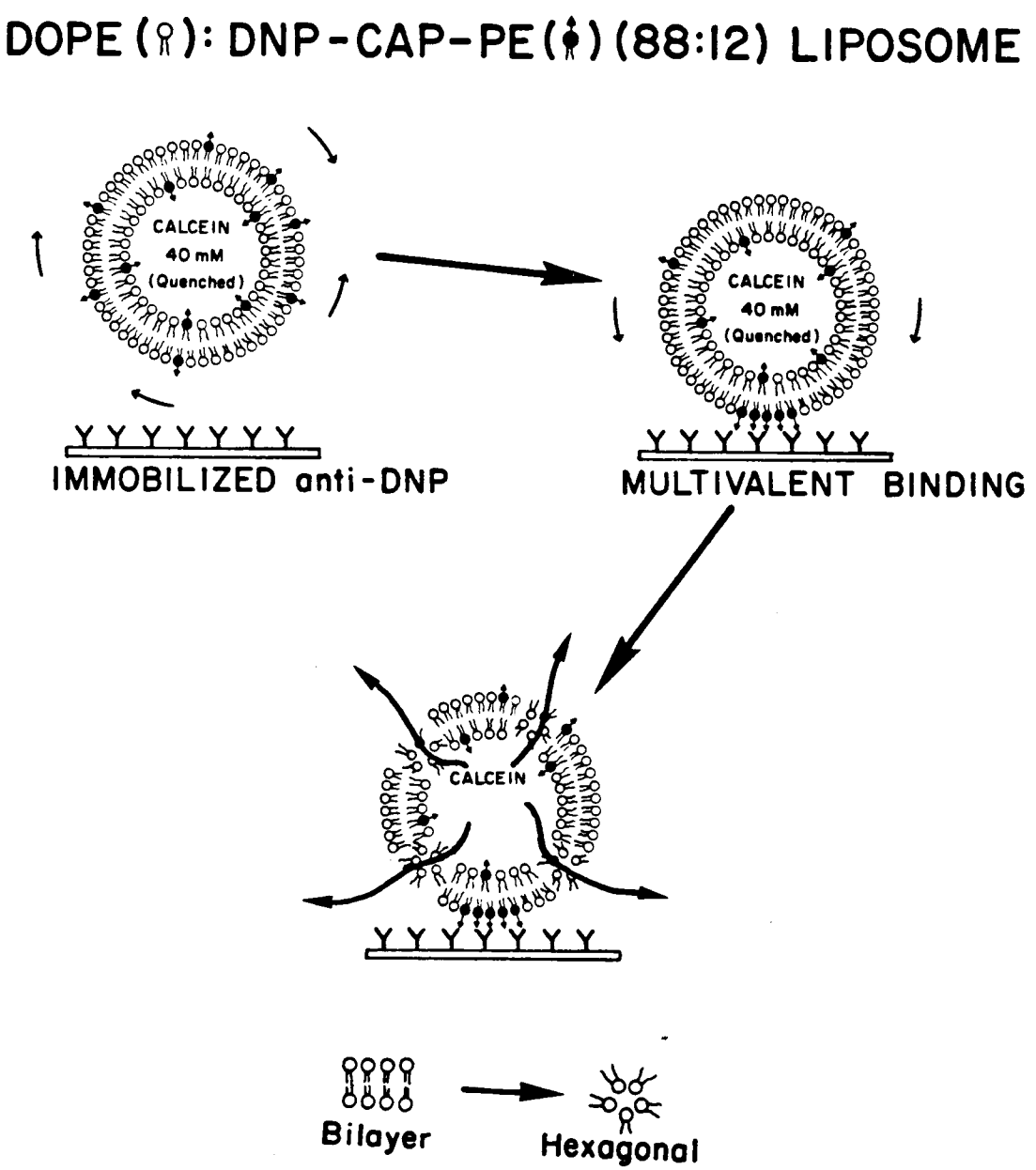

Figure 8. Schematic Representation of Solid-Phase Immunoliposome Assay.

process will take a few seconds or less at room temperature. Although this mechanism remains speculative, it is consistent with the observation that free bivalent antibody does not induce liposome leakage, because binding of the bivalent antibody to either the haptenated lipid or glycophorin A cannot bring about extensive phase separation. In fact, aggregation of the liposomes crosslinked by the bivalent antibody was observed.

Whether the liposome destabilization involves the formation of the hexagonal (H_{II}) phase, although highly possible, is not clear at present. However, such a phenomenon has been observed in unsaturated PE bilayer stabilized by the presence of glycophorin A, a transmembrane glycoprotein of erythrocyte membranes. When glycophorin A molecules are laterally aggregated by the binding of lectin, extensive H_{II} phase formation occurs with subsequent leakage of the encapsulated contents (Taraschi et al., 1982). Our observation is closely analogous to this situation.

Although the exact molecular mechanism of liposome lysis remains to be investigated, the fact that lysis can be blocked by free antigen or free antibody forms the basis for an immunoliposome assay for either antigen or antibody. Because the assay is a membrane lytic type, it is very sensitive. We have shown that 14 pmol of free hapten can inhibit 50% of the release (Fig. 5). This sensitivity compares favorably with other membrane lytic assays (Cole, 1982). The antibody used in that part of the study was a IgG fraction of a polyclonal antiserum. As we have expected, increase in sensitivity of about three orders of magnitude was observed with the use of a monoclonal antibody in the

glycophorin A assay. In fact, inhibition assay performed with the free anti-human-glycophorin A IgG can easily detect 1ng of protein in 5μL incubation medium (Fig. 7B). Furthermore, the sensitivity may also be improved by trapping of enzymes such as peroxidase or phosphatase in the liposomes and using colorigenic substrates for a photometric assay. Moreover, the procedure may be further simplified by using latex, glass, or other polymer beads with covalently immobilized antibody. Finally, because the lysis event is a direct result of immune complex formation and does not involve any other membrane lytic molecules, such as complement, the assay is simpler and may be more reproducible than other immunoliposome assay (Litchfield et al., 1984; Uemura & Kinsky, 1972).

In principle, the assay can be designed in a reciprocal manner such that the stabilizing factor for the DOPE liposomes is the antibody itself and the leakage occurs when the antibody binds to a multivalent antigen. Native IgG does not stably bind to liposomes; however, IgG conjugated with fatty acids (Huang et al., 1982) or phospholipids (Sinha & Karush, 1979; Jansons & Mallett, 1981) may serve this purpose. The advantage of this design is that the liposomes should be directly sensitive to such multivalent antigens as intact bacteria, viruses, and other pathogens. No competition or inhibition assay should be necessary.

In summary, we have described the interactions of liposomes containing antigen with immobilized antibody. In particular, the interactions lead to the lysis of liposomes composed of DOPE. This observation may be used to design a simple immunoliposome assay. With

appropriate modifications, the assay should be useful in quantitating a wide variety of antigens and antibodies in biologic fluids and other sources.

Acknowledgments

I thank Dr. Stephen J. Kennel for providing the antiDNP antiserum, and Dr. Timothy Heath for providing the anti-human-glycophorin A antibody.

CHAPTER IV

TARGET-SENSITIVE IMMUNOLIPOSOMES: PREPARATION AND CHARACTERIZATION

A novel target-sensitive immunoliposome was prepared and characterized. In this design, target specific binding of antibody-coated liposomes was sufficient to induce bilayer destabilization, resulting in a site-specific release of liposome contents. Unilamellar liposomes were prepared by using a small quantity of palmitoyl IgG (pIgG) to stabilize the bilayer phase of the unsaturated phosphatidylethanolamine (PE) which by itself does not form stable liposomes. A mouse monoclonal IgG antibody to the glycoprotein D (gD) of Herpes Simplex Virus (HSV) and dioleoyl PE were used in this study. A minimal coupling stoichiometry of 2.2 palmitic acid per IgG was essential for the stabilization activity of pIgG. In addition, the minimal pIgG to PE molar ratio for stable liposomes was 2.5×10^{-4}. PE immunoliposomes bound with HSV-infected mouse L929 cells with an apparent K_d of 1×10^{-8} M which was approximately the same as that of the native antibody. When 50 mM calcein was encapsulated in the PE immunoliposomes as an aqueous marker, binding of the liposomes to HSV-infected cells resulted in a cell concentration dependent lysis of the liposomes as detected by the release of the encapsulated calcein. Neither uninfected nor Sendai virus-infected cells caused a significant amount of calcein release. Therefore, the release of calcein from PE immunoliposomes was target specific. PC immunoliposomes were not lysed upon contact with infected cells under the same conditions, indicating that PE was essential for the target-specific liposome destabilization. Since 70% of palmitic

acid was located on the Fc portion of the pIgG molecule, pIgG was proposed to stabilize the PE liposomes by inserting either the acylated Fc portion or the Fc-linked palmitic acid into the lipid bilayer and leaving the Fab portion available at the surface for antigen binding. Destabilization of the liposomes upon binding with a multivalent antigen may involve a local aggregation of pIgG at the contact area (contact capping). These liposomes may be useful for site-specific drug delivery and liposome-based immunoassays.

Introduction

Target-specific liposomes have been studied extensively for their use as inert carriers for drugs, enzymes, hormones, DNA and other biomedically important substances (for recent reviews see Connor et al., 1985). Immunoliposomes have been shown to bind specifically to their target cells *in vitro* and *in vivo*. The subsequent events which follow cell binding are currently of great interest since liposome binding may not necessarily be followed by the delivery of the encapsulated drug into the the target cell.

One approach to ensure target-specific delivery of drugs into the cell is to fuse liposomes with intact cells. This approach has been demonstrated using a variety of fusogens such as Sendai virus (Okada, 1962, Nakanishi et al., 1985), synexin (Hong et al., 1979), and polyethyleneglycol (Boni et al., 1984), as well as fusion by means of pulsed electric field (Vienken & Zimmerman, 1985). However, all of these fusion protocols suffer from a relatively high cytotoxicity to the target cells. An alternative approach for drug intake is to

exploit cellular endocytosis. Using pH-sensitive immunoliposomes, which become fusion competent at pH below 6.5 and fuse with endosomes, this approach was successful for cytoplasmic delivery of antitumor drugs (Connor & Huang, 1985a). However, an endocytosis-dependent drug delivery system may not be applicable to target cells with low endocytotic activity.

To overcome this constraint, heat-sensitive immunoliposomes were developed (Sullivan & Huang, 1985). This cell delivery system depends on the uptake of drug released from briefly heated cell bound liposomes. For example, enhanced uptake of [^{3}H]uridine encapsulated in heat-sensitive immunoliposomes composed of dipalmitoylphosphatidylcholine (DPPC) was reported recently (Sullivan & Huang, 1986). Although the system was quite efficient, the localized heating of the liposome-treated cells may not be feasible for many applications, particularly for in vivo situations.

In this chapter, we have described a new approach in the design of liposomes with a built-in mechanism to release encapsulated drugs at the surface of the target cell. These target-sensitive (TS) immunoliposomes require the antibody not only for specific target cell recognition but also to stabilize the otherwise unstable liposomes. Since the IgG antibody is not sufficiently hydrophobic to be incorporated into the liposome membrane (Huang & Kennel, 1979), palmitic acid was covalently coupled to IgG (Huang et al., 1980) and the palmitoyl IgG was used to prepare the TS immunoliposomes.

Materials and Methods

Materials

Dioleoyl PE and dioleoyl PC were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Calcein, and papain were purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents were analytical grade.

Antibody

Mouse monoclonal IgG_{2a} antibody 4.2 against Herpes Simplex Virus (HSV) antigen gD (hereafter called IgG) was isolated from mouse ascities fluid and was kindly provided by Dr. Steve Norley. The antibody was originally isolated by Dr. Melvin Trousdale. IgG was purified by protein A-sepharose affinity chromatography followed by DEAE Sephadex A25 ion-exchange chromatography. The purified antibody was stored in PBS at -20°C. When required, IgG was radiolabelled with ^{125}I using chloramine T (Hunter & Greenwood, 1962) to a specific activity of 1 X 10^3 to 5 X 10^5 cpm/μg.

Derivatization of IgG with Palmitic Acid

Coupling of N-hydroxysuccinimide ester of palmitic acid (NHSP) to IgG was done following the procedure of Huang et al. (1980). Briefly, ^{125}I-labeled IgG was added to the [^3H]NHSP or NHSP in PBS such that the final deoxycholate (DOC) concentration was 2%. The coupling was performed at 37°C for 12 hr. Palmitic acid, the hydrolyzed product of NHSP, in the reaction mixture was separated from derivatized IgG using Sephadex G75 column and eluted with PBS containing 0.15% DOC as

described previously (Huang et al.,1980). The derivatized IgG was concentrated with a Centricon 30 microconcentrator (Amicon Co, MA) and dialyzed against PBS containing 0.15% DOC. Binding activity of palmitoyl-IgG (pIgG) was tested by a radioimmunoassay method using the [^{125}I]pIgG. Briefly, 4 X 10^6 pfu of HSV in 50µL of PBS pH 7.6 was incubated at 4^{o}C overnight in an Immulon Removawell (Dynatech Lab Inc.). After washing, the Removawells were incubated with 50µL of 5% goat serum in PBS for 1 hr to block the nonspecific binding sites. The goat serum was then removed and replaced with [^{125}I]pIgG (0.1 to 10µg/mL) in 40µL of PBS containing 0.15% DOC. The incubation of pIgG was performed at 4^{o}C for 1 hr. After washing with PBS, pH7.4, the Removawells were counted for ^{125}I-radioactivity to determine the [^{125}I]pIgG bound to the HSV adsorbed on the wells. All of the measurements were done in duplicate. The dissociation constants K_d's were determined by Scatchard analysis (Scatchard, 1949).

Distribution of Palmitic Acid on pIgG

[^{3}H]palmitic acid conjugated pIgG (coupling stoichiometry, palmitic acid: IgG = 2.2) was subjected to papain digestion to prepare Fab and Fc fragments according to Porter (1959). Resulting mixture of Fab and Fc fragments were separated on a 18% SDS-PAGE (Laemmli, 1970). Gel samples were done in triplicate and the protein bands in two sets of the gel were electrophoretically transferred to nitrocellulose paper for western blot analysis. The third set was stained with coomassie blue to reveal the protein bands which were then cut out from the dried gel and solubilized in Protosol (NEN Inc.) to determine ^{3}H-

radioactivity (Nicoli et al.,1974). After normalizing with respect to the amount of protein and the molecular weight of each band, the distribution of [^{3}H]palmitic acid was determined as the percent of total palmitic acid in pIgG. Western blot analysis (Burnette, 1981) was used to identify the positions of the Fab and Fc bands by using goat anti-mouse-Fab and goat anti-mouse-Fc (Koppel, Inc.), followed by binding of [^{125}I]protein A and detection with radioautography.

Liposome Preparation

Routinely, PE or PC (1-4μmol) and a trace amount of hexadecyl [^{3}H]cholestanyl ether (CE) (Pool et al., 1982) (final specific activity 4-11 x 10^{13} cpm/mol total lipid) were mixed and evaporated free of solvent with a gentle stream of N_2 gas. The dry lipid was vacuum desiccated for a minimum of 30 min. Two hundred μL PBS containing varying amounts of derivatized IgG, 0.09% DOC, pH 8.0 was added to hydrate the lipid. For the liposome lysis experiments, 50 mM calcein was included during the hydration step as a fluorescence marker . The mixture was sonicated in a bath sonicator (Laboratory Supplies, Inc., Hicksville, NY) for two five-min cycles with an intervening 30 min rest period at room temperature. The liposome suspension was then chromatographed on a Biogel A-0.5M column to remove the untrapped calcein as well as DOC. The liposomes, eluted with PBS pH 8.0 in the void volume, were detected by ^{3}H-radioactivity. These fractions were pooled and stored at 4^{o}C. In order to determine the concentration-dependent quenching of liposome-encapsulated calcein fluorescence, sonicated PC liposomes were prepared in PBS containing various calcein

concentrations. After chromatography to remove the untrapped dye, calcein fluorescence was measured with a Perkin Elmer LS5 spectrophotometer at λ_{ex} = 490nm and λ_{em} = 520nm. Total calcein fluorescence was determined by the addition of DOC to a final concentration of 0.12%. The percent quenching was calculated using the following formula:

$$\% \text{ Quenching} = [1 - F_o/F_t] \times 100$$

where F_o and F_t are the fluorescence of the liposome samples before and after the addition of DOC. We found 50 mM liposome-entrapped calcein gave approximately 70% fluorescence quenching.

Sucrose Density Gradient Centrifugation

Analytical centrifugation of linear 5-20% sucrose gradient was done as described previously (Huang et al., 1980). Briefly, 5mL of linear 5-20% sucrose gradient in PBS, pH 7.4 was overlayed on 0.5mL of 65% sucrose cushion. Liposome samples were loaded in 200µL and centrifuged in a SW50.1 rotor at 200,000xg for 5 hr at 4°C. The gradients were fractionated from the bottom using a peristalic pump.

Infecting L929 Cells with Viruses

L929 cells were grown as monolayer cultures in 35mm six-well Linbro plates (Flow Inc.) using McCoy's medium containing 10% fetal calf serum (FCS). Cells were infected with HSV or Sendai with MOI=10 in McCoy's with 2% FCS in 1mL. After one hour, the infection media was removed and replaced with normal culture media. Incubation was

continued at 37°C for five more hours before the cells were used for the analysis of liposome binding and liposome lysis.

Binding of Immunoliposomes to Cells

Virus-infected or normal L929 cells in 35mm plates were cooled to 4°C and were incubated in 5% goat serum containing McCoy's medium for 1 hr to block the nonspecific binding sites. Subsequently, immunoliposomes containing [^{125}I]pIgG and [^{3}H]CE in 1mL were added at various concentrations and incubation continued for 2 hr at 4°C. In free antibody inhibition experiments, the cells were preincubated with free IgG for 30 min before the addition of immunoliposomes. After incubation of immunoliposomes with cells, the incubation medium was removed and cells were washed 3 times with 2mL PBS. The cells were solubilized with 0.5mL 1% Triton X-100 at room temperature for 2 hr, followed by an additional 0.5mL Triton wash. The solubilized cells were counted for both ^{3}H and ^{125}I-radioactivity. Measurements were done in duplicate.

Cell-induced Lysis of Immunoliposomes

The infected and normal L929 cells were scraped with a rubber policeman and washed three times with PBS. Two µL liposomes with entrapped calcein were added to the Linbro Titertek plates containing 20µL of cell suspension in various concentrations and the mixture was incubated at 20°C for 30 min with gentle mixing. After incubation, the liposome-cell suspensions were transferred into a quartz cuvette and the volume was brought to 2mL with PBS. For inhibition experiments, the inhibiting liposomes were added together with the calcein-containing liposomes. The total calcein fluorescence in the incubation

mixture was measured after the addition of DOC to a final concentration of 0.12%. The percent dye release was calculated according to:

$$\% \text{ Release} = [(F - F_o)/(F_t - F_o)] \times 100$$

where F_o and F are the calcein fluorescence of the sample before and after the interaction with the cells. F_t is the total calcein fluorescence after lysis of liposomes with DOC. Light scattering due to cells was less than 1.5% of the total calcein fluorescence. The measurements of calcein fluorescence were reproducible within $\pm$10%.

Electron Microscopy

Immunoliposomes (0.42μmol/mL) were negatively stained with 0.5% aqueous uranyl acetate and viewed in a Hitachi 600 electron microscope operating at 75 KV. The size of liposomes was determined on photographically enlarged micrographs.

Papain-induced Release of Calcein from Immunoliposomes

Immunoliposomes (41μM lipid) with entrapped calcein were added to PBS containing 1mM EGTA, 1mM cysteine and various amounts of papain, in a quartz cuvette housed in a temperature controlled cell holder. The initial rate of calcein release was determined as the percent of total fluorescence increase per minute. The temperature of the cell holder was controlled by an Endocal refrigerated circulating water bath (NESLAB Inc., NH) with a variation of $\pm$ 0.5^{o}C.

Results

Derivatization of Anti-HSV-gD-IgG with Palmitic Acid

We first examined the degree of palmitic acid coupling to the IgG by varying the input ratio of [^{3}H]NHSP to [^{125}I]IgG from 0 to 44. The final molar ratio of palmitic acid to IgG was determined from the pooled IgG fractions (void volume) of a Sephadex G75 column (Huang et al., 1982). Therefore, these values represent the average ratio since the pooled samples contained both derivatized and underivatized antibodies. As can be seen in Table 3, increasing input ratio of NHSP/IgG resulted in more palmitic acid coupled per IgG without significant change in the antigen binding affinity as revealed by the dissociation constant K_d of the antibody. Therefore the coupling condition was sufficiently mild and the coupling stoichiometry can be controlled by varying the input NHSP/IgG ratio. To determine the palmitic acid distribution on the pIgG, we used papain to cleave a pIgG preparation with the coupling stoichiometry of 2.2 in the presence of 0.15% DOC according to Porter (1959). When the resulting mixture was subjected to SDS-PAGE, two distinct protein bands with apparent molecular weights of 32.5kd and 26.5kd were detected. They were identified in western blots (Burnette, 1981) as Fc and Fab, respectively. By counting radioactivity of ^{3}H-palmitic acid, the distribution of palmitic acid on Fab and Fc were determined to be approximately 30% and 70%, respectively.

Table 3

Derivatization of Anti-HSV-gD-IgG with Palmitic Acid

Input molar ratio NHSP/IgG (mol/mol)	Coupling stoichiometry, Palmitic Acid/IgG (mol/mol)	K_d ($\times 10^{8}$ M)
0	0	0.75[a]
11	1.4	ND[b]
14	2.1	ND
20	2.2	1.17
25	5.14	ND
30	6.66	ND
44	14.6	1.90

[a] K_d for native anti-gD-IgG was 0.48×10^{-8} M.

[b] not determined.

Stabilization of PE Liposome Bilayer by Palmitoyl Anti-HSV-gD-IgG.

Formation of stable liposomes was monitored by their ability to encapsulate 50mM self-quenching fluorescence dye, calcein. At this concentration, calcein fluorescence was about 70% quenched. Fluorescence was greatly enhanced as the dye was diluted upon release from liposomes. In order to determine the optimal palmitic acid to IgG coupling stoichiometry for PE liposome formation, we prepared the liposomes by sonication in the presence of 50mM calcein with various pIgG to PE ratios for each pIgG preparation. A wide range of coupling stoichiometry (0-14.6) was used. After chromatographed to remove the untrapped dye, liposome formation was detected by analyzing the total amount of calcein encapsulated per mole of PE. In order to demonstrate the encapsulation of calcein, quenching of calcein fluorescence was

also determined (Fig. 9). As shown in figure 9A, we found the optimal coupling stoichiometry of palmitic acid to IgG was 2.2 to 5.1, as evidenced by the highest amount of total calcein encapsulated per mole of lipid. In this particular experiment, pIgG to lipid ratio was kept constant at 2.5×10^{-4}. Similar coupling stoichiometric optima was also found with a pIgG to lipid ratio of 4.7×10^{-4}.

We further analyzed these PE immunoliposomes by sucrose gradient centrifugation. A typical gradient profile is shown in figure 10. With palmitic acid to IgG coupling stoichiometry of 2.2 to 5.1, practically no underivatized IgG was detected. In addition, the pIgG was all incorporated into the PE immunoliposome composed of pIgG:PE = 1:4000, as evidenced by the absence of aggregated pIgG at the bottom of the gradient. Furthermore, we found heterogeneity of pIgG distribution in the liposome population in the liposomes enriched with more pIgG migrated further into the gradient than the ones with fewer pIgG. For the gradient shown in figure 10, the pIgG:PE ratio in the population (fraction no. 13-17) was calculated to be $1.2\text{-}4.3 \times 10^{-4}$. This result indicates that the liposomes that sedimented further into the gradient contained up to 3.6 times more pIgG per mole of lipid than the ones found at the top of the gradient. However, the constant quenching of calcein fluorescence across the peak indicates that the concentration of calcein encapsulated was approximately the same, i.e. 50 mM. Therefore, despite the heterogeneity, the PE immunoliposomes prepared under the chosen conditions can stably encapsulate a small molecular weight marker, calcein.

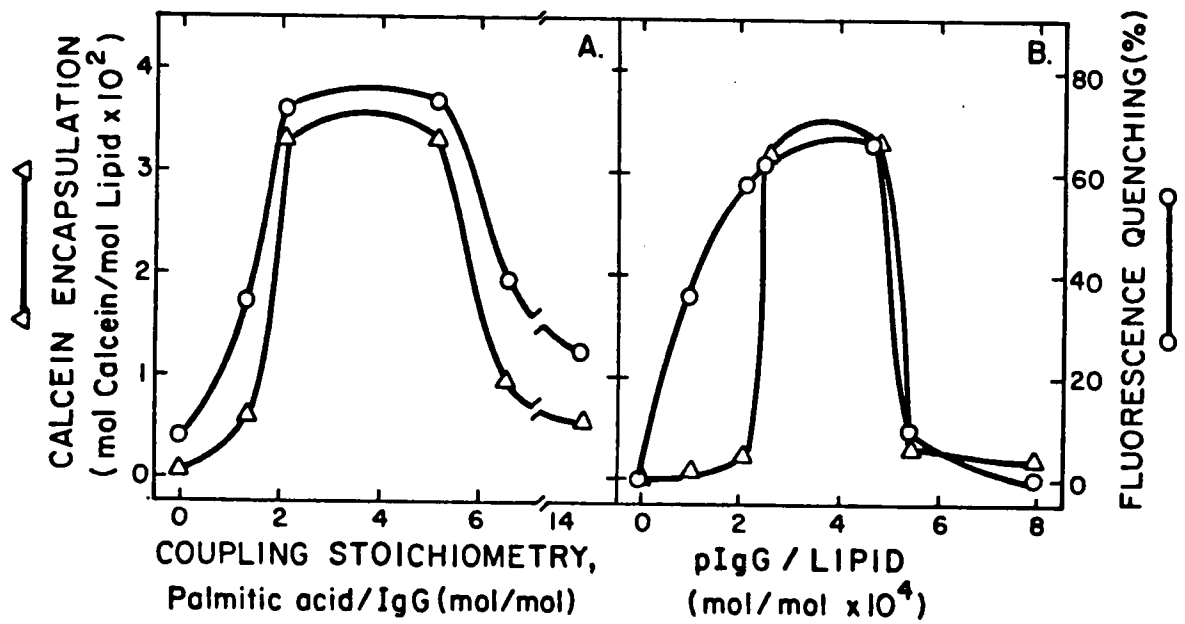

Figure 9. Stabilization of PE Liposome Bilayer by Palmitoyl-Anti-gD-IgG. Calcein encapsulation (△) and fluorescence quenching (○) were measured with various coupling stoichiometry, palmitic acid/IgG (A), or with various pIgG to PE ratio (B).

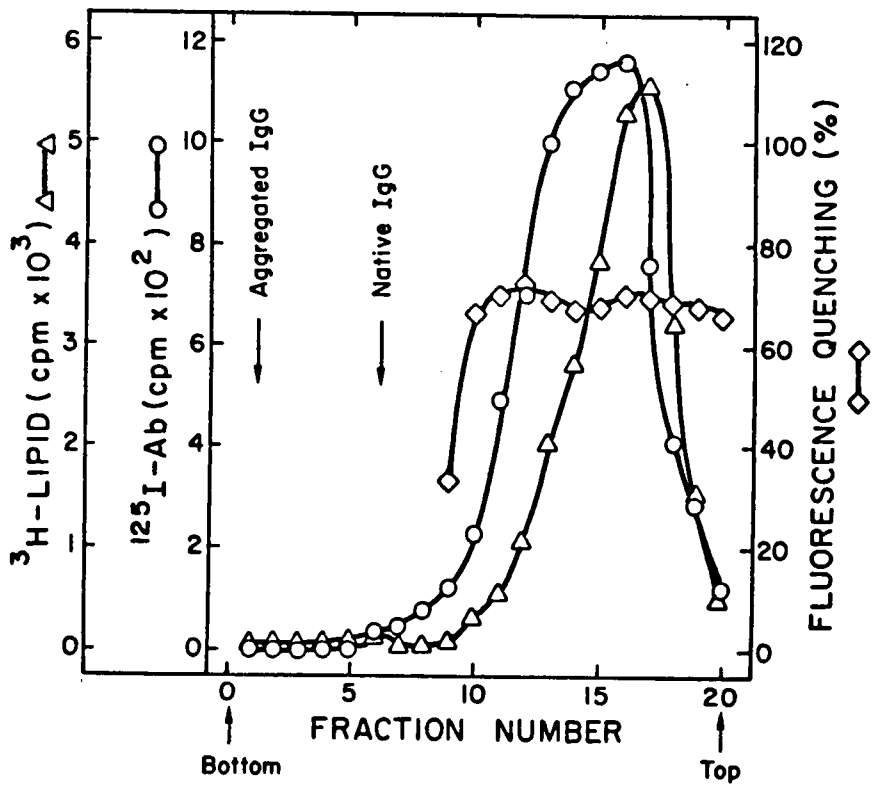

Figure 10. Sucrose Gradient Centrifugation Analysis of Antibody Stabilized PE Liposomes. PE immunoliposomes were fractionated with 5-20% linear sucrose gradient centrifugation as described in materials and methods. Each collected fraction was analyzed for [^{3}H]lipid (△), [^{125}I]IgG (◯), and fluorescence quenching (◇). The arrows indicate where self-aggregated pIgG and native IgG sediment.

In order to determine the optimal PE to pIgG ratio, we analyzed the calcein encapsulation and the percent quenching of the PE immunoliposomes with various ratios of pIgG to PE. In this experiment, the coupling stoichiometry of the pIgG used was 2.2. As shown in figure 9B, the optimal pIgG to PE was found be 2.5 to 4.7×10^{-4}. Within this range, the highest amount of total calcein fluorescence (which was quenched up to 70%) per mole of lipid was detected. Either increasing or decreasing the pIgG to PE ratio resulted in a decrease of calcein encapsulation. With pIgG to PE ratio less than 2.5×10^{-4}, a sharp decrease was detected in the calcein encapsulation without significant decrease in quenching. This could be attributed to the heterogeneity of the liposome population. A small number of pIgG stabilized liposomes in the population could give rise to a high degree of calcein quenching. At pIgG to PE ratios greater than 4.7×10^{-4}, the excess pIgG were self aggregated as revealed by sucrose gradient centrifugation (data not shown).

Our results indicate that a minimum coupling stoichiometry of 2.2 palmitic acid per IgG was required for the pIgG to stabilize the PE liposomes (Fig. 9A). In addition, the minimum pIgG to PE ratio was 2.5×10^{-4}. This combination was used to prepare the PE immunoliposomes for all subsequent experiments. Under these conditions, we calculate that liposomes of 500 Å average diameter have five pIgG molecules per PE immunoliposome.

Size of Liposomes

Liposomes composed of pIgG:PE (1:4000) were unilamellar and the average diameter of liposomes was 500Å ± 130Å as determined by negative-stain electron microscopy (micrograph not shown).

Binding of Immunoliposomes to L929 Cells

In order to demonstrate the binding specificity of the immunoliposomes, we infected the mouse fibroblast L929 cells with HSV and measured the binding of the immunoliposomes at 6 hr post infection which is the optimal time for gD expression (Cohen et al., 1980, Balachandran et al., 1982, Johnson & Spear, 1984). By using [^{3}H]CE as a lipid marker and [^{125}I]pIgG, the binding of immunoliposomes to the cells was detected by counting ^{3}H and ^{125}I-radioactivity. With increasing concentration of liposomes added, increasing amount of both PC and PE immunoliposomes were bound specifically to the HSV-infected cells (Fig. 11). On the contrary, very few PE or PC immunoliposomes were bound to the uninfected cells. Furthermore, this immunoliposome binding could be inhibited by preincubation of the HSV-infected cells with free IgG in three-fold excess of the highest concentration of liposome associated pIgG used.

In addition, we analyzed the affinity of these immunoliposomes using the Scatchard analysis (Scatchard, 1949). We found that the apparent dissociation constant, K_d, for pIgG and lipid was $1x10^{-8}$M and $4x10^{-5}$M, respectively. The K_d of pIgG incorporated in the immunoliposomes ($1x10^{-8}$M) was very similar to the K_d of free pIgG ($1.17x10^{-8}$M, Table 3), indicating that significant denaturation of the antibody did

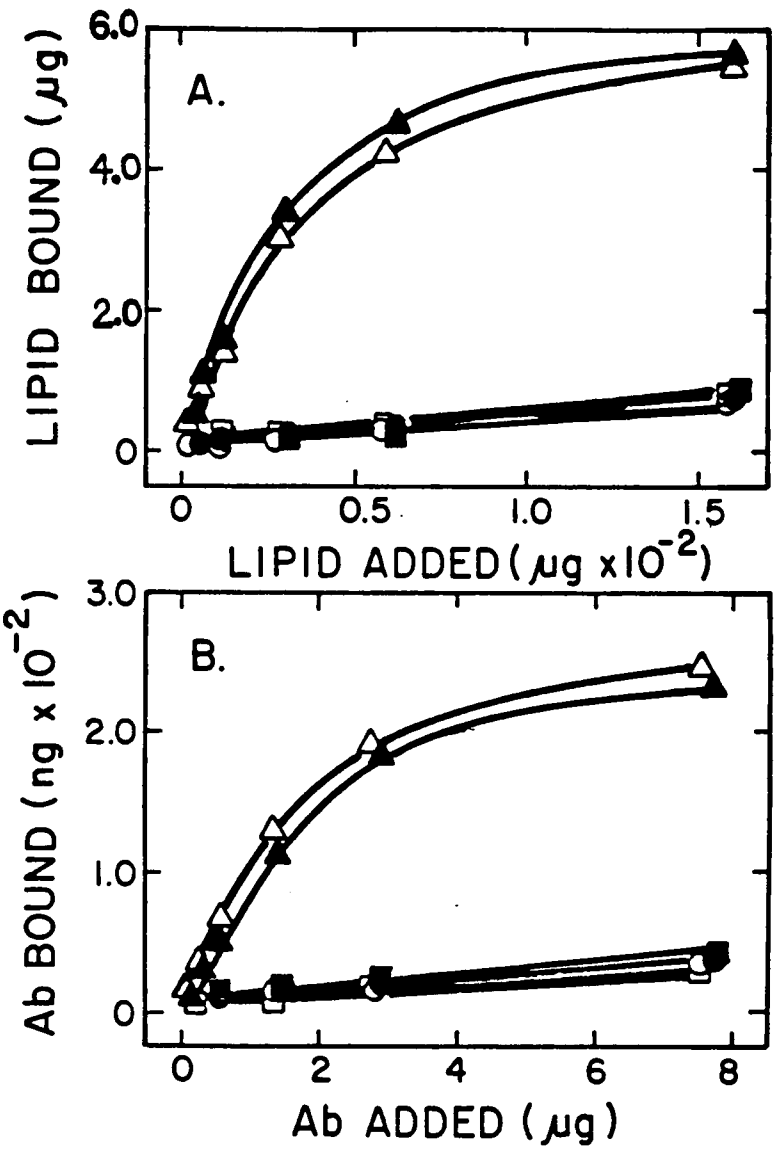

Figure 11. Binding of Immunoliposomes to L Cells. Uninfected (○&●), HSV-infected (△, ▲, □ & ■) L cells were incubated with varying amount of PE liposomes (○, △ & □) and PC liposomes (●, ▲ & ■). The amount of lipid (A) and IgG (B) bound to the cell was plotted against the amount of lipid and IgG added in the form of immunoliposomes. Twenty ug of free anti-HSV-gD-IgG was used to block PE (□) and PC (■) immunoliposome binding to the HSV-infected L cells.

not occur during the preparation of immunoliposomes. Since no significant increase in the apparent binding affinity of antibody was observed upon incorporation into the liposomes, the binding of these liposomes to the virus-infected cells is probably not a cooperative event (Babbitt et al., 1985). By comparing the apparent K_d for pIgG and lipid, pIgG to lipid ratio of the cell-bound liposomes was determined to be 1:4020 (mole/mole). This finding indicates that the lipid and antibody in the liposomes were binding to cells as a unit since the pIgG to lipid ratio of the applied liposomes was 1:4000. From the X-intercept of the Scatchard plot, we had determined the maximum number of liposome bound to the HSV-infected L929 cells to be 1.3×10^5 per cell.

Cell-Induced Lysis of Immunoliposomes

We have investigated the ability of HSV-infected L929 cells to lyse immunoliposomes containing calcein. As shown in figure 12, PE immunoliposomes were lysed specifically in a concentration dependent manner by exposure to HSV-infected cells. In contrast only partial (up to approximately 30%) lysis occurred with uninfected cells. Approximately 90% of the entrapped calcein was released at the infected cell concentration of 6×10^6 cells/mL after 30 min incubation. In addition, the HSV-infected cell-induced calcein release could be blocked by incubating PE immunoliposomes with a three-fold excess of the PC immunoliposomes containing no calcein. Since no significant lysis of liposomes was observed with Sendai virus-infected cells, this type of immunoliposome is target specific. Furthermore, when PC was used to substitute for PE in immunoliposomes, the lytic activity was lost. The

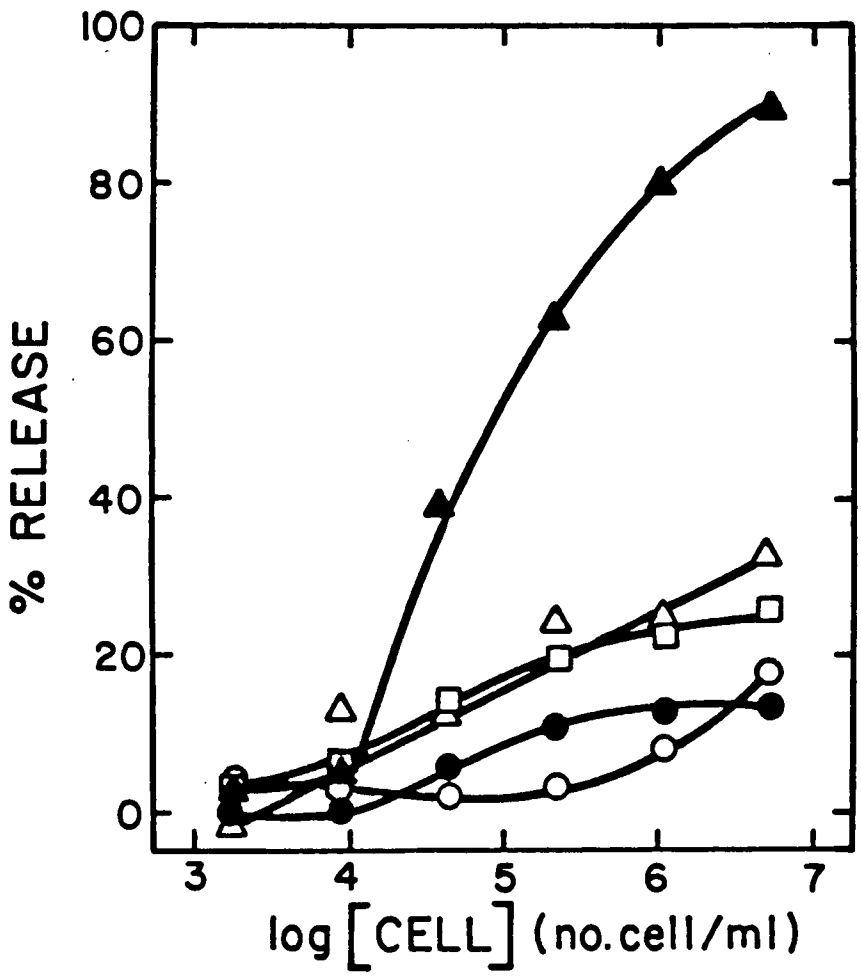

Figure 12. Cell-Induced Lysis of Immunoliposomes. To uninfected (○), HSV-infected (●, △& ▲), or Sendai virus infected (□) L cells in suspension, 0.58 nmole of calcein encapsulated PE (○, □, △ & ▲) or PC (●) immunoliposomes were used. To inhibit the lysis of PE immunoliposomes by HSV-infected L cells, 1.8nmoles of PC immunoliposomes containing no calcein were also added (△).

inability of HSV-infected cells to lyse PC containing immunoliposomes could not be attributed to the lack of liposome binding to the cells since both the PC and PE immunoliposomes bound equally well to the HSV-infected cells (Fig. 11A and B). In order to determine whether the cell-induced increase of calcein fluorescence was due to the calcein released into the medium or into the cell, cells were pelleted and the fluorescence in the supernatant and the cell pellet were measured. Approximately 15% of total calcein was found in the pellet and the fluorescence was not quenched, indicating most of the calcein was released extracellularly.

Finally, it was of interest to determine if target-sensitive immunoliposomes could detect virus-infected target cells in the presence of cells which lacked the target antigen. When varying fractions of HSV-1 infected cells (6 hr post infection at high multiplicity) were diluted with uninfected control cells, a linear increase of liposome lysis was seen with increasing target antigen-expressing cells in the mixture. The data was fitted with a least square analysis that gave the slope of 1.03 and correlation coefficient of 0.998. The total infected cell concentration was kept at $3X10^6$ cell/mL.

Papain-induced Lysis of Immunoliposomes

In order to determine the essential domain of the IgG in stabilizing PE liposomes, we digested the pIgG on calcein encapsulated PE immunoliposomes with papain. Destabilization of immunoliposomes was detected by following the initial rate of calcein leakage. With increasing concentration of papain added, elevating rate of leakage up to

8% per minute at 37°C was observed with PE immunoliposomes (Fig. 13). This papain-induced leakage was specific for PE immunoliposomes since PC immunoliposomes were not sensitive to papain. In addition, papain-induced leakage was temperature dependent as evidenced by essentially no release of calcein from the PE immunoliposomes at 4°C regardless of the amount of papain added. Inability of papain to induce liposome leakage at low temperature could not be accounted for by the decrease in enzyme activity since at least 15% activity remains when the temperature is reduced from 37° to 4°C (Stockell & Smith, 1957). In addition, the papain induced leakage rate decreased as the liposomes were diluted (data not shown). This finding is in agreement with the observation of Ellens et al. (1984) that destabilization of PE bilayers was concentration dependent because of the bilayer contact requirement.

Discussion

Unsaturated PE, which does not form stable bilayers under physiologic conditions (Gruner et al., 1985, Cullis & DeKruijff, 1979), can be made to do so in a variety of ways. These have included mixing with another type of lipid such as dinitrophenyl-caproyl PE (Ho & Huang, 1985), N-succinyldioleoyl PE (Nayar & Schroit, 1985) or various types of mixed-lipids (for a review see Cullis & DeKruijff, 1979); gangliosides such as GD_{1a} (Tsao and Huang, 1986); fatty acids and derivatives such as oleic acid (Duzgunes et al. 1985; Huang & Liu, 1984), cholesteryl hemisuccinate (Ellens et al., 1984) and palmitoylhomocysteine (Connor et al., 1984); and finally proteins such as glycophorin A (Taraschi et al., 1982; Ho & Huang, 1985). The present report

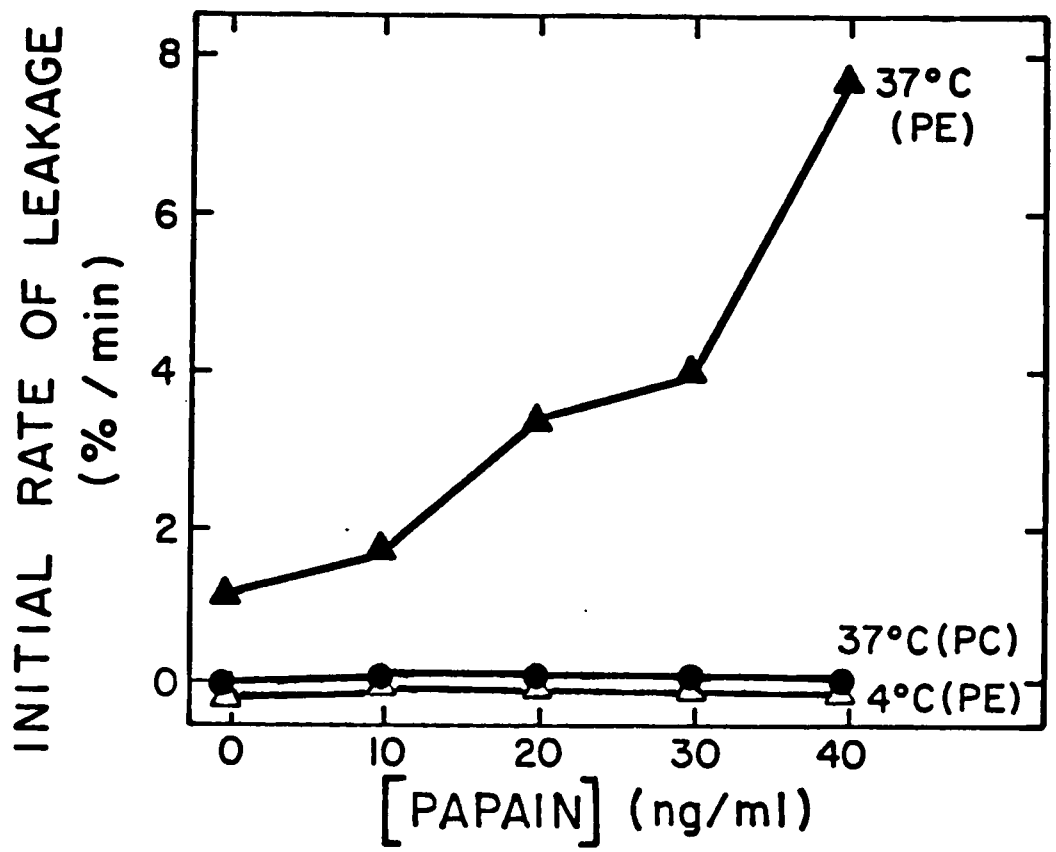

Figure 13. Papain-Induced Lysis of Immunoliposomes. Various concentrations of papain was added to PE (△ & ▲) and PC (●) immunoliposomes at 37°C (▲ & ●) and 4°C (△) and the initial rate of calcein leakage was measured.

documents the first time that immunoglobulin is used as a stabilizer for an unsaturated PE bilayer. Since these PE immunoliposomes are triggered to release encapsulated contents upon binding to the target (Fig. 12), we refer to them as target-sensitive immunoliposomes.

The exact stabilization mechanism of the PE bilayer by palmitoyl antibody is not known at present. However, the orientation of pIgG in the bilayer is probably a major contributing factor. The preferential derivatization of palmitic acid to the Fc region (70%) of the IgG indicates that either the Fc-linked palmitic acids or the acylated Fc portion of the antibody is inserted into the bilayer with Fab region outside and available for binding (Fig. 14). Although we do not know the extent of the pIgG insertion, the destabilization of PE immunoliposomes by papain, which cleaves the hinge region of IgG (Fig. 13), clearly demonstrates that an intact Fab domain is essential for stabilization of PE bilayers. Since the hinge region is available for the papain cleavage, the Fab portion of the pIgG is most likely exposed to the aqueous phase. This is further supported by the experimental finding that the Fab portion of the liposome bound pIgG is available for antigen binding with an apparent K_d approximately the same as that of the native IgG (Table 3 and Fig. 11). Our model of stabilization is also consistent with the physical principles involved in membrane organization. According to the "hydration" theory (Marcelja & Radic, 1976), PE, which has a relatively low head-group charge content, will not attract a significant number of water molecules at neutral pH. This results in a small head group (Rand et al., 1971, Harlos, 1978) that gives an average dynamic shape of an inverted cone (Israelachvili et

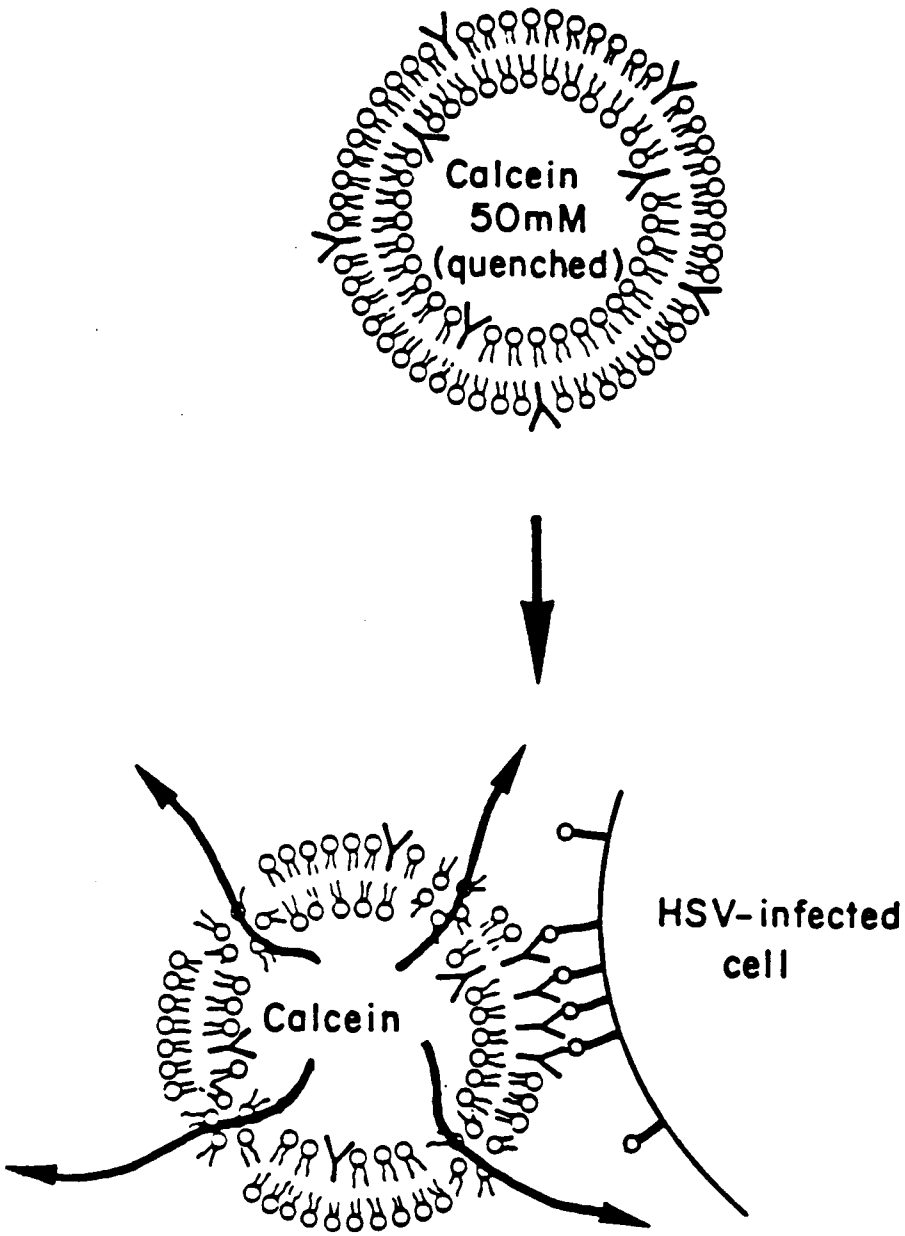

Figure 14. Schematic Representation of the Specific Lysis of the Target-Sensitive Immunoliposomes. Note that molecules are not drawn to scale.

al., 1980). To maintain bilayer structure, PE requires complementary molecules which assume a dynamic truncated cone or wedge shape. The membrane-bound pIgG in our system probably complements the inverted cone-shaped PE by a combination of attracting interfacial water, due to the hydrophilic Fab portion at the bilayer surface, and the intrinsic molecular conformation of pIgG that gives the average dynamic shape of a truncated cone. In addition, membrane bound pIgG may serve as a repulsion force against the PE bilayer contact, thereby preventing the already formed PE bilayer reverting to the hexagonal phase. It is also interesting to note that only five pIgG molecules were required to stabilize a 500Å PE liposome. For glycophorin-PE liposomes of comparable size, 100 molecules of glycophorin were required for bilayer stabilization (Taraschi et al., 1982; Ho & Huang, 1985), indicating that the Fab portion of pIgG is probably more hydrophilic than glycophorin and so attracts more interfacial water to the bilayer surface. Hence, the pIgG exhibit a markedly higher degree of stabilizing activity on PE bilayers than does glycophorin.

Our data clearly demonstrate that PE immunoliposomes can be destabilized upon specific binding to target cells (Fig. 12). Although the detailed molecular mechanism of target cell-induced destabilization of immunoliposomes requires definition, they probably involve a lateral phase separation of the liposome membrane components. As schematically presented in figure 6, pIgG molecules are likely to freely diffuse in the fluid bilayer with a diffusion coefficient of 1.87×10^{-8} cm^2/sec (Huang, 1985). Consequently, only a fraction of a second is required for the surface bound pIgG to diffuse to the contact area between

liposome and cell. This process eventually causes a multiple immune complex formation at the contact area (contact capping) (Bell, 1979). Although there was no decrease in dissociation constant of liposome bound pIgG due to multiple immune complex formation (Table 3 & Fig. 11), the possibility of multivalent binding cannot be ruled out at present. It is likely that appreciable multivalent binding is cancelled by the steric factors which increase the dissociation constant for all the interactions between cells and membrane bound IgG. As a result, the membrane bound pIgG does not exhibit a lower apparent K_d than that of free IgG. Furthermore, it is also possible to have transient multivalent binding that is sufficient to provide a release of liposomal content, before the multivalent interaction relaxes to the monovalent form. This process may also result in the similar dissociation constant measured at the equilibrium condition.

Regardless of the nature of multivalent binding, the resulting lateral pahse separation may decrease the effective concentration of pIgG in the bulk lipid bilayer which destabilizes the PE immunoliposomes. As a result of lateral phase separation, the effective concentration of pIgG in the bulk lipid bilayer decreases resulting in liposome destabilization. Although this hypothesis remains speculative, it is consistent with our previous finding that multivalent antibody-binding is essential for lysis of antigen-stabilized PE liposomes. In this example, bivalent antibody binding only results in PE liposome aggregation but not lysis (Ho & Huang, 1985). In addition to lateral phase separation of bilayers, contact between the individual PE immunoliposomes is probably required for the destabilization of the PE

bilayer (Ellens et. al., 1984). This contact requirement could be satisfied either by collision between adjacent liposomes bound to the same cell or between liposomes on separate cells. Although the kinetics of this process remains to be investigated, the overall destabilization rate of the TS liposomes is likely dependent on collision rate which controls the formation of the immune complex between a TS liposome and a target cell.

The possibility of a hexagonal (H_{II}) phase intermediate in the destabilization of TS liposomes should not be overlooked, because a similar phenomenon has been observed by Taraschi et al. (1982). When lectins were added to glycophorin A-stabilized PE bilayers, extensive hexagonal phase formation was detected.

Potential use of the target-sensitive immunoliposomes as a site-specific drug delivery system depends on the following considerations. First, the target cell must express a sufficient antigen density to promote contact capping of the liposome following binding. Second, the drug released from liposomes at the cell surface should be rapidly taken up by the target cell. Cytotoxic and antiviral drugs of nucleoside analogs such as fluorodeoxyuridine, iododeoxyuridine, acyclovir or cytosine arabinoside should be good choices (Plagemann & Wohlhueter, 1980). A closely related nucleoside, uridine, was recently used in the site-specific drug delivery using heat-sensitive DPPC immunoliposomes via the nucleoside transporter of the target cell (Sullivan & Huang, 1986). A rapid uridine uptake was observed when the immunoliposome bound target cells were briefly heated to release the encapsulated uridine at the cell surface. For our system, nucleoside

analogs of anti-HSV drugs, such as iododeoxyuridine and acyclovir, should serve the same purpose. Thus, selective uptake of the antiviral drugs by the infected cells could be mediated by the TS liposomes described here. Since drug delivery by TS liposomes depends only on antigen-antibody binding, there is no additional requirement of the target cell metabolism such as endocytosis. Accordingly, this targeting design could be effective for cells that do not actively endocytose as long as sufficient antigen density and functional drug transporters are available at the cell surface.

In principle, any IgG that is monospecific for the target antigen can be used to prepare the TS immunoliposomes. Furthermore, one would expect that TS immunoliposomes lyse when binding occurs with other types of multivalent antigens such as intact viruses, bacteria and other pathogens. If a suitable reporter molecule is encapsulated in the liposome, a simple liposome-based immunoassay could be designed.

Acknowledgments

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CHAPTER V

INTERACTIONS OF TARGET-SENSITIVE IMMUNOLIPOSOMES WITH HERPES SIMPLEX VIRUS

Interactions between target-sensitive (TS) immunoliposomes and herpes simplex virus (HSV) were investigated. Target sensitivity of phosphatidylethanolamine (PE) immunoliposomes is a result of the ability of acylated monoclonal anti-HSV glycoprotein D (gD) to stabilize the bilayer phase of PE, whereas, by itself PE does not form stable liposomes [Ho et al., Biochemistry (1986) 25, 5500]. Upon binding of these immunoliposomes to HSV virions expressing surface gD, destabilization of PE immunoliposomes was observed. By encapsulating either a self quenching fluorescent dye, calcein, or alkaline phosphatase inside the liposomal compartment, the HSV-induced destabilization of TS immunoliposomes was shown to be target specific. Neither Sendai, Semliki Forest, nor Sindbis virus could significantly destabilize the TS immunoliposomes. Moreover, HSV-induced liposome destabilization could be inhibited by free anti-gD (the same antibody used in TS immunoliposomes), but not by monoclonal anti-HSV glycoprotein B, indicating that the interaction was antigen specific. Destabilization could also be induced by binding to truncated gD (tgD) but only when in a multivalent form immobilized on latex beads. Truncated gD is a cloned, 312 amino acid-fragment of HSV-gD that lacks the transmembrane segment. Preincubation of soluble tgD with the TS immunoliposomes failed to induce destabilization and in addition, abolished the tgD-bead induced destabilization. This finding strongly indicated that multivalent binding

is essential for TS immunoliposome destabilization. Using alkaline phosphatase encapsulated in the liposomes, TS immunoliposomes could be used to detect HSV in fluid phase with 50% signal recorded at 5 μL of 3.2×10^3 pfu/mL; at least 10-fold more sensitive than the standard double-antibody sandwich ELISA. The interactions described here may be useful in designing a homogeneous and sensitive immunoliposome assay.

Introduction

Although liposomes can be made by hydration of bilayer forming lipids such as phosphatidylcholine (PC), it is well documented that unsaturated phosphatidylethanolamine (PE) cannot form stable liposomes at physiological temperature and pH (Reiss-Husson, 1967). Rather, a nonbilayer structure known as hexagonal phase is the equilibrium phase of PE at neutral pH and physiological temperature. However, the bilayer phase of PE can be stabilized by the presence of a second lipid (Cullis & DeKruijff, 1979) or some transmembrane proteins such as glycophorin (Taraschi et al.,1982; Ho & Huang, 1985). Stabilization by a second component (stabilizer) requires even mixing of the stabilizer molecules in the PE bilayer; lateral phase separation of the stabilizer can cause the reversion to the hexagonal phase (Taraschi et al., 1982; Ho & Huang, 1985). This polymorphic behavior is unique to PE and other hexagonal-phase forming lipids.

We previously documented that an otherwise unstable membrane bilayer containing dioleoyl PE (DOPE), could be stabilized with acylated antibody against HSV-glycoprotein D (gD) (Ho et al., 1986a & 1986b). The resulting antibody-stabilized immunoliposomes were readily

destabilized and released the entrapped marker molecules upon exposure to a target membrane, i.e. herpes simplex virus (HSV)-infected L cells expressing antigen gD (Ho et al., 1986a). Such immunoliposomes were referred to as target-sensitive (TS) immunoliposomes. We further suggested that similar interactions were likely to occur between TS immunoliposomes and HSV virions. Our recent report, which demonstrated the ability of HSV to induce TS immunoliposome lysis (Ho et al., 1986b), prompted us to further study the detail interactions of HSV virions and TS immunoliposomes.

In this chapter, we demonstrate the direct HSV binding of TS immunoliposomes that leads to liposome destabilization. Our results also suggest that multivalent binding is essential for the destabilization of TS immunoliposomes. Finally, the potential use of TS immunoliposome in HSV detection is demonstrated in comparison with conventional enzyme-linked-immunosorbent assay (ELISA).

Materials and Methods

Materials

DOPE and dioleoyl PC (DOPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL.). Calcein, alkaline phosphatase, and alkaline phosphatase-conjugated goat anti-mouse IgG were purchased from Sigma Chemical Co (St. Louis, Mo). p-nitrophenyl-phosphate was purchased from Calbiochem (San Diego, CA). Carboxylated latex beads of 0.99μm (#8828) were purchased form polysciences, Inc. (Worrington, PA) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide-hydrochloride (EDCI)

was from Chemical Dynamic Co. (South Plainfield, NJ). All other reagents were analytical grade.

Antibody

Monoclonal antibody IgG_{2a} against the HSV glycoprotein D (gD) (clone D4.2) was kindly provided by Dr. M. Trousdale and isolated from ascities fluid by Dr. S. Norley. This particular monoclonal was previously characterized by Lathey et al. (1986). The IgG fraction of ascities was purified by protein A-Sepharose affinity chromatography, followed by DEAE Sephadex A-25 ion-exchange chromatography. The purified IgG was stored in PBS at $-20^{o}C$. When necessary, antibody was radiolabelled with ^{125}I using chloramine T (Hunter & Greenwood, 1962) to a final specific activity of $1x10^{3}$ to $5x10^{5}$ cpm/μg.

Virus

The HF strain of HSV-1 and strain 186 of HSV-2 were propagated in Hep-2 cells. After three cycles of freeze-thawing, the virus was isolated from cell debris by centrifugation at 1000xg for 10 min. The virus in the supernatant was subsequently aliquoted and stored at $-70^{o}C$ (Norley et al., 1986). Virus stocks contained 10^{6}-10^{9} pfu/mL on the average. When protein concentration was used to express HSV, sucrose density gradient centrifugation step was performed for further virus purification (Tsao & Huang, 1985) before the protein content was determined (Lowry et al., 1951). Freeze-fracture and negative stain electronmicrograph showed that isolated virus was mainly intact.

Preparation of Truncated Glycoprotein D-conjugated Latex Beads

Cloned HSV gD (Nar) [a gift from Dr. Berman and Dr. Lasky, Genentech Inc.] which is a 312 amino acid, soluble fragment of gD which lacks the transmembrane segment (truncated gD) was used in this study. The truncated gD (tgD) was purified by affinity chromatography using the anti-gD (D 4.2) Sepharose column according to Lathey et al. (1986). Covalent attachment of tgD to carboxylated latex beads were done according to Kung et al. (1985). Briefly, the 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide-hydrochloride (EDCI) activated polycarboxylated latex bead of 0.99 μm was mixed with the tgD to a final concentration of 2.5% latex bead, 100 μg/mL tgD and 2.4mg/mL EDCI in 0.5mL of 15mM sodium phosphate buffer, pH 8.0. The mixture was agitated at $4^{o}C$ for 24 hr. Then, the unreacted tgD was removed by washing the latex beads with 0.1M glycine, 0.14M NaCl, pH 8.0. Finally, the resulting tgD conjugated beads (tgD-beads) were resuspended to a final concentration of 0.5% tgD-beads with 2mg/mL of BSA in PBS containing 0.02% NaN_3, pH 8.0. The same procedure was used to prepare the BSA conjugated latex beads. Under such condition, about 80% of tgD was conjugated to the latex beads. The tgD conjugated on latex exhibited a specific binding to ^{125}I-anti-gD IgG with a dissociation constant, K_d, of $1.5x10^{-8}M$, a value which is similar to the K_d of the same antibody (D4.2) binding to the membrane bound gD [$K_d = 0.5x10^{-8}M$ (Ho et al., 1986a)]. Additionally, Scatchard analysis (Scatchard, 1949) showed that each tgD-bead contain about $2.4x10^{8}$ tgD molecules which are available for antibody binding.

Derivatization of IgG with Palmitic Acid

Anti-gD IgG was conjugated to palmitic acid using a 20 fold mole excess of N-hydroxysuccinimide ester of palmitic acid following the procedure described by Ho et al. (1986a), modified from Huang et al. (1982). Under such conditions, the resulting palmitoyl IgG (pIgG) contained about 2.2 acyl chains per IgG, of which approximately 70% located on the Fc portion of the IgG molecule, and K_d for pIgG binding to HSV was determined to be 1.2×10^{-8} M (Ho et al., 1986a). These pIgG were previously shown to be acylated optimally for membrane incorporation and stabilization of PE bilayer (Ho et al., 1986a).

Liposome Preparation

In routine experiments, DOPE or DOPC (1-4 μmole) and a trace amount of [^{3}H]cholestanyl ether (CE) (Pool et al., 1982) (final specific activity $4\text{-}11 \times 10^{13}$ cpm/mole lipid) were mixed and solvents were evaporated with a stream of N_2 gas. The dry lipid was desiccated under vacuum for 30 min. Two hundred μL of PBS containing 50mM calcein, pIgG and 0.09% deoxycholate (DOC), pH 8.0 was added to hydrate the lipid. An appropriate amount of pIgG was added, such that the final lipid to pIgG molar ratio was 4×10^3. In preparing alkaline phosphatase encapsulated liposomes, 10 mg/mL alkaline phosphatase in 20mM Tris-10mM Glycine buffer (TGB), pH 8.0, with 0.09% DOC was added in the process of hydration. The mixture was vortexed, and subsequently sonicated in a bath sonicator (Laboratory Supplies, Inc., Hicksville, NY) for two five-min cycles with an intervening rest period of 30 min. The resulting liposome suspension was fractionated by using

a Biogel A-0.5m column chromatography to remove untrapped calcein or alkaline phosphatase, eluted with either PBS, pH 8.0 or TGB, pH 8.0, respectively. In a separate experiment, the efficiency of DOC removal by this method was determined to be at least 99.9%. That is, a maximum of $5x10^{-4}$ mole ratio of DOC to PE remained in the liposome preparation. Liposomes eluted with a flow rate of 0.3 mL/min in the void volume as determined by counting the tritium radioactivity, and were pooled in appropriate buffer and stored at $4^{o}C$. Liposomes containing calcein showed about 70% quenching of calcein fluorescence, and liposomes containing alkaline phosphatase showed about 85% latency of the enzymatic activity, indicating that these liposomes were intact (Ho et al., 1986a). Both calcein and alkaline phosphatase encapsulated liposomes were stable in physiological saline at $4^{o}C$ for at least six months.

HSV-induced Destabilization of Immunoliposomes

In order to analyze HSV dependent liposome lysis, 5 µL of liposomes containing 0.82 nmole lipid was added to 5 µL of HSV-1 in Linbro Titertek 96 well plates. After incubation at $20^{o}C$ for 30 min with gentle mixing, the suspension was transferred into a quartz cuvette and diluted with PBS to 2mL final volume. For inhibition experiments, various concentrations of protein in 5µL PBS were incubated with HSV for 20 min prior to the liposome addition. Total calcein fluorescence in the incubation mixture was measured after addition of DOC to a final concentration of 0.12%. Percent calcein release was calculated as follows:

$$\% \text{ Release} = [(F - F_o)/(F_t - F_o)] \times 100$$

where, F_o and F are the calcein fluorescence of the sample before and after interaction with virus. F_t is the total calcein fluorescence after destabilization of the liposomes with DOC. In the experiments where alkaline phosphatase encapsulated liposomes were used, 5μL of virus sample was incubated with 5 μL (0.15 nmoles) of the liposomes in Dynatech Removawells. Determination of the total release was accomplished by using 5 μL of 10% NP40 added to achieve total destabilization of liposomes. NP40 instead of DOC was used as a detergent because DOC interfered with the alkaline phosphatase assay. The HSV-immunoliposome mixtures were incubated at room temperature for 30 min. Subsequently, 100 μL of 60μM p-nitrophenyl-phosphate in TGB, pH 8.0, was added, and incubation continued with gentle agitation for 60 min before detecting the product, p-nitrophenol, at 405nm with a Biotek EL 310 ELISA reader.

Interaction of Target Sensitive Immunoliposomes with HSV Glycoprotein D

To 2 nmoles lipid in 2mL of calcein encapsulated liposomes in PBS, various amount of tgD in the form of either soluble or immobilized on latex beads, was added. After 15 min incubation at room temperature, the destabilization of liposomes were detected by the enhancement of calcein fluorescence as they were released from liposomes. For antibody inhibition experiments free anti-gD IgG was added to the tgD-beads and incubated for 30 min prior to liposome addition. Similarly, inhibition of soluble tgD was performed by preincubation of liposomes with soluble tgD for 30 min prior to tgD-bead addition. Finally, the tgD-beads and liposome mixture was incubated for 15 min

before detection of the calcein fluorescence. Degree of liposome destabilization was presented as % calcein release as described above. Data were expressed as the average of duplicates which were within 5% of the average value.

Liposome-virus Binding

Calcein encapsulated liposomes samples of 14.5nmoles lipid in 50 µL were incubated at room temperature with either 100 µL of 10^7 pfu/mL of HSV-1 or PBS pH 8.0. In the experiments where no liposome was added, 50 µL of PBS was added to HSV. After 1 hr incubation, the mixture was applied on a 5mL linear gradient containing 5-20% sucrose with 0.5mL 65% sucrose cushion. The samples were centrifuged in a SW50.1 rotor at 200,000xg for 5 hr at 4°C. Subsequently, the gradients were fractionated from the bottom using a peristalic pump (Huang et al., 1982). Fifty µL of each fraction was counted for ^{3}H-CE radioactivity to detect lipid content, 100 µL of each fraction was used to determine the 90° light scattering ($\lambda_{ex}=\lambda_{em}$=660 nm) as well as calcein fluorescence in a LS5 spectrofluorometer (Ho & Huang, 1985). Calcein fluorescence measurements were done before and after the total destabilization of liposomes with DOC as described above. The data presented was corrected for volume increase due to detergent addition.

HSV Detection with ELISA

A double-antibody sandwich ELISA technique was used. Rabbit anti-HSV-1 sera (1/20 dilution) in PBS pH 8.0 was adsorbed on microtitre wells overnight. After extensive washing with PBS, HSV containing samples were introduced in 50 µL. Incubation continued at room

temperature for 1hr. Then unbound HSV was removed by washing 3x with TGB, pH 8.0. Then 50 µL of 20 µg/mL monoclonal anti-HSV-gD IgG (the same D4.2 antibody used for immunoliposome preparation) was added and incubated for another hr. The microtitre wells were washed 4x with TGB. Immediately, the alkaline phosphatase conjugated goat-anti-mouse IgG was added and again, incubated for 1 hr. Finally, the unbound secondary antibody was removed by washing 6x with TGB. One hundred µL of 3 mM p-nitrophenyl-phosphate was added and incubated at room temperature for 1 hr. The product p-nitrophenol was measured with a Biotek EL 310 ELISA reader at 405 nm. The readings at saturating concentrations of virus was taken as the maximum signal. All the data were expressed as % of maximum signal. ELISA assays were done in quadruplicate and variation was expressed as standard deviation.

Results

Specificity of Virus-induced Target-sensitive Immunoliposome Destabilization

In order to optimize the conditions for detecting HSV-induced liposome destabilization, it is essential to determine the minimum time required to provide a maximum signal. To determine the kinetics of HSV-induced destabilization of TS immunoliposomes, calcein, a self quenching fluorescent dye, was encapsulated and virus induced-liposome destabilization was followed by detecting the enhancement of calcein fluorescence. The total time required to ensure complete reaction of the 3.1 µM TS immunoliposome with various HSV-1 concentrations was 20 min. Liposome concentrations below 1µM caused a decrease in reaction

rate (data not shown). All the subsequent experiments were done using 3.1 μM liposome for 30 min to assure complete liposome destabilization.

To demonstrate the specificity of the target-sensitive immunoliposome destabilization, reactions with unrelated viral antigens were investigated. Specificity was evident since only HSV-1 bearing the gD antigen caused destabilization with the total release of calcein occurring over a 30 min incubation time (Fig. 15). Under the same conditions, neither Semliki Forest, Sendai, nor Sindbis virus caused a significant degree of destabilization. HSV-1 induced destabilization was concentration dependent, as demonstrated by the fact that 50% release was detected with 5 μL of 8 μg/mL purified HSV-1.

Inhibition of HSV-Induced Liposome Destabilization by Antibody

When HSV-1 was preincubated with antibody, only the anti-gD antibody, which is the same antibody (clone D4.2) used in preparing TS immunoliposome, could inhibit the liposome destabilization (Fig. 16). This inhibition by anti-gD was concentration dependent, and 50% inhibition was achieved with 5ng of protein in 5 μL. Monoclonal anti-gB antibody which recognizes the glycoprotein B of HSV, failed to inhibit HSV-induced liposome destabilization; neither did bovine serum albumin in control experiments. However, inhibition was observed with the mouse anti-HSV serum (data not shown). Hence, destabilization of these TS immunoliposomes required the presence of exposed gD on the HSV surface.

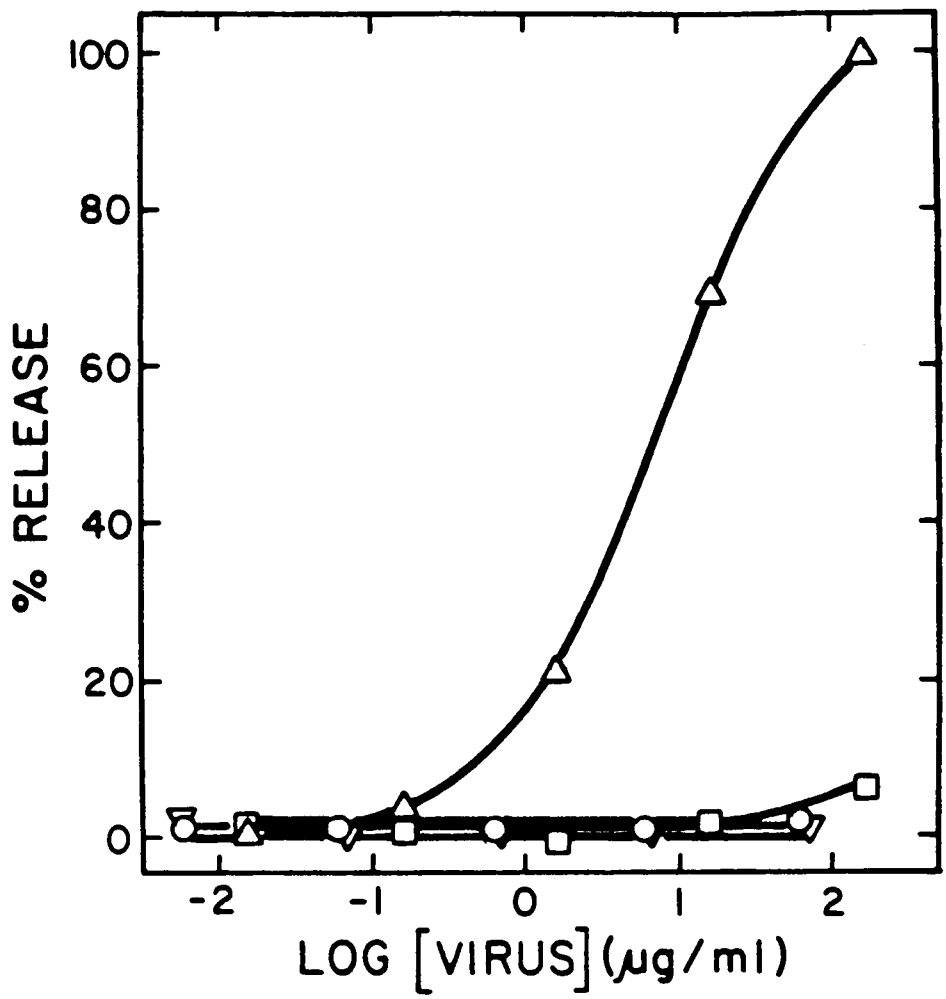

Figure 15. Interactions of Target-Sensitive Immunoliposomes with Viruses. 0.81 nmoles of liposomes were added to indicated concentration of HSV-1 (△), SFV (▽), Sendai (○), or Sindbis (□). Destabilization of liposomes were detected after 30 min incubation by measuring the release of the entrapped calcein.

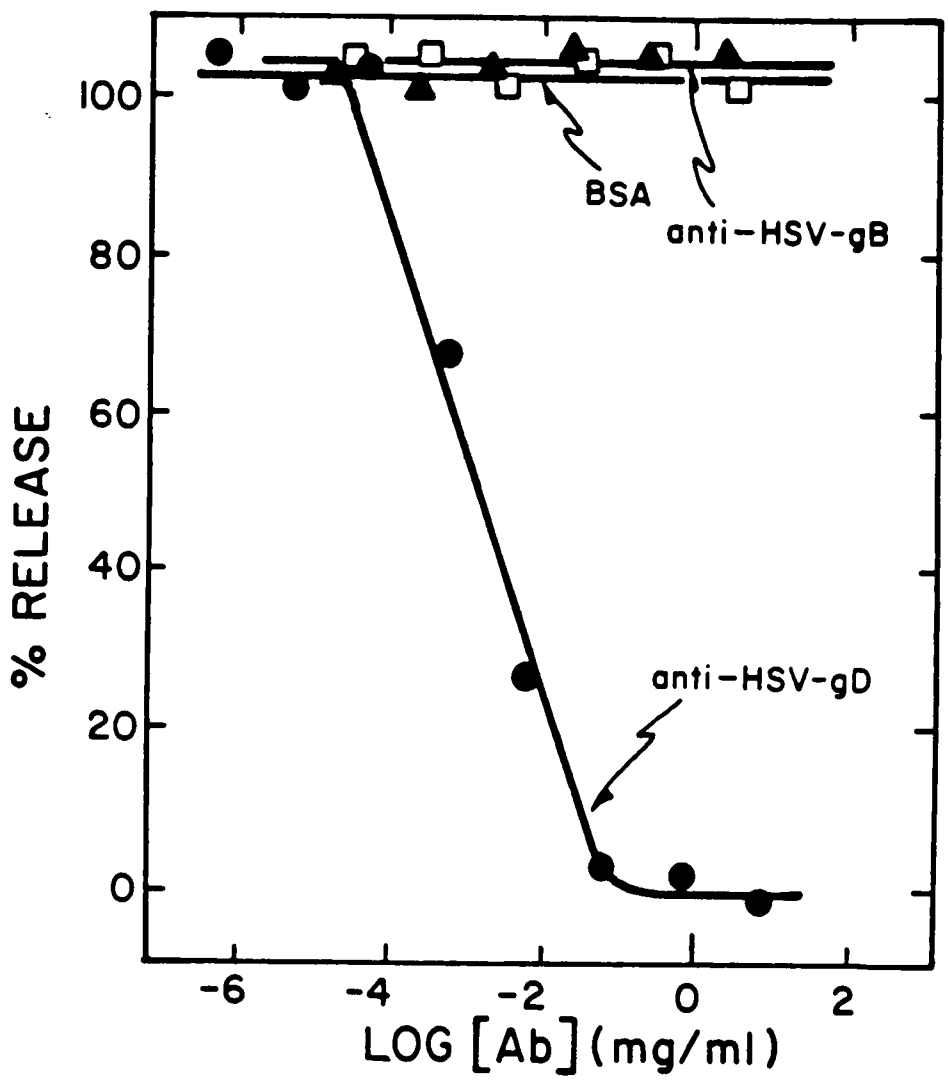

Figure 16. Inhibition of HSV-Induced Target-Sensitive Immunoliposome Destabilization by Free Antibody. HSV-1 was preincubated with free anti-HSV gB (▲), anti-HSV gD (●), or bovine serum albumin (□), at indicated concentrations before addition of TS immunoliposome bearing anti-HSV gD.

Interactions of Targetsensitive Liposomes with HSV Glycoprotein D

To investigate whether the presence of multivalent binding is required to destabilize the TS immunoliposomes, liposomes were reacted with either monovalent, soluble tgD, or multivalent tgD covalently conjugated onto latex beads. As shown in table 4, the tgD-beads caused the release of about 90% of liposome encapsulated calcein whereas release after reaction with soluble (monovalent) tgD was insignificant. Preincubation of soluble tgD with TS immunoliposomes abolished the subsequent destabilization of TS liposomes by the tgD-beads. Additionally, the tgD-bead-induced destabilization was antigen specific. Thus, either substitution of BSA in place of tgD on latex bead or the reaction of the tgD-beads with cognant but not irrelevant antibody abolished the liposome destabilization activity of the tgD-beads (Table 4). Taken together, our results strongly indicate that multivalent binding is essential for the destabilization of TS immunoliposomes.

Table 4

Interactions of Target-sensitive Immunoliposomes with Truncated Glycoprotein D

	(μg/mL)	Preincubation	%Release[a]
tgD-beads[b]	0.4	none	89.4
tgD-beads[b]	0.4	soluble tgD[c]	1.1
tgD-beads[b]	0.4	anti-gD IgG[d]	0.8
tgD-beads[b]	0.2	none	47.0
BSA-beads[e]	2.0×10^3	none	-2.3
soluble tgD	3.5	none	0.4

[a]Release of calcein from TS immunoliposome was measured at 15 min after the addition of tgD.

[b]tgD was covalently coupled to latex beads as described in materials and method section: the amount of tgD on tgD-beads was expressed as the final protein concentration.

[c]TS immunoliposomes were preincubated with 3.5μg/mL of soluble tgD prior to tgD-beads addition.

[d]tgD-beads were preincubated with 70μg/mL of anti-gD IgG prior to liposome addition.

[e]BSA covalently coupled to latex beads was used and the amount of BSA immobilized on the latex was expressed as the final protein concentration.

Analysis of Liposome Binding to HSV

HSV-1 specific binding resulting in liposome lysis was further explored to determine whether liposomes remained associated with virus, or detached from the virus after lysis. As shown in figure 17E, when the incubation mixture, containing calcein entrapped TS immunoliposomes and HSV-1, was separated in a sucrose gradient, all the liposomes were associated with the virus at the bottom of the gradient, whereas the free liposomes were distributed at the top (Fig. 17B). TS

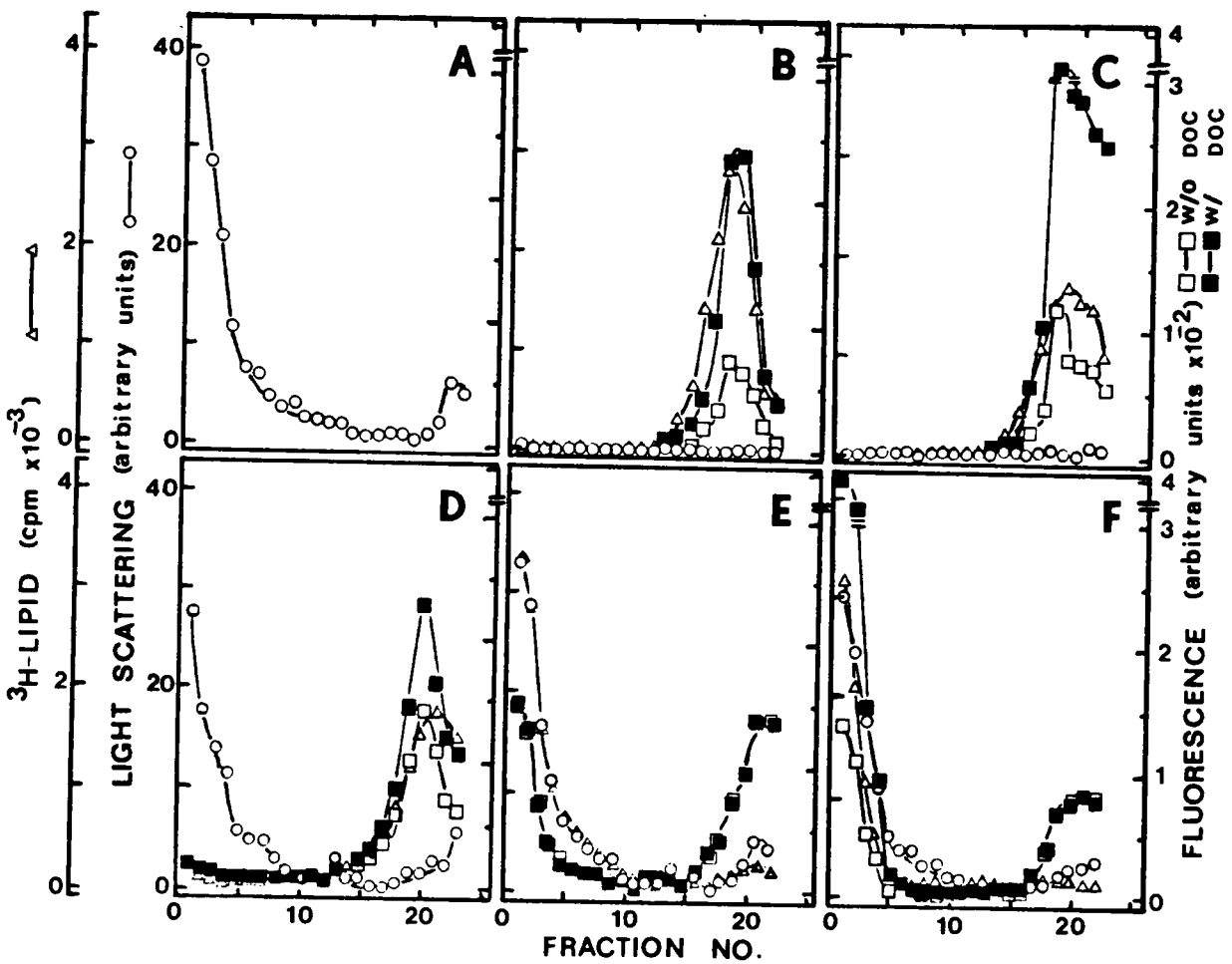

Figure 17. Analysis of Liposome Binding to HSV. HSV was incubated with none (A), PC liposomes (D), target-sensitive, PE immunoliposomes (E), or PC immunoliposomes (F). PE immunoliposomes (B) and PC immunoliposomes (C) were incubated with PBS. The resulting mixtures, separated on sucrose gradients by ultracentrifugations were fractionated from the bottom of the tube. Each fraction was analyzed by 90° light scattering (◯), [^{3}H]lipid counting (△), and calcein fluorescence in the absence (□), or presence (■) of detergent DOC.

immunoliposome binding to HSV-1 was specific in that a similar analysis using non-targeted liposomes (without antibody) did not bind to the HSV, as evidenced by antibody-free liposome sedimenting at the top of the gradient (Fig. 17D). The virus bound TS immunoliposomes shown in figure 17E contained about half of the total calcein fluorescence which was not quenched, indicating that at least 50% of the calcein fluorescence, after release from the TS liposomes, was retained by a virus-liposome complex. Control immunoliposomes containing phosphatidylcholine (PC) which do not exhibit target-sensitivity showed a similar sedimentation without a significant amount of calcein released from the liposomes (Fig. 17F). This is evident by the latency of the calcein fluorescence associated with HSV-bound PC immunoliposomes. A small amount of unquenched fluorescence found at the top of the gradient was probably due to the binding as well as mechanical sheering effect of the complex during the centrifugation. In the absence of HSV, either PE immunoliposomes (Fig. 17B) (target-sensitive), or PC immunoliposomes (Fig. 17C) (target-insensitive) retained their membrane integrity, as shown by the self quenching of calcein within the liposomal compartment. Accordingly, the HSV-specific destabilization of TS immunoliposomes was characterized by the physical association of HSV with liposome, and at least 50% of the total unquenched calcein associated with the virus-liposome complex. Such marker retainment may be peculiar for calcein. When alkaline phosphatase was used as a liposome-entrapped marker, virus-liposome interactions resulted in the full expression of liposome-entrapped enzyme activity (see below), indicating either a total release of enzyme molecules from the virus-liposome

complex or a ready penetration of the substrate molecules into the enzyme containing complex.

Comparison of a Double Antibody Sandwich ELISA and the Use of TS Immunoliposomes in HSV Detection.

To determine whether the principle of HSV induced destabilization of TS immunoliposomes can be used in constructing an immunoassay, the enzymatic detection system as an indicator of the liposome destabilization was adapted. Catalytic activity of the enzymatic detection permitted the use of a smaller amount of liposome per assay than the calcein fluorescence assay (0.82 nmole lipid for fluorescence assay vs. 0.15 nmole lipid for enzyme assay). These alkaline phosphatase containing TS immunoliposomes were used to assay for both HSV-1 and HSV-2. The same anti-gD was also used as a primary antibody in the double antibody sandwich ELISA in order to compare the two methods. As shown in figure 18, the TS immunoliposomes detected both HSV-1 and HSV-2, with 50% maximum signal recorded at 3.2×10^3 pfu/mL and 1.8×10^4 pfu/mL of crude virus preparation, respectively. The differences in detection sensitivity between HSV-1 and HSV-2 was expected since this particular monoclonal D4.2 was previously shown to be more specific for HSV-1-gD (Lathey et al., 1986). In comparison, much higher HSV concentrations were required for the sandwich ELISA method to obtain a similar signal level for either HSV-1 or HSV-2, indicating that the TS immunoliposome assay was more sensitive in detecting HSV than the ELISA. In addition, the entrapment of alkaline phosphatase in the TS immunoliposome improved the virus detection limit by at least three orders of

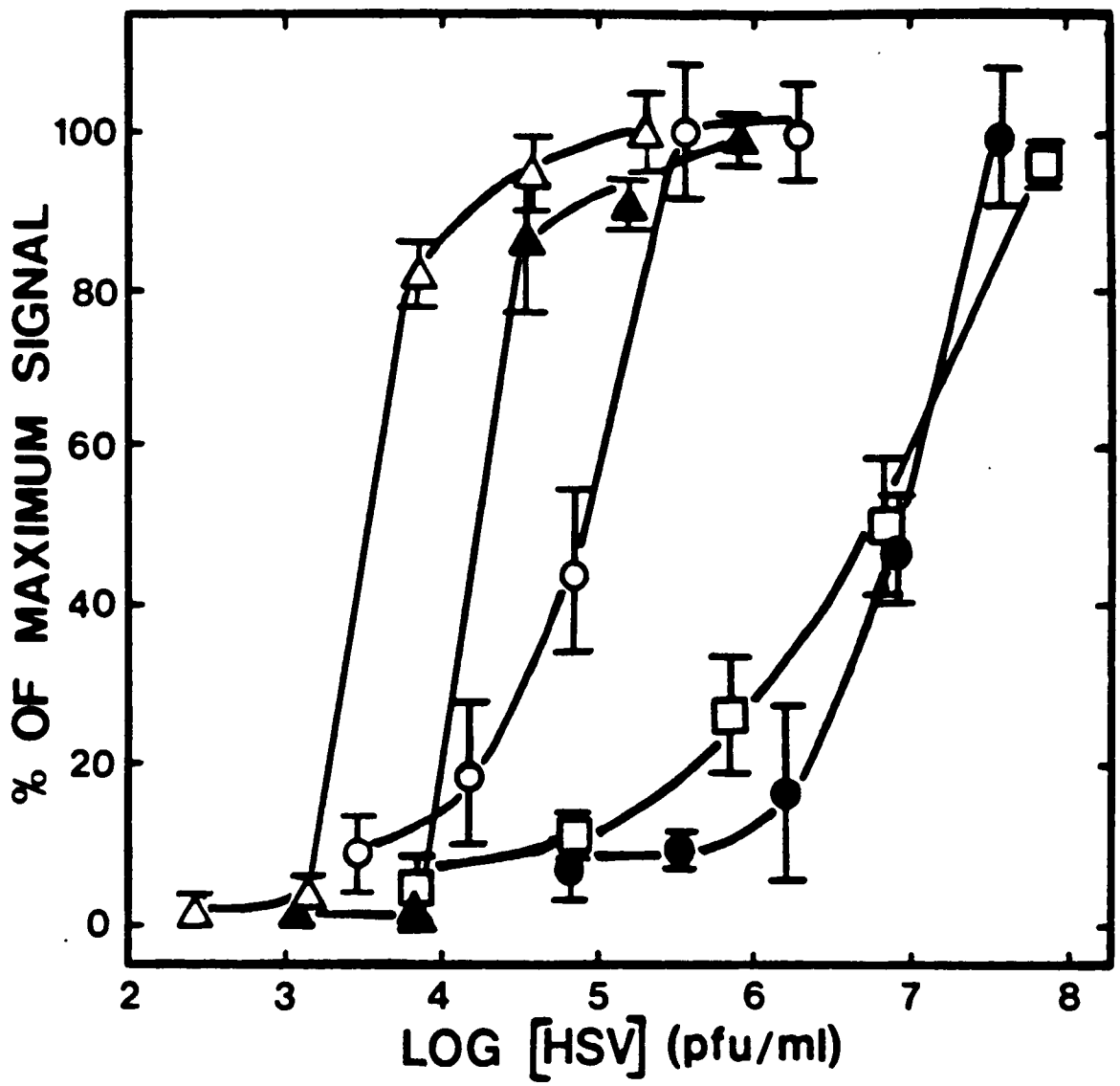

Figure 18. Comparison of Double Antibody Sandwich ELISA and the Use of TS Immunoliposome in HSV detection. A double antibody sandwich ELISA was used to detect HSV-1 (○) or HSV-2 (●) at indicated virus concentration. Similarly, alkaline phosphatase encapsulated TS immunoliposomes were used to detect HSV-1 (△) or HSV-2 (▲). Additionally, calcein encapsulated TS immunoliposomes was also performed to detect HSV-1 (□).

magnitude over that of calcein entrapment. Thus, the detection limit of the immunoliposome-based assay was reduced to 3.2×10^3 pfu/mL.

Discussion

We previously reported that HSV infected cells could cause TS immunoliposome destabilization(Ho et al., 1986a & b). This report further established the target sensitivity of TS immunoliposomes by studying their interaction with HSV virions and glycoprotein D which also resulted in the destabilization of TS immunoliposomes. Target specific lysis of TS immunoliposomes was highly specific in that no viruses other than HSV-1 or HSV-2 could cause significant liposome destabilization within the concentration range of our experiments (Figs. 15 and 19). Furthermore, antigen specificity was demonstrated by the ability of the same antibody, in soluble form, used in liposomes (anti-gD) to inhibit HSV-induced liposome destabilization (Fig. 16). In contrast, antibody to HSV glycoprotein B failed to inhibit TS immunoliposome destabilization, even though this antibody could bind to the virions. Consequently, TS immunoliposome destabilization was target antigen specific. Furthermore, TS immunoliposome remained associated with HSV even after the destabilization of liposomes (Fig. 17).

Our results with tgD clearly indicate that the availability of multivalent antigen on the target is essential for the destabilization of TS immunoliposomes to occur (Table 4). In our model, only tgD made multivalent by immobilization on latex beads could destabilize the TS immunoliposomes; monovalent soluble tgD could not bring about this effect (Table 4). Apparently multivalent binding between the liposome

bound pIgG and the gD molecule on either the cell surface (Ho et al, 1986a), virus envelope membrane (Fig. 15 & 16, Ho et al., 1986b) or latex beads (Table 4), is necessary for destabilization. These findings are consistent with our previous observation using an antigen-stabilized PE liposomes (Ho & Huang, 1985). When the dinitrophenyl-caproyl-PE stabilized DOPE liposomes were interacted with immobilized anti dinitrophenyl antibody, destabilization of liposomes was shown to be dependent on the density of immobilized antibody. Dilution of the antibody on the solid support decreased the level of destabilization supporting the requirement of multivalent binding between the antigen on liposome and the antibody on the solid support (Ho & Huang, 1985).

Detailed mechanisms of stabilization and destabilization of TS immunoliposomes were proposed previously (Ho et al., 1986a & b). Briefly, as shown in figure 19, we envision that pIgG molecules in the PE bilayer are freely diffusable, with a diffusion coefficient of approximately $1x10^{-8}$ cm^2/sec (Huang, 1985), due to the fluid nature of PE bilayer at room temperature. Upon binding of TS immunoliposome to HSV, random migration of antigen and antibody to the contact area leads to multiple immune complex formation, or contact capping (Bell, 1979). As a result, PE enriched domains are formed which can no longer maintain bilayer structure when depleted of the stabilizer pIgG. Therefore, the liposome reverts to hexagonal phase. Although this may be an oversimplification of the destabilization mechanism, specific binding of TS immunoliposomes to HSV caused the immune specific lysis of TS immunoliposomes (Fig. 17). Only the PE immunoliposome which is target-sensitive could release the liposome encapsulated calcein, despite the

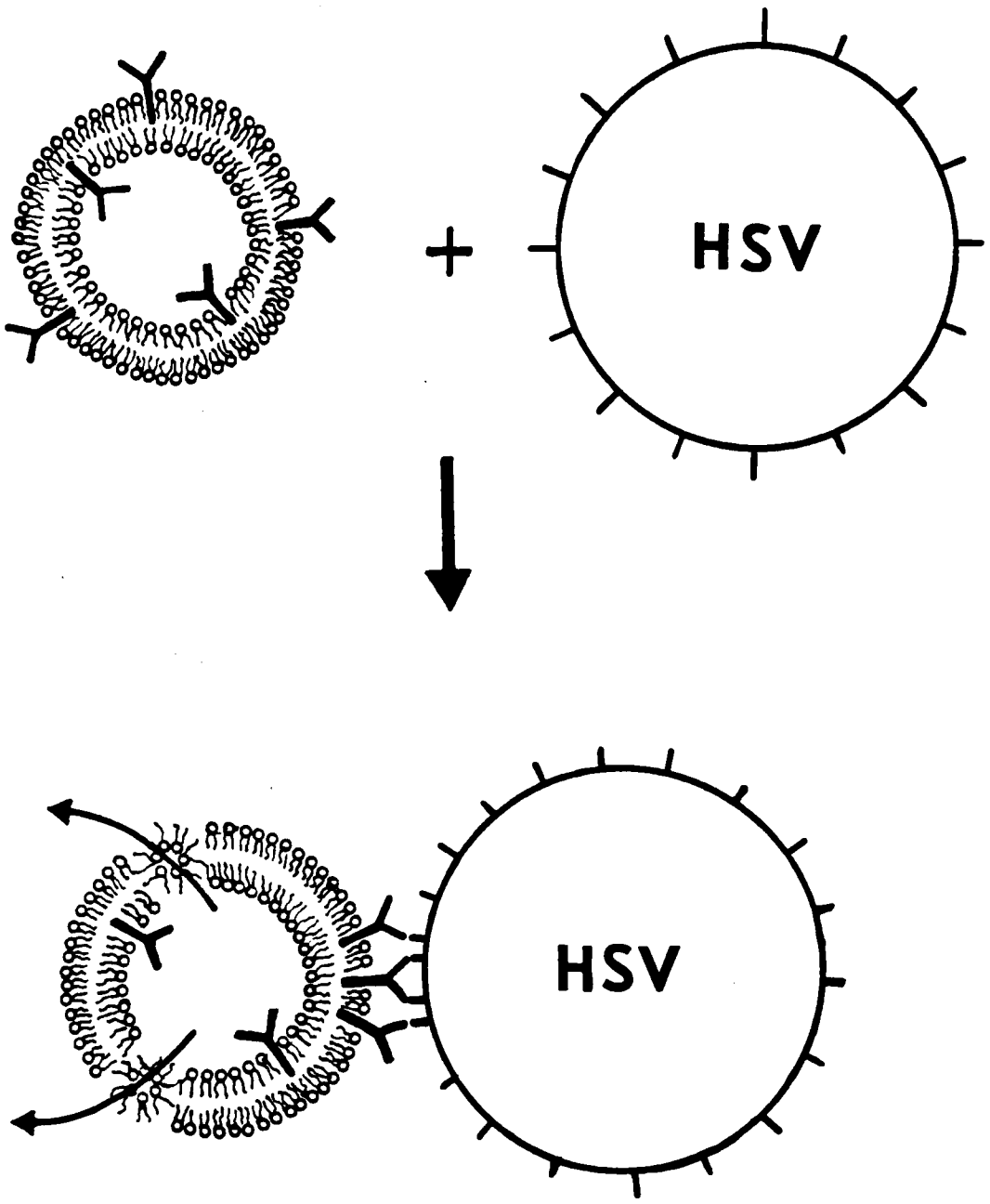

Figure 19. Schematic Representation of Target-Sensitive Immunoliposome Assay for HSV. Destabilization of liposome membrane may involve the formation of hexagonal phase. Sizes of the molecules are not drawn to scale.

ability of PC immunoliposome to bind to HSV (Fig. 17). PE liposome destabilization may additionally require collisions among HSV bound TS immunoliposomes. It has been shown that destabilization of PE liposomes depends on liposome contact (Ellens et al., 1984; Hu et al., 1986). Another possible mechanism is liposome-virus fusion involving some unstable membrane intermediates, leading to the release of calcein. The hexagonal phase has been proposed as an important intermediate structure during membrane fusion (Siegel, 1986). Liposomes enriched with PE are prone to fuse (Cullis & DeKruijff, 1979). It has also been suggested that HSV can fuse with the cell membrane as part of the infection mechanism (Noble et al., 1983; Johnson, et al., 1984). These and other possibilities are under our current investigation.

While the mechanism of TS immunoliposome lysis remains to be tested, the target specific interactions of these immunoliposomes can potentially be used in designing a sensitive immunoliposome assay to detect HSV. The protocols using TS immunoliposomes described here, which requires neither separation nor washing, took only a total of 1.5 hr for HSV detection. This is a significant improvement in the time efficiency over that of HSV culture tests which requires 24 to 72 hr. However, the culture test with a practical detection limit of $5x10^2$pfu/mL (Zhao et al., 1986) is about 6 fold lower than our current limit of $3.2x10^3$ pfu/mL with the liposome assay. In comparison the biotin and streptavidin enhanced sandwich ELISA which requires extensive washings and a 4.5 hr assay time, give a similar detection limit of $5x10^3$ pfu/mL (Nerukar et al., 1984). Similarly, the approach using

DNA probes as HSV detection which could detect 10,000 pfu (Leary et al., 1983; Richman et al., 1984) in 2-3 hr, also required multiple washing steps. Although the stability of liposomes and interfering factors in biological samples remained to be tested, current HSV detection system using TS immunoliposome provides an encouraging performance which includes higher sensitivity, rapidity, and total avoidance of elaborate washing procedures.

Finally, it is important to point out that the principle of TS immunoliposome-virus interactions described here is not limited to detecting HSV. It can be applied for the detection of any multivalent antigen for which a monospecific antibody is available. Since the IgG acylation procedures are well-characterized (Ho et al., 1986a & Huang et al., 1982), one should be able to readily use pIgG to engineer TS immunoliposomes specific to the target of interest (i.e. virus, bacterium or cell). According to the demand, the detection limit could be further improved by (1) using a relatively larger liposome (of 1-2000Å in diameter) to carry more signal molecules per unit of lipid (Deamer & Uster, 1983), (2) using an enzyme substrate that gives fluorescent product (Ho & Huang, 1986), (3) using an enzymatic product which can mediate chemiluminescence production (Seitz, 1978), or (4) using any combination of the above.

Acknowledgments

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CHAPTER VI

KINETIC AND ULTRASTRUCTURAL STUDIES OF INTERACTIONS OF TARGET SENSITIVE IMMUNOLIPOSOMES WITH HERPES SIMPLEX VIRUS

Interactions of target-sensitive (TS) immunoliposomes with the target, glycoprotein D (gD) of herpes simplex virions , were further characterized by analyzing the kinetics of lipid mixing, liposomal content release, and by ultrastructural studies using electron microscopy. Employing a resonance energy transfer assay, lipid mixing between TS immunoliposomes and virions was shown to be very rapid. Lipid mixing, which immediately followed an increase in light scattering, produced with an overall second order rate constant k_{app} of 0.173 $(min)^{-1}(\mu g/mL\ virus)^{-1}$ under our experimental conditions. In comparison, content release from TS immunoliposomes was much slower and exhibited multiple-phase, mixed-order kinetics, indicating that liposome destabilization involved fusion of liposomes with HSV. Analyses of virus-induced liposomal content release indicated that the extent and the apparent rate of liposome destabilization were strongly dependent on liposome concentration. This was evidenced by the fact that only one to two liposomes were destabilized by each virus particle at a low liposome concentration (0.1μM). For higher liposome concentrations (1-10μM), this value was 14-41. This finding implies that collision among the virus bound liposomes is essential for the eventual collapse of TS immunoliposomes to form the hexagonal (H_{II}) equilibrium phase which was observed using freeze-fracture electron microscopy. Studies employing immobilized truncated-gD coupled to latex beads (tgD-beads)

indicated that a multivalent antigen source is essential for TS immunoliposome destabilization. This experimental finding further supports the hypothesis that multivalent binding mediates a lateral phase separation of the phosphatidylethanolamine (PE) bilayer as an initial step of liposome destabilization. Taken together, this evidence indicates that HSV-induced liposome destabilization involved multivalent binding between anti-gD IgG on TS immunoliposomes and gD on herpes virions. The result is lateral phase separation of the PE lipid bilayer that subsequently fuses with the adjacent viral membrane. Upon growth of these fusion complexes, which increase to 14-41 liposomes for each virus, an eventual collapse of the structures results, probably driving the PE to its equilibrium structure of H_{II} phase.

Introduction

We have recently reported preparation of novel, antibody-coated phosphatidylethanolamine (PE) liposomes that can be readily destabilized by binding to target membranes expressing the antigen [target-sensitive immunoliposome (Ho et al., 1986a & b)]. Target-sensitive (TS) immunoliposomes were constructed by employing palmitoyl IgG (pIgG) antibody, against the glycoprotein D (gD) of herpes simplex virus (HSV) to stabilized a PE bilayer (Ho et al., 1986a). By itself, PE assumes a non bilayer, hexagonal (H_{II}) structure under physiological conditions [See Cullis & DeKruijff, (1979); Siegel (1987); Isrealachvili et al., (1980); Verkleij (1984) for reviews]. Using both intact virions and viral antigen expressing cells, we have shown that a specific

interaction exists between TS immunoliposomes and immobilized, multivalent antigen gD which results in the destabilization of the liposomes. This was readily detected by the release of liposome-entrapped calcein, and further demonstrated using antiviral drugs (Ho et al., 1986a & b; Ho et al., 1987; Chapter VII).

All of the reported measurements were done at equilibrium or near the end point of the destabilization process (i.e., 30 min after liposome-virus mixing); and hence, we do not yet know about intermediate steps. Because the understanding of such molecular events is crucial both to optimize TS immunoliposome mediated antiviral drug delivery and to optimize the TS immunoliposome-based immune detection assay, we have further investigated the interactions of TS immunoliposomes with target herpes simplex virions. In this report we elucidate the mechanism of TS immunoliposome and HSV-gD interactions based on kinetic studies of lipid mixing, liposomal content release, and ultrastructural studies of intermediates. The results showed that binding of TS immunoliposomes to target HSV is characterized by fast lipid mixing, fusion between liposome and virus, and a subsequent slower, multiple-phase content release, eventually leading to the conversion of PE bilayer to hexagonal (H_{II}) equilibrium phase.

Materials and Methods

Materials

DOPE, TPE, PC, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE), and N-(lissamine rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). Carboxylated latex beads of

0.99μm (#8828) were purchased from Polysciences, Inc. (Worrington, PA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide-hydrochloride (EDCI) was from Chemical Dynamic Co. (South Plainfield, NJ), and all other reagents were analytical grade. Monoclonal antibody (D4.2) to the glycoprotein D (gD) of herpes simplex virions (HSV) was purified and acylated with palmitic acid as described (Ho et al., 1986a).

Liposome Preparation

A self-quenching fluorescence marker, calcein was entrapped in target sensitive immunoliposomes composed of DOPE and palmitoyl IgG (4000:1, m/m), Liposomes were prepared by sonication according to Ho et al., (1986a). Further details and modifications can be found in Ho et al., (1987). Liposomes used in the lipid mixing experiment were prepared by the addition of 1 mole percent each of Rh-PE and NBD-PE without calcein. These liposomes were passed through a Biogel A-0.5M column to remove the trace amount of deoxycholate (DOC) in the pIgG buffer (Ho et al., 1986a).

For ultrastructural studies, the sonicated immunoliposomes composed of DOPE, TPE, or PC without calcein were extruded through a 0.1μm polycarbonate membrane and residual DOC in the mixture was removed by dialysis against several changes of PBS containing 1mM EGTA and 0.02% NaN_3.

Virus Preparation

The HF strain of HSV-1 was propagated in Hep-2 cells. After 3 cycles of freeze-thawing the infected monolayer, HSV was isolated from cell debris by an initial centrifugation at 10^3g for 10 min. The virus

in the supernatant was pelleted by ultracentrifugation at 10^5g for 1.5 hr. Resulting crude HSV virus was resuspended in a small volume of PBS and enveloped virus was further purified with a linear 20-50% potassium tartrate gradient centrifugation at $8x10^4$g for 4 hr according to Lopez and O'Rielly (1977). The resulting gradients were fractionated from the bottom of the tubes with a peristaltic pump, and fractions containing enveloped virus were diluted 50 times with PBS and the virus was pelleted by centrifugation at 10^5g for 1.5 hr. The virus pellet was resuspended in PBS and protein concentration was determined to be 140µg/mL (Lowry et al., 1951). Purified virus, which contains mainly enveloped HSV, was infectious, and was stored in PBS at -70°C.

Preparation of Latex Beads Conjugated with Truncated HSV Glycoprotein D

Affinity purified, genetically engineered HSV-gD (Nar) (a gift from Dr. Berman and Dr. Lasky, Genetech Inc.; a 312 amino acid, soluble fragment of gD lacking the transmembrane segment referred to as tgD) was conjugated to carboxylated latex according to Kung et al. (1985), with modifications (Chapter V). The final suspension contained 0.5% of tgD-beads, which is equivalent to 16µg/mL of tgD immobilized on latex. The resulting tgD-beads were previously shown to bind specifically to anti-gD IgG with a dissociation constant K_d of $1.5x10^{-8}$M. This is similar to the K_d of the same IgG binding to the membrane bound HSV gD [K_d=$0.5x10^{-8}$M (Ho et al., 1986a)].

Kinetics of Lipid Mixing between TS Immunoliposomes and HSV

TS immunoliposomes containing 1 mole percent each of NBD-PE and Rh-PE were used in these experiments. The resonance energy transfer

efficiency between the donor fluorophore NBD-PE and the acceptor fluorophore Rh-PE incorporated in the TS immunoliposome is dependent on the surface density of the probe in the membrane. Upon fusion of these liposomes with HSV, the surface density of the fluorophores decreases, giving rise to an increase in the fluorescence intensity of the donor lipid NBD-PE (Struck et al., 1981; Hoekstra, 1982). A varying amount of HSV was added to the liposome suspension of 1μM (final concentration of lipid) in PBS containing 1mM EGTA, and the increase in NBD fluorescence was continuously monitored at λ_{ex} = 465 nm and λ_{em} = 530 nm with a Perkin-Elmer LS5 spectrofluorometer. Total volume of the reaction mixture was 2mL. The fraction of liposomes remaining unfused with viral membrane was determined using the following formula:

$$\text{fraction of unfused liposomes} = (1 - F/F_t)$$

where F is the additional fluorescence induced by the presence of virus; and F_t is the background corrected total fluorescence after the prolonged sonication to mechanically induce lipid mixing between virus and liposomes. The data were analyzed graphically by plotting Ln $(1 - F/F_t)$ vs. time to determine the first order, apparent rate constant, k_{app}.

Kinetics of Virus-Induced Liposome Destabilization

To a liposome suspension containing 1mM EGTA in PBS, at the indicated lipid concentration, an appropriate volume of virus was added and the increase in calcein fluorescence due to release of calcein from destabilized liposomes was detected continuously with a Perkin-Elmer

LS5 spectrophotometer at λ_{ex}=490nm and λ_{em}=520nm. Fluorescence values were presented as % of total calcein fluorescence. The total volume of the reaction mixture was kept constant at 2mL. Using either tgD or anti-gD IgG in inhibition experiments, inhibitors were added to either the liposome suspension (tgD), or the HSV suspension (anti-gD IgG), 2 min prior to the mixing of liposomes with virus. All the experiments were done at 22^{o}C.

Liposome destabilization based on calcein release data was analyzed with the following assumptions. First, the change in calcein fluorescence over time is due to the destabilization of calcein-entrapped liposomes. The fraction of the total fluorescence is directly proportional to the concentration of destabilized liposomes in the suspension. Finally, virus-induced destabilization of liposomes yields a total release of the calcein entrapped in each liposome. Using these assumptions, the fraction of stable liposomes remaining at a given time can be estimated using the following formula:

$$\text{fraction of stable liposomes} = (1 - F/F_t)$$

where, F is the fluorescence value due to virus addition at indicated time; and, F_t is the total fluorescence increase on total liposome lysis by the addition of 0.15% DOC in the absence of virus. The data was analyzed by plotting the Ln $(1 - F/F_t)$ vs. time for first order kinetic analysis; and, $(1- F/F_t)^{-1}$ vs. time for second order kinetic analysis with respect to liposome concentration.

Kinetics of Interactions of TS Immunoliposomes with tgD-Beads

To calcein-entrapped liposomes of 1μM in PBS (containing 1mM EGTA), varying amounts of tgD-beads were added and destabilization of the TS immunoliposomes was monitored continuously with the LS5 spectrofluorometer as described above. The final volume was 2mL. The fraction of stable liposomes remaining was also determined as above, and the resulting data were plotted as Ln $(1 - F/F_t)$ vs. time, and the apparent first order rate constant with respect to liposome concentration was determined. The replot of these rate constant with the virus concentration gave an apparent overall second order rate constant.

Electron Microscopy

Liposomes (20μM) and virus (24μg/mL) were mixed to initiate the reaction. At indicated times, 5μL of the mixture was diluted to 50μL with PBS and was immediately loaded onto a carbon coated copper grid. With the exception of figure 25B, all of the samples were negatively stained with 0.5% (w/v) potassium phosphotunstate. The liposome sample from the electronmicrograph presented in figure 25B was stained with 0.5% uranyl acetate.

A much higher final concentration of virus (1mg/mL) and liposomes (5mM) were used in freeze-fracture studies. One microliter of liposome-virus mixture was loaded between two copper strips and were rapidly frozen in excess of 10^{4o}C/sec by plunging the resulting sample into liquid propane at -190^{o}C (Costello, 1980; Costello et al., 1982). Frozen samples were mounted in a double replica device adapted for use in a Balzer 360 M freeze-fracture apparatus (Balzers Co., Nashua, NH)

and fractured at -150°C with a vacuum not less than 10^{-7} Torr. The fractured samples were immediately replicated with Pt/C at a 45° angle followed by C at a 90° angle. Methods of floating and digestion of the copper support have been published elsewhere (Costello et al., 1982; Ting-Beall et al., 1984). The resulting replicas were cleaned with Clorox and picked up on 400-mesh uncoated grids.

The replicas as well as the negatively stained samples on the copper grids were examined with a Phillip 301 electron microscope at 80kV and 50μm objective aperture.

Results

Kinetics of Lipid Mixing Between TS Immunoliposomes with HSV

In order to analyze membrane fusion between TS immunoliposomes and the envelope membrane of HSV, we employed a resonance energy transfer assay (Struck et al., 1981; Hoekstra, 1982). One percent each of NBD-PE and Rh-PE were incorporated into the bilayer of TS immunoliposomes and the increase of NBD fluorescence was followed. The efficiency of resonance energy transfer between NBD-PE and Rh-PE decreases with the sixth power of the mean distance between the donor (liposomes) and acceptor (virus) (Förster, 1949) when the probes were diluted by lipid mixing. As can be seen in figure 20A, the increase of NBD fluorescence was dependent on the concentration of virus added and was completed within a few minutes. No further increase of NBD fluorescence was observed even after a prolonged period of sonication to mechanically induce complete lipid mixing. Under our experimental conditions, viral lipids appeared to be limiting. This is further evidenced by graphical

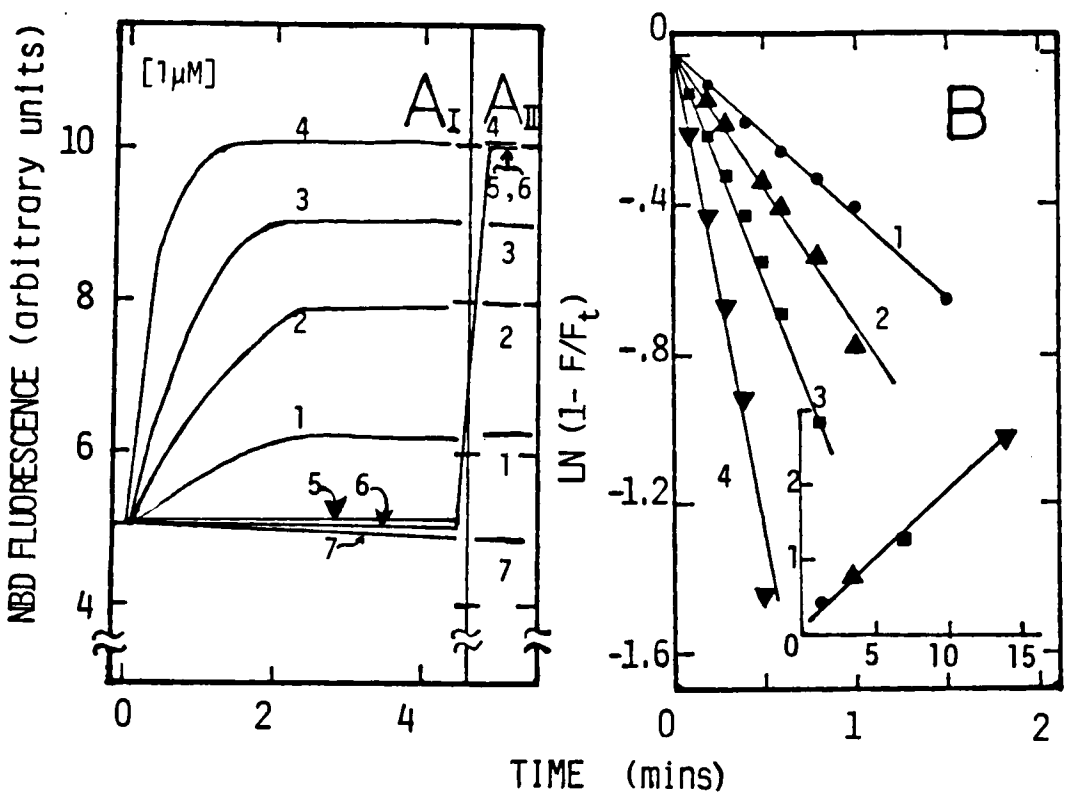


Figure 20. Kinetics of Lipid Mixing between TS Immunoliposomes and Herpes Simplex Virions. To 1 μM of TS immunoliposomes containing 1% NBD-PE and 1% Rh-PE, varying concentrations of HSV were added and the increase of NBD fluorescence due to lipid mixing between liposomes and virus was recorded (A_I). Lines 1, 2, and 3 represent 1.4, 3.5 and 7.0 μg/mL of HSV, respectively; lines 4, 5, and 6 represent 14 μg/mL of HSV; control experiment with no virus was represented by line 7. Inhibition by excess (70μg/mL) tgD (line 5), and excess (70μg/mL) anti-gD IgG (line 6) were also shown in panel A. NBD fluorescence after sonication to induce total lipid mixing was shown in A_{II}. The data from panel A was further analyzed by a first order kinetic plot in panel B and the same numbers as panel A were used to represent the virus concentration. In the insert of panel B, the apparent first order rate constants (determined from slopes of the lines in panel B) were replotted against virus concentration in deducing the overall apparent second order rate constant.

analysis of Ln $(1 - F/F_t)$ vs. time (Fig 20B) which demonstrate an apparent first order reaction. The overall k_{app}, the second order rate constant of lipid mixing, is determined from the slope of straight line in figure 20B insert to be $0.173(\mu g/mL)^{-1}(min)^{-1}$. A similar rate of increase in (90^{o}) light scattering (an indication of liposome virus binding) further indicated that TS immunoliposomes binding to virus is very rapid and immediately followed by a complete mixing of lipid (data not shown) The similarity in kinetics between binding and lipid mixing did not allow us to separate the two events under our experimental conditions. Therefore, the rapid lipid mixing between TS immunoliposomes and target HSV, immediately following the light scattering increase, indicated the rapid fusion between liposomes and virus.

Kinetics of HSV-Induced TS Immunoliposome Destabilization

We have investigated the kinetics of HSV induced destabilization of TS immunoliposomes by following the increase of calcein fluorescence upon the addition of HSV to the reaction mixture. When 50mM of a self quenching dye, calcein, was stably entrapped inside these TS immunoliposomes, about 65-70% of the fluorescence was self quenched. Upon release of calcein as the liposomes were destabilized, the increase of calcein fluorescence due to dilution can be used as a measure of membrane destabilization (Connor et al., 1984; Allen & Cleland, 1980). As can be seen in figure 21A-C, the rate of calcein fluorescence increase was dependent on both liposome and HSV concentrations. In order to compare liposome-destabilization rates, % total calcein-fluorescence value in figure 21A-C were converted to equivalent amount (i.e. mole)

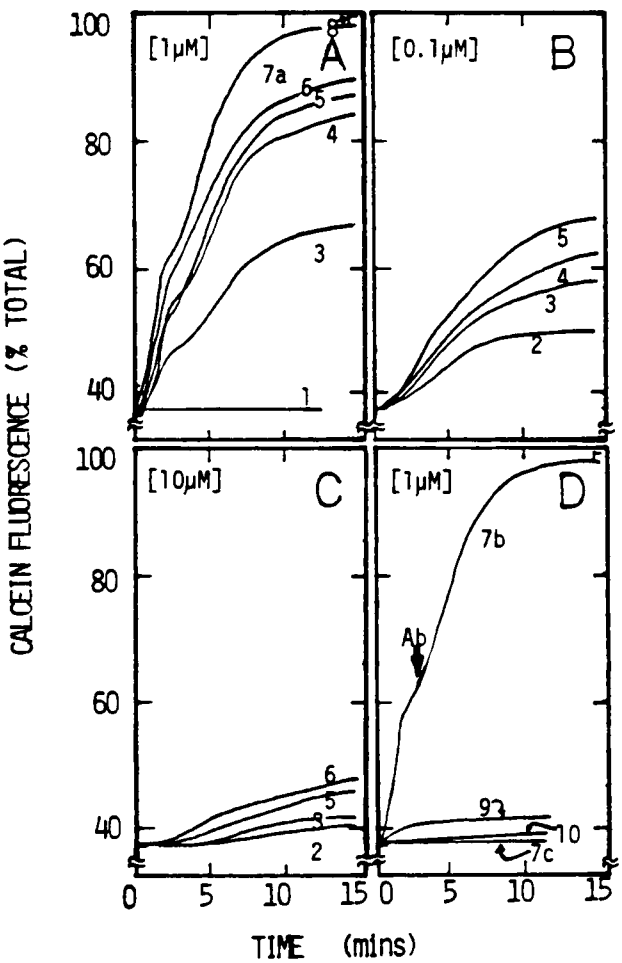


Figure 21. HSV-Induced TS Immunoliposome Destabilization. To TS immunoliposome suspensions of 0.1 μM (B), 1 μM (A&D), and 10 μM (C) lipids, various amounts of HSV were added and the increase of calcein fluorescence due to liposome destabilization was monitored. Final virus concentrations were 0 (line 1), 0.35 (line 2), 0.7 (line 3), 1.05 (line 4), 1.4 (line 5), 2.1 (line 6), and 2.5 (line 7a-c, 9 & 10) μg/mL, respectively. Line 8 represents the total liposome-entrapped calcein fluorescence which was obtained by the addition of DOC. In panel D, the arrow indicates the addition of excess (70 μg/mL) anti-gD IgG to 2.5 μg/mL of HSV containing liposome-virus mixture (line 7b). In line 7C, 70 μg/mL of anti-gD-IgG was preincubated with HSV before the addition of TS immunoliposomes. Control experiments were done with immunoliposomes composed of TPE (line 9) and PC (line 10).

of calcein released. Using such conversion, the 1 & 10μM concentrations of TS immunoliposomes gave the maximum rate as well as the amount of calcein released for the same amount of HSV added. Decreasing the liposome concentration to 0.1μM gave a much lower fraction of the total fluorescence value at the end point. Furthermore, a non-exponential calcein-fluorescence increase was also noted with the 1μM liposome concentration. This concentration of liposomes gave multiple kinetic phases which took place over the time span of 15 min. This phenomenon was reproducible and was eliminated by either increasing or decreasing the liposome concentration (Fig. 21A compared to Fig. 21B & C). Further analysis showed that HSV-induced TS immunoliposome destabilization can be inhibited specifically by either preincubation of excess free anti-gD IgG (the same antibody incorporated in TS immunoliposomes) (Fig. 21D) or by the addition of tgD to the liposomes (data not shown). However, the addition of excess anti-gD IgG after the completion of the first phase (see the arrow in Fig. 21D) could not inhibit the subsequent increase of calcein fluorescence (Fig. 21D). These findings indicate that the second phase of calcein release was not dependent on exposed antigenic sites, accessible to antibody binding at that instant (Fig. 21A & D).

Additionally, we have noted that as the concentration of HSV was increased, the time required to reach the end point of first phase (for 1μM liposome suspension) was decreased although this time delay was not directly proportional to the virus concentration (Fig. 21A).

The requirement for DOPE bilayer was also studied. As shown in figure 21D, substitution of DOPE with DOPC in the TS immunoliposome

bilayer eliminated the ability of virus to induce liposome destabilization as indicated by the elimination of the virus induced increase of calcein fluorescence. Replacement of DOPE with TPE, however, decreased the ability of virus to induce liposome destabilization, resulting in a lower amount of total calcein fluorescence observed under the same set of conditions (Fig. 21D). These data indicate that under our experimental conditions, DOPE is the most efficient type of lipid for constructing TS immunoliposomes.

The calcein release data from figure 21 was analyzed graphically by using first order (Fig. 22A-C) and second order (Fig. 22D-F) plots (Benson, 1960). For the 1μM concentration of liposomes, at least three distinct phases could be discerned. From 0-2.4 mins, destabilization of liposomes appeared to follow second order kinetics with a subsequent delayed step that was prominent between 2.4-3 mins for lower concentrations of virus (Fig. 22E line 2, 4 & 5). This delayed step was eliminated by increasing the concentration of virus (line 6 & 7a of Fig. 22E). Between 4 to 6 min, the release of calcein appeared to follow a first order process at virus concentrations between 1.05 and 2.1μg/mL (see lines 4-6 of Fig. 22B). Either at higher or lower concentrations of liposomes, liposome destabilization appeared to follow at least two distinct second order processes (Fig. 22A, C, D & F) indicating that the overall reaction was a composite of several processes.

Therefore the HSV-induced destabilization of TS immunoliposomes, detected by the increase of calcein fluorescence, exhibited a strong dependence on liposome concentration which followed mixed order kinetics with respect to liposome concentration. This mixed order kinetics

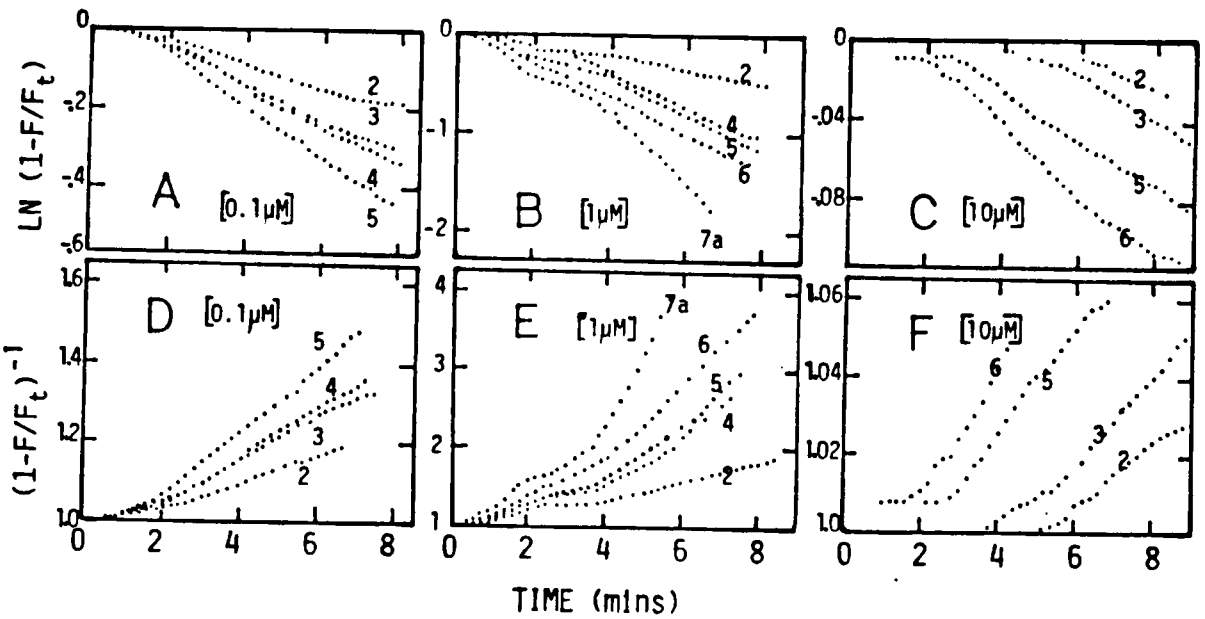


Figure 22. Kinetic Analyses of HSV-Induced TS Immunoliposome Destabilization. Calcein release data from figure 21 were further analyzed employing first order kinetic plots (A-C) and second order kinetic plots (D-F). Panels A and D represent 0.1 μM, B and E represent 1 μM, and C and F represent 10 μM liposomal lipids, respectively. The same numerical representations for concentration as those of figure 21 were used.

was also dependent on the virus concentration but could not be accounted for solely by the concentration of virus present.

The Number of TS Immunoliposomes Destabilized by Each Virus

Liposome destabilization data from figure 21 were analyzed further in an attempt to estimate the number of liposomes that could be destabilized by each virus particle. Since the size of this herpes virion is 1500-2000Å in diameter (Hsuing, 1982 and this report), and assuming the virus is spherical with an average diameter of 1800Å, the surface area can be estimated to be $1.02 \times 10^{-9} cm^2$. Using the estimated liposome diameter of 500Å (Ho et al., 1986a and this report), the average cross- sectional area of the liposome is $1.96 \times 10^{-11} cm^2$. Based on these numbers we estimated that each virus particle can accommodate a maximum of 52 liposomes (one layer thick).

In order to measure experimentally the number of liposomes destabilized by each virus particle, we made an assumption that there were 5×10^5 phospholipid molecules per 500Å TS immunoliposome (Enoch & Strittmatter, 1979) or 2.4×10^{10} liposomes in 2mL of the reaction mixture of 1μM liposome suspension . An additional assumption was made according to Gentry and Randall (1973) in estimating herpes simplex virus protein content to be 3×10^{-15}g/virion or 3.33×10^8 virions/μg of viral protein. Based on these assumption, the experimental number of liposomes destabilized per virion was calculated from the data presented in figure 21A-C (at the 14 min time point) and presented in table 5. The number of liposome destabilized by each virus was much less at lower liposome concentrations and began to reach a maximum at

about the 1μM concentration of liposomal lipid. While the number of liposomes destabilized by each virion was shown to be about 10 fold lower for the 0.1μM liposome concentration for the same amount of virus added, liposomes did not appear to be limiting since only a fraction of total liposome-entrapped calcein was released after 14 min (see Fig. 22B and table 5). This result indicated that at least 14 liposomes per virus was necessary to induce full release of entrapped calcein, and hence, total collapse of the liposome-virus complex. Additionally, 14-41 liposomes per virus appeared to be the optimum ratio for liposome destabilization that exhibited the highest initial rates. Experimental estimation of the saturation number was in reasonably good agreement with the theoretical value of 52 liposomes per virion which did not correct for the steric hindrance effects. Thus, we conclude that each virus can destabilize a maximum of 29-41 TS immunoliposomes of 500Å in size.

Table 5

Estimation of the Number of TS Immunoliposomes Destabilized by Each Herpes Simplex Virion

[HSV][a]	Liposome Concentration[b]		
(μg/mL)	(0.1μM)	(1.0μM)	(10μM)
0.35	2.1	-	41.3
0.70	1.7	24.2	36.2
1.05	1.3	25.5	33.6
1.40	1.3	20.9	29.2
2.1	-	14.3	-
2.5	-	14.0	-

[a]Initial concentration of virus added.

[b]Initial concentration of liposomal lipid in the reaction mixture.

Kinetics of Interactions of TS Immunoliposomes with Immobilized antigen

We next addressed the questions of multivalent antigen requirement for destabilization of TS immunoliposomes and if antigen immobilized on latex beads acted the same as did native antigen expressed on virions. The antigen conjugated to latex beads was a genetically cloned HSV-gD without transmembrane segment (a soluble form of gD, tgD)(Berman et al., 1984). When tgD immobilized on latex beads (tgD-beads) was added to the liposome suspension at 1μM, the extent (Fig. 23A) and the rate (Fig. 23B) of TS immunoliposome destabilization was shown to be dependent on the concentration of tgD-beads. Kinetic analyses showed that the rate of liposome destabilization followed

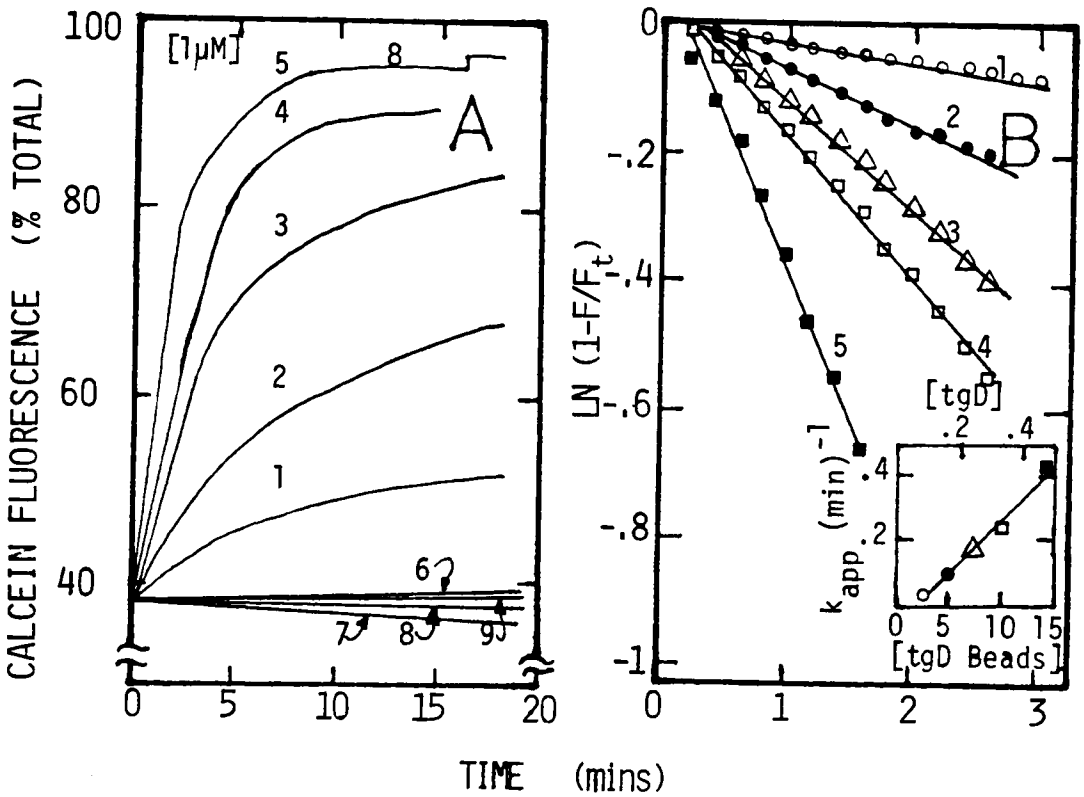


Figure 23. Kinetics of TS Immunoliposomes Destabilization by Immobilized Antigen tgD-Beads. Panel (A): In a quartz cuvette containing 1 μM TS immunoliposome (encapsulated with calcein), various amounts of tgD-Beads were added to a final tgD-bead concentration of 0.0025% (line 1), 0.005% (line 2), 0.0075% (line 3), 0.01% (line 4), and 0.015% (lines 5-7). This is equivalent to tgD protein concentrations of 0.08, 0.16, 0.24, 0.32, and 0.48 μg/mL, respectively. For inhibition experiments, TS immunoliposomes were preincubated with 14 μg of tgD (line 6) or tgD beads were preincubated with 70 μg of anti-gD IgG (line 7) before the initiation of reactions. Additional control experiments were done with BSA immobilized beads (line 8) or PC immunoliposomes (line 9). Panel (B): The calcein release data from panel A were plotted on a first order kinetic plot using the same numerical representation of tgD or tgD-bead concentration as that of panel A, and the insert shows the replot of the slopes that give apparent second order rate constant.

apparent first order kinetics with respect to liposome concentration as well as tgD-bead concentration and it gave an overall apparent second order rate constant of k_{app} = 31.62 $(min)^{-1}$ $(\%tgD\text{-}beads)^{-1}$ or $0.988(min)^{-1}(\mu g\ tgD/mL)^{-1}$ (Fig. 23B). In addition, the tgD-bead induced destabilization of TS immunoliposomes can be inhibited by preincubation of tgD with TS immunoliposomes while the soluble tgD alone could not bring about destabilization of TS immunoliposomes (Fig 23A). Similar inhibition was also observed with preincubation of tgD-beads with anti-gD IgG indicating that the interaction was gD epitope specific. In order to rule out the possibility that a contact between latex and bilayer alone can promote liposome destabilization, immunoliposomes composed of PC were used as a control. As can be seen in figure 23A, tgD-beads could not induce destabilization of such liposomes. Therefore, destabilization of TS immunoliposome by tgD required a multivalent antigen source, exhibited an apparent second order reaction process, and was distinctively different from the mixed-order process observed with herpes simplex virions.

Ultrastructural Studies of TS Immunoliposomes-HSV Interactions

Due to the multiple steps involved in the HSV-induced release of calcein from the TS immunoliposomes (Fig 21 & 22), and due to the fact that this phenomenum was eliminated with tgD-beads-induced liposome destabilization, we reasoned that there may be several intermediate events involved in virus-liposome interactions. If this is the case, these intermediates might be observed by ultrastructural techniques. Hence, both negative-stain and freeze-fracture electron microscopy were

employed to detect liposome-virus intermediates. As shown in figure 24, upon mixing of TS immunoliposomes (Fig. 24B) with HSV (Fig. 24A), a fusion intermediate can be detected (Fig. 24C). These fusion intermediates were short lived ($t_{1/2}$ < 30 sec) and quickly grew to much larger multiple fusion complexes (Fig 25A-D). The viral nucleocapsids appeared to be disassembled in the fusion intermediates (Fig. 24C) and total collapse of the nucleocapsid structure was seen in the larger fusion complexes (Fig. 25A-D). When DOPE in TS immunoliposomes was replaced with either TPE or PC, only grossly aggregated liposome-virus complexes were seen (micrograph not shown).

When these samples were observed by freeze-fracture electron microscopy, we could not detect the intermediate structure before the TS immunoliposomes were converted to H_{II} equilibrium phase (Fig 26C & D) probably due to a much higher concentration (about 500 fold) of liposomes and virus used. As shown in figure 26, when TS immunoliposomes (Fig. 26A) and HSV (Fig. 26B) were mixed, freeze-fracture electron micrograph showed that the final equilibrium phase consisted of extensive tubules characteristic of the hexagonal phase (Fig. 26C & D) which apparently excluded viral nucleocapsid proteins (Fig. 26D).

Taken together, negative-stain and freeze-fracture electron micrographs showed that HSV-induced destabilization of TS immunoliposomes involved multiple steps which include initial binding, fusion intermediates that becomes multiple fusion intermediates which appeared to dissemble the viral nucleocapsid and finally, the rearrangement of the lipids to become the H_{II} equilibrium phase that excludes proteins. At present, we cannot quantitatively define the time frame and the

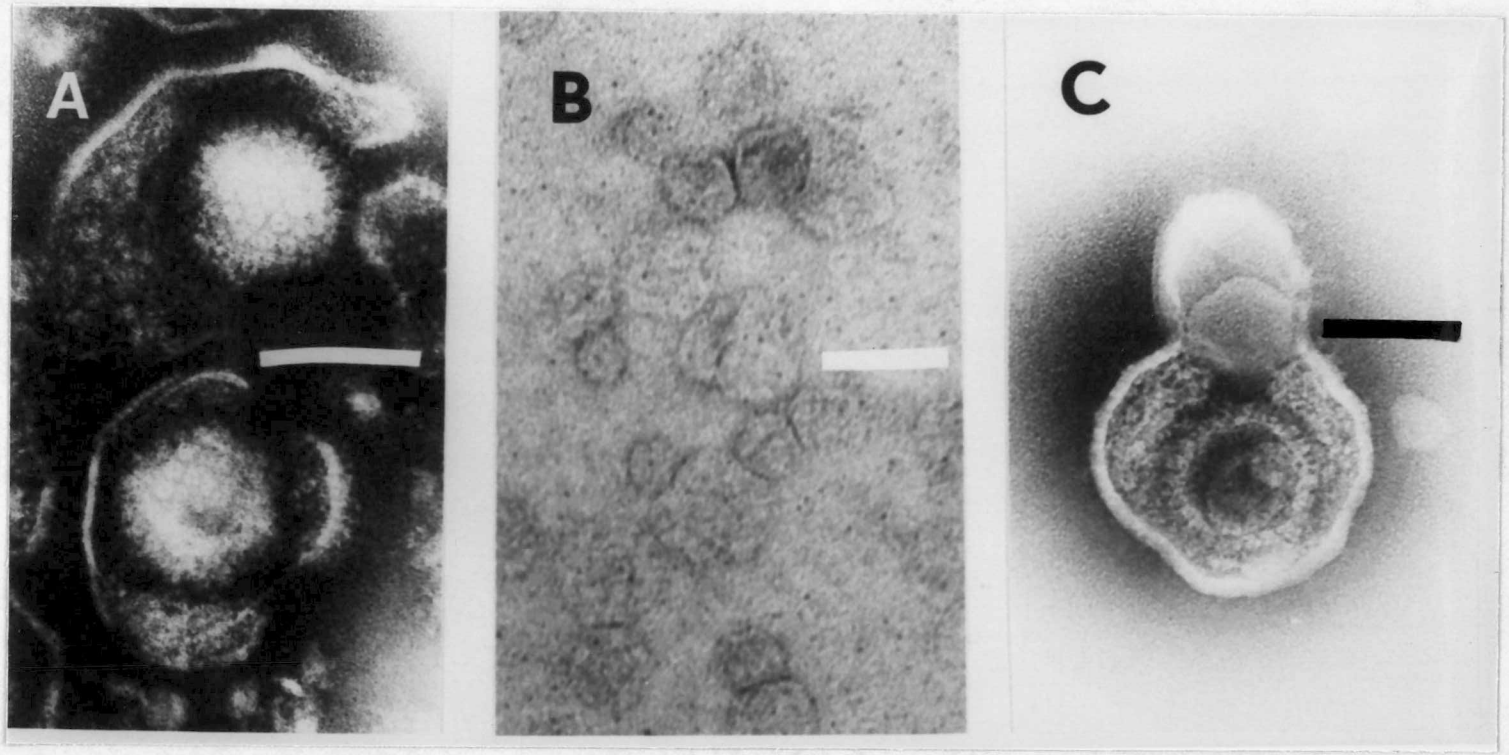


Figure 24. Negative-Stain Electron Micrograph of HSV, TS Immunoliposomes and Liposome-Virus Fusion Intermediates. After mixing of herpes virions (A) and TS immunoliposomes (B) for 30 sec, fusion intermediates such as the one shown in panel C were detected. The bars indicate 0.1 μm.

Figure 25. Negative-Stain Electron Micrographs of TS Immunoliposome HSV Fusion Complexes. After 2 min of the reaction, TS immunoliposomes-virus complexes A-D were detected. Arrows in panels A-D indicate the membrane stacking structures which might lead to the formation of the tubular structure of hexagonal phase (arrow head). Dissembly of nucleocapsid can be seen in area 1 & 2 in panel A & D. The bars indicate 0.5 μm.

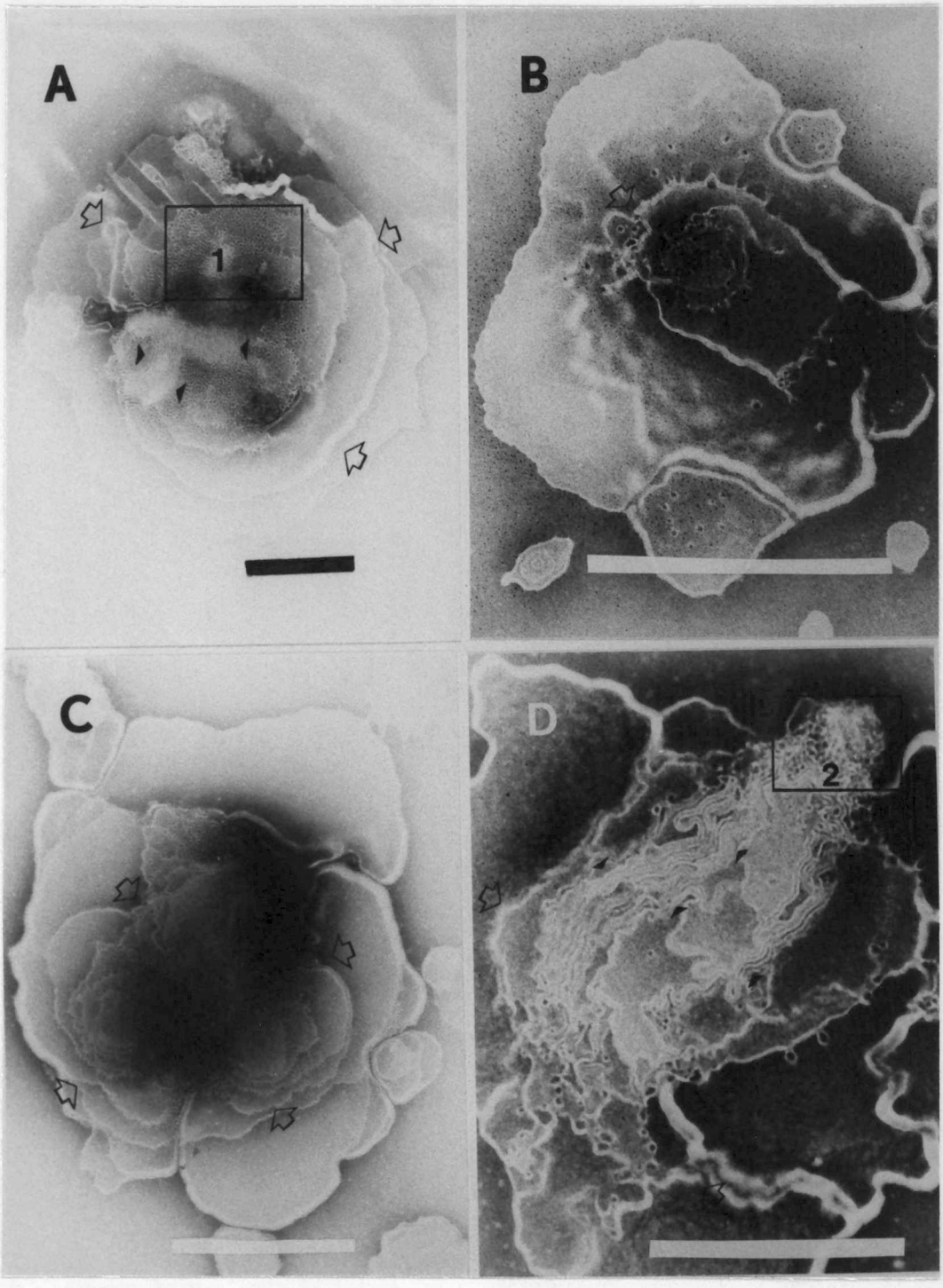
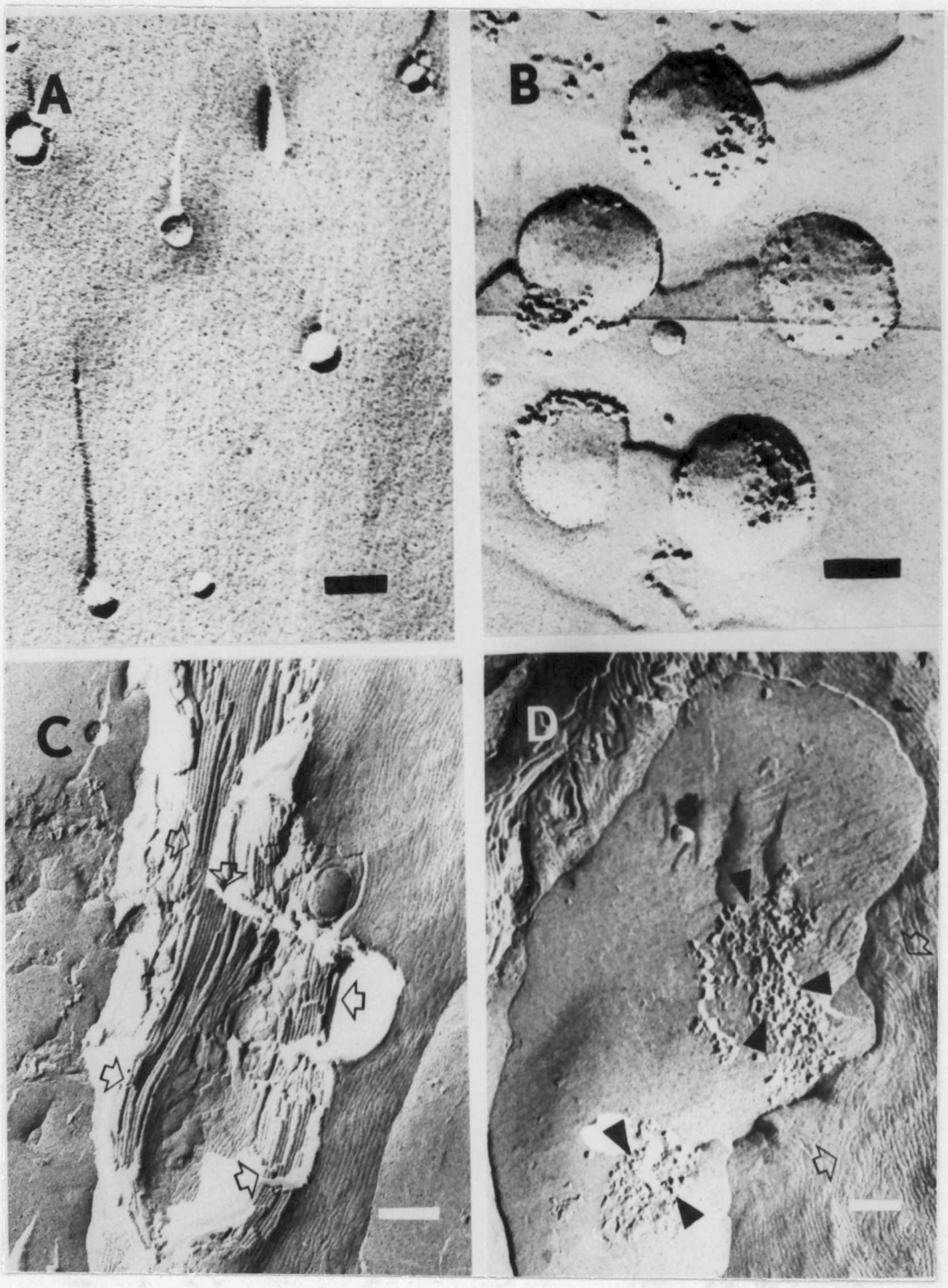

A
1
B
C
D
2

Figure 26. Freeze-fracture electron micrographs of TS immunoliposome-HSV interactions. Addition of herpes simplex virion (B) to immunoliposomes (A), resulted in the structures with an apparent discontinuous tubular lipids shown in panels C and D. The arrows indicate the discontinuous tubular structure of H_{II} phase and the arrow heads indicate the viral protein particles that appear to be excluded from tubular structures of lipids. The bars indicate 0.1μm.


A
B
C
D

extent of each event which corresponds to multiple kinetic steps observed in the calcein release experiments shown in figure 21A.

Discussions

In our previous reports, we have established that target sensitive (TS) immunoliposomes can be destabilized specifically by the target antigen gD which is multivalently expressed on either herpes simplex virions or HSV infected cells (Ho et al., 1986a & b). Additionally, we have shown that the lipid from TS immunoliposomes remains associated with the HSV even after the destabilization (Ho et al., 1987). In this report we have further characterized these interactions by studying the kinetics of lipid mixing , content release from the liposomes, and the requirement for multivalency of the antigen. We observed a much faster apparent rate (Fig. 20) of lipid mixing compared to rate of calcein release (Fig. 21 & 22) indicating that binding and fusion alone were not the only events of the liposome destabilization which resulted in calcein release; but rather, that the initial steps led to the final collapse of the TS immunoliposomes in allowing PE to form the H_{II} equilibrium phase (Fig 24-26). In addition to the ultrastructural evidence for fusion intermediates, multiple phase calcein release kinetics further supported our hypothesis.

Since we do not yet have a reasonable estimate as to the fraction of calcein fluorescence contributed by binding, fusion, growth of fusion intermediates and finally the bilayer collapse to form H_{II} phase, estimation of the rate constant for each event using the mass action method is not possible at present. In addition, the use of

higher concentrations of the virus was limited experimentally by the increase in the light scattering which interfered with fluorescence measurements. As a result, attempts to deduce kinetic constants for the release of entrapped markers (i.e., calcein) from TS immunoliposome-HSV interactions have met with little success. However, the total number of liposomes destabilized by each virion was shown to be dependent on liposome concentration. This is evidenced by the fact that only a small number (1-2) of liposomes were destabilized by each virion for 0.1μM liposomes, under conditions where the liposome concentration was not limiting (table 5 and Fig. 21B). Since we could not measure lipid mixing due to the relatively low sensitivity of the resonance energy transfer assay at this particular concentration of liposomes, we could not rule out the possibility that non-leaky fusion intermediates did not contribute to an increase of calcein fluorescence. Nevertheless, the findings of at least 10 fold higher number of liposomes destabilized per virion (in the range of 14-41, Table 5), at higher liposome concentrations indicated that binding of a minimum of 14 liposomes is likely required to totally destabilize the virus-liposome fusion complexes. This hypothesis further implies that collision among the adjacent liposomes on the same virion may be essential for release of liposome-encapsulated contents. TS immunoliposome destabilization by liposome collision is further supported by the finding that calcein fluorescence increase after 2 min was not inhibitable by the addition of excess antibody (see Fig. 21 D).

The requirement of liposome collision for the destabilization of PE liposomes was proposed previously (Ellens et al., 1984; Bentz et

al., 1985; Hu et al., 1986). However all these studies used at least 100μM liposomal lipid concentrations. Inclusion of antibody in the PE liposomes in our case has allowed us to use lower concentrations of liposomes (≤1μM) to detect PE bilayer destabilization by the target HSV membrane. This is probably due to the specific binding of the liposomes to targets that can bring the membranes into close contact. In our system, membrane contact is probably required, but not sufficient to cause TS immunoliposome destabilization.

Initial destabilization of TS immunoliposomes requires a multivalent binding between anti-gD IgG (on TS immunoliposomes) and gD (on virus or tgD-beads). This requirement is evidenced by the fact that a soluble antigen alone could not bring about the destabilization of TS immunoliposomes, whereas the same antigen, tgD, immobilized on latex (tgD-bead) was a potent destabilizer (Fig 23). Due to the complex kinetic data observed with herpes virions (Fig. 21-22), we do not have a reasonable rate constant to compare k_{app} of tgD-beads with that of HSV. The lipid mixing k_{app} of $0.173(min)^{-1}(\mu g\ HSV\ protein/mL)^{-1}$ could not be used for comparison since only a small fraction of liposomes may actually be destabilized to release calcein, and furthermore, only a small fraction of viral protein is gD. Nonetheless, the studies of interactions between tgD-beads and TS immunoliposomes strongly indicated that multivalent and/or immobilized antigen, but not soluble antigen is essential for the destabilization of TS immunoliposomes. These findings are consistent with our hypothesis, published elsewhere (Ho et al., 1986 a&b), that lateral phase separation of the TS immunoliposome components is required for PE membrane destabilization. Taken

together, our data indicate that initial destabilization by multivalent binding may promote liposome-virus fusion products that eventually collapse as the liposome-virus-fusion complexes grow to larger size.

Due to the experimental limitations at present, we can not deduce absolute constants for each event that may be taking place following TS immunoliposomes binding to their target HSV. However, we have observed some intermediates using ultrastructural methods which showed that specific binding of the TS immunoliposomes to target HSV is followed by formation of fusion intermediates which grow to become larger fusion complexes and eventually collapse and lead to formation of the H_{II} phase as the equilibrium structure (Fig. 24-26). Whether the same events take place in TS immunoliposome binding to other membranes such as the HSV-infected cell membrane, however, remains to be elucidated.

In summary, we have shown that HSV-induced TS immunoliposomes destabilization involves a rapid lipid mixing and complex fusion between liposomes and virions that results in complex multiple kinetics of calcein release. These observations may be applicable in designing a site-specific liposome mediated drug delivery system as well as constructing liposome-based assays for immune detection.

Acknowledgments

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CHAPTER VII

EVALUATION OF TARGET-SENSITIVE IMMUNOLIPOSOME ASSAY AS A HERPES TEST: A COMPARISON WITH COMMERCIAL ELISA AND IMMUNOPEROXIDASE STAINING OF INFECTED CELL CULTURE

An immunoliposome assay (ILA) for herpes simplex virus (HSV) was evaluated using target sensitive (TS) immunoliposomes that can be destabilized specifically by the presence of HSV (Ho et al., 1986a & b). Peroxidase enzyme was encapsulated in the TS immunoliposomes by the fast extrusion method and these liposomes were used as a principle reagent to detect HSV. Numerically coded clinical specimens were tested with ILA in comparison with a commercial enzyme-linked-immunosorbent (ELISA) assay kit from Ortho Diagnostic Systems, Inc. ILA scored positive for all the clinical HSV isolates while the ELISA method failed to detect four out of five positive specimens. These Ortho test-negative specimens were confirmed to be HSV culture positive by an immunoperoxidase staining (IP) method. In comparison with the IP method, ILA scored 100% on both sensitivity and specificity, whereas Ortho-ELISA exhibited 20% sensitivity and 100% specificity. In addition to the sensitivity and specificity, ILA is very rapid, with the assay completed within 1 hr. Hence, an ILA for HSV test which showed a parallel sensitivity and specificity to that of the IP method, should be useful as a rapid, direct and homogeneous assay for HSV.

Introduction

Herpetic infections are often controllable by antiviral therapy providing treatment is begun early. A rapid, sensitive and accurate test to detect viral antigen or infected cells would be of great value especially if the test could be performed without the assistance of a sophisticated laboratory as is necessary to culture virus in detecting the cytopathic effect (CPE). Numerous enzyme-linked-immunosorbent assays (ELISA) have been developed with the intention of improving the time efficiency (Warford et al., 1984a). However, many ELISA tests lack the required sensitivity and often give false positive results (Warford et al., 1984a). Although the low sensitivity of ELISA can be compensated for by viral amplification of specimens in enriched cell culture (Warford et al., 1984b), the required extensive washing protocol is still an obstacle.

A better approach that requires no washing steps for the diagnosis of viral infections could be immunoliposome assays (ILA) which provide rapid and sensitive means of detecting a wide variety of analytes (Chapter II). Whereas previous designs of ILA were based on the use of lytic molecules, such as complement components (reviewed in Ho & Huang, 1987) and melittin (Litchfield et al., 1984), we previously reported a new design of liposome composed of an otherwise unstable phosphatidylethanolamine (PE) membrane bilayer stabilized by the addition of either a membrane bound hapten, dinitrophenyl-caproyl-PE, or by an erythrocyte transmembrane glycoprotein, glycophorin A. Upon exposure of these antigen-stabilized liposomes to multivalent antibody

(immobilized on glass surface), lysis occurred (Ho & Huang, 1985a & b). This was inhibitable by free antibody or free antigen and was sensitive to sub-ng quantities of protein or hapten (Ho & Huang, 1985a & b).

We have recently demonstrated further that PE can be stabilized with acylated antibody (IgG) providing immunoliposomes that can be destabilized to release indicator molecules upon exposure to targets containing multivalent antigen (Ho & Huang, 1986a & b). Such immunoliposomes were referred to as target-sensitive (TS) immunoliposomes. In this chapter, we evaluate the TS immunoliposome approach as a mean of detecting herpes simplex virus in clinical samples and compared the results with commercial ELISA as well as immunoperoxidase staining of tissue culture cells.

Materials and Methods

Materials

Dioleoyl phosphatidylethanolamine (DOPE), transphosphatidylated egg PE (TPE) were purchased from Avanti Polar Lipid (Birmingham, AL.), double antibody sandwich format ELISA antigen test kit for HSV was purchased from Ortho Diagnostic System Inc. (Raritan, NJ.) and all other reagents were of analytical grades.

Sample Handling

Clinical samples were sent to us with a numerical code as blind samples by Dr. R. Courtney (Louisiana State University School of Medicine). These samples were previously tested by immunoperoxidase

staining of tissue culture infected cells. Samples were kept frozen in dry ice throughout transportation as well as storage. Immediately before performing the assays, samples were thawed and diluted five fold (v/v) with Dulbecco's modified Eagle medium (DMEM) without serum. Two hundred microliter and 20μL of the diluted samples were used for ELISA and ILA, respectively. All dilutions as well as sample handling were done in a sterile hood.

HSV Antigen Test With Ortho-ELISA

A double antibody sandwich ELISA kit (Ortho Diagnostic System Inc.; Warford et al., 1984a) designed for testing HSV antigen was used for comparison. The assay was performed in capture antibody coated microwells according to the manufacturer's recommendations and the detailed procedure has been published elsewhere (Warford et al., 1984a). The presence of the HSV antigen was determined by the absorbance reading (λ_{ex} = 490) with a Bio-Tek microwell reader. Samples with 0.15 absorbance value above background (of transport medium control) were scored as positive according to the manufacturer's specifications. All other internal controls met specifications.

Target Sensitive Immunoliposome Preparation

A liposome formulation of TPE:DOPE (1:1 m/m) with acylated IgG to lipid ratio of 1:4000 (m/m) was used. The specificity and binding activity of the acylated IgG toward HSV glycoprotein D (gD) has been characterized in our previous reports (Ho et al., 1986a & b). Routinely, 2.5μmole each of TPE and DOPE, mixed with a trace amount of [^{3}H]cholestanyl ether (CE) were dried under N_2 gas and vacuum

dessiccated for no less than 30 min. The lipid marker [^{3}H]CE was added such that the final specific activity was $5x10^{13}$ cpm/mole of lipid. Then the acylated IgG [which contained an average of 5 acyl chains per IgG (Ho et al., 1986a)] in 0.15% deoxycholate (DOC) was added in a small volume and vortexed to hydrate the lipid film deposited at the bottom of the tube. This was followed by the addition of peroxidase (horseradish, Chemical Dynamic Co.) in PBS containing 2mM EGTA and 0.02% NaN_3. The final concentration of DOC was kept at 0.02% and peroxidase was 20mg/mL. The mixture was immediately vortexed to suspend the lipid protein mixture. Hydrated lipid was allowed to stand at room temperature for 1-2 hr. Then, the suspension was extruded quickly and forcefully through 28-gauge needle using a 0.5mL insulin syringe (B-D) for 20 times. Two additional cycles of extrusion were performed with a 1 hr resting interval. Negative-stain electronmicrographs showed that the resulting liposomes were unilamellar with an average diameter of 2000Å (Fig. 27). We proposed that this unique method of liposome preparation be called the fast extrusion method. Untrapped peroxidase in the liposome suspension was removed by gel filtration on a Sepharose 4B column with a flow rate of 0.3 mL/min. The peak samples containing [^{3}H] were pooled, diluted to 100μM and stored at 4°C. The stock liposomes were diluted to 7.5μM immediately before performing the ILA.

Immunoliposome Assay

Twenty μL of the diluted clinical samples were added to Immunlon (Dynatech) micro wells. Then 20μL of peroxidase encapsulated TS immu-

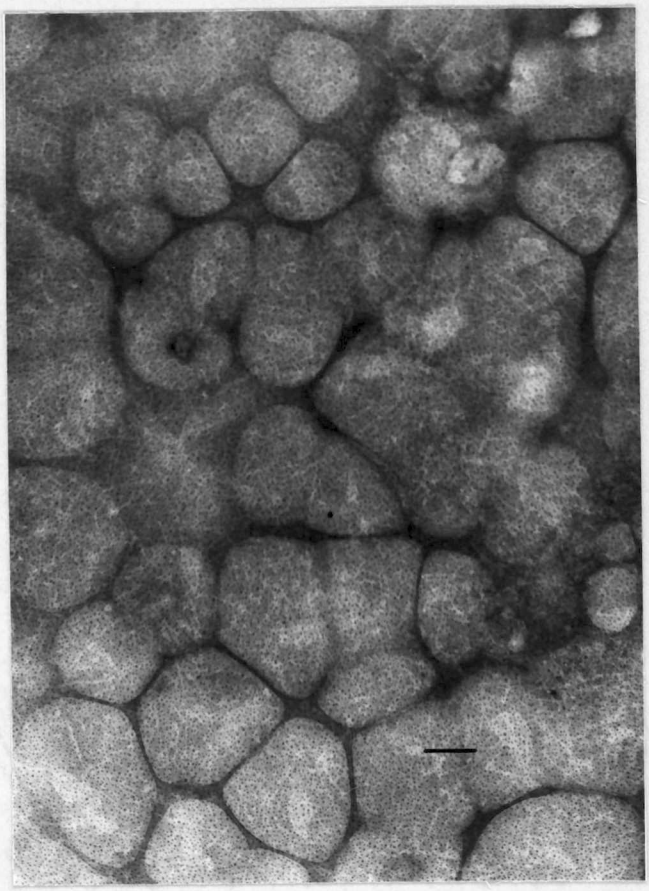

Figure 27. Electron Micrograph of Target-Sensitive Immunoliposomes Prepared by the Fast Extrusion Method. 0.7μmole/mL of liposome suspension was negatively stained with 0.5% aqueous uranylacetate and viewed with a Hitachi 600 electron microscope. The bar indicates 1000Å.

noliposome suspension [expressing specific antibody for HSV-1 antigen, glycoprotein D (gD)] containing 0.15nmole of lipid was added to initiate the reaction. After 30 min of incubation at room temperature, 200μL of peroxidase substrate solution containing 0.1mg/mL of O-phenylenediamine (OPD), 0.01% H_2O_2 in PBS was added and incubation was continued for another 30 min. Subsequently, 20μL of 2N HCL was added to stop the peroxidase activity. The product formation was detected at 490nm employing a Bio-Tek micro-well spectrophotometer. Any samples with an absorbance value 2 times greater than that of the transport medium, which gave the highest absorbance value among the negative control tested, was scored as a positive. Using this criteria, samples with absorbance value greater that 0.191 were scored as positives of HSV test.

Results and Discussion

Numerically coded clinical specimens were used to evaluate the ILA and the ELISA. Data collected by immunoassays were compared with results from the traditional HSV test using the method of immunoperoxidase staining of viral antigen on tissue culture cells. As shown in table 6, ILA could sensitively detect the clinical samples containing HSV as evidenced by the agreement in scoring of clinical isolates with the immunoperoxidase staining technique. On the contrary, Ortho-ELISA detected only one out of five positive clinical isolates. Neither ILA or Ortho-ELISA gave any false positive value (table 6). Hence, the enhanced sensitivity of ILA (compared to ELISA) was not at the expense

Table 6

Comparison of HSV Assays

Code #	Sample #	IP[a] Score	Ortho test A_{490}	Ortho test score[b]	ILA A_{490}	ILA Score[c]
88	Ci[d]	+	0.100	-	1.462	+
89	Ci	+	0.109	-	1.338	+
90	TM	-	0.063	-	0.079	-
91	Ci	-	0.053	-	0.083	-
92	EM	-	0.046	-	0.078	-
93	EM	-	0.061	-	0.084	-
94	Ci	-	0.062	-	0.138	-
95	TM	-	0.062	-	0.077	-
96	Ci	+	off scale	+	2.072	+
97	TM	-	0.062	-	0.079	-
98	Ci	-	0.056	-	0.177	-
99	EM	-	0.060	-	0.085	-
100	TM	-	0.052	-	0.082	-
101	TM	-	0.054	-	0.072	-
102	Ci	+	0.070	-	1.131	+
103	Ci	+	0.092	-	1.110	+
104	EM	-	0.051	-	0.081	-
105	Ci	-	0.061	-	0.079	-
106	EM	-	0.060	-	0.077	-
107	Ci	-	0.062	-	0.131	-
	Transport Medium (TM)	A	0.066		0.090	
		B	0.059		0.101	
	Eagle (EM) Medium (+ serum)	A	0.050		0.079	
		B	0.051		0.086	
	Ag Standard	A	0.801		1.735	
		B	0.856		1.423	
	DMEM (-serum)	A	0.054		0.059	
		B	0.054		0.055	

[a]Data based on tissue culture amplified-immunoperoxidase staining technique.

[b]cut-off for Ortho = BKG_{avg} + 0.15 = 0.0625 + 0.15 = 0.213

[c]cut-off for ILA = (BKG_{avg}) X 2 = 0.191

[d]Ci, clinical isolate.

of the specificity of HSV detection. Table 7 compares the performance of ILA and Ortho-ELISA with the immunoperoxidase staining technique. While Ortho-ELISA exhibit the same specificity of 100% as in ILA, ELISA was much less sensitive in detecting positive HSV samples. Our ILA results on this small sample size, clearly showed excellent correlation with the traditional immunoperoxidase staining method which takes several days to complete. The sensitivity of the Ortho-ELISA has been reported to be about 52% with 96% specificity (Warford et al., 1984a). The difference in the absolute value in these results may be due to the relatively small number of samples as well as the titre of HSV in the clinical samples. Nevertheless, the higher sensitivity of the ILA over ELISA which has been demonstrated in our previous report (Chapter V), was evident.

This report demonstrates the potential of TS immunoliposomes stabilized by acylated monoclonal antibody to gD for the detection of HSV in clinical samples. Virus type specificity was previously shown by the fact that neither semliki forest, sindbis nor sendai viruses was able to induce liposome lysis. In addition, the antigen specificity has also been shown by the ability of soluble anti-gD but not anti-gB (same Ig subclass) to inhibit target induced lysis (Ho et al; 1986b & 1987). Thus the HSV immunoassay using TS immunoliposomes promises to be valuable for the rapid diagnosis of HSV infections. In addition, this ILA is simple and convenient to use. Neither separation nor washing is required and the assay can be completed within 1 hr. This is a significant improvement over the ELISA test which required washing steps and a total of 4.5 hr assay time (Table 7). In comparison, viral

Table 7

Performance of ILA and Ortho-ELISA

	TIP[a]		%	%	Total Correlation	Total Time	Washing
	Positive	Negative	Sensitivity	Specificity	(%)	Required	Required
ILA			100	100	100	1 hr	no
Positive	5	0					
Negative	0	15					
Ortho-ELISA			20	100	80	4.5 hr	yes
Positive	1	0					
Negative	4	15					

[a]Data based on HSV test using tissue culture-amplified immunoperoxidase staining technique.

culture tests require up to three days, and a recently reported sensitive biotin and streptavidin enhanced sandwich ELISA method requires 4.5 hr and involves extensive washing steps (Nerurkar et al., 1984).

We anticipate that the sensitivity of current ILA can be additionally improved by perfecting a better reporter system. The present limitation of our ILA is that the intensity of the signal is low at very low virus concentrations. We expect improvements will result from using a more sensitive reporter system that would permit the use of fewer liposomes per assay such that a lesser amount of antigen, virus or infected cells, is needed to generate a detectable signal. Enzyme systems with high turnover numbers could be used. Additionally, the use of substrates that give products of higher molar absorptivity, products that emit fluorescence, or products that mediate chemiluminescence would also serve the same purpose. Other means of improving immunoassay sensitivities have also been discussed elsewhere (Ho & Huang, 1986).

In summary, we have shown that the ILA employing TS immunoliposomes can be used as a rapid, homogeneous and simple test to detect HSV in clinical samples with excellent sensitivity and specificity. This technology which has been described elsewhere in detail (Ho et al., 1986a & b), can be extended in principle to detect any membrane bound or surface antigen for which a monospecific antibody is available. Therefore, ILA employing TS immunoliposomes may be used to design homogeneous immunoassays for a wide variety of analytes in biological samples and other sources.

Acknowledgments

I thank our collaborator Dr. Richard Courtney for his gracious contributions to this work by providing clinical samples and evaluating the results.

CHAPTER VIII

TARGET-SENSITIVE IMMUNOLIPOSOMES AS AN EFFICIENT DRUG CARRIER FOR ANTIVIRAL ACTIVITY

We have shown previously that target-sensitive (TS) immunoliposomes composed of palmitoyl-antibody stabilized phosphatidylethanolamine (PE) bilayers, could be destabilized by binding to the target cells [Ho et al., Biochemistry (1986) 25,5500]. TS immunoliposome-encapsulated and free cytotoxic drugs of nucleoside analogs cytosine-β-D-arabinoside (AraC) or acycloguanosine (acyclovir, ACV) were compared for their antiviral efficacy and cell cytotoxicity. Target-insensitive immunoliposomes and non-targeted liposomes were also investigated. When mouse fibroblast L929 cells were infected at low multiplicity with herpes simplex virus (HSV), AraC encapsulated in TS immunoliposomes, composed of transphosphatidylated egg PE (TPE), effectively inhibited virus replication and had far less cell cytotoxicity than free drug. As a measure of cytotoxicity, the drug concentration required to inhibit 50% of [^{3}H]dT incorporation 42 hr later (CD_{50}) was determined. For free AraC, this value was 0.3ng/mL, whereas for TS immunoliposome encapsulated AraC, the CD_{50} exceeded 1μg/mL. However, TS immunoliposome encapsulated AraC was virus inhibitory (50% effective dose = ED_{50}) at 1.8ng/mL. A free drug concentration of at least 1000 fold greater was required for comparable antiviral activity. A similar phenomenon was observed when ACV was administered via TS immunoliposomes. The CD_{50} of the free and TS immunoliposomes encapsulated ACV was 12.5ng/mL and 1.4μg/mL respectively, while the ED_{50} of the free and TS

immunoliposomes encapsulated ACV was 1.1μg/mL and 125ng/mL respectively. Consequently, our results indicated the superiority of TS immunoliposomes at drug delivery, especially when drugs were cytotoxic to cells. The use of liposomes of the target insensitive variety provided some enhancement of activity, but this was several fold less than that observed with TS immunoliposomes. In addition, the nucleoside-transport inhibitors, p-nitrothiobenzylinosine and dipyridamole, were shown to inhibit liposome mediated antiviral activity of AraC. This findings indicated that site-specific cytosolic delivery of nucleoside analogs by TS immunoliposomes involves a cellular nucleoside transport system. A mechanism of action is proposed.

Introduction

Liposomes have been extensively studied as inert carriers for the delivery of protein, DNA and biologically active agents into cells (Gregoriadis & Allison, 1980; Ostro, 1983; Knight, 1981). It was widely held that liposomes (composed mainly of phosphatidylcholine, PC) adsorbed on cell membranes could subsequently fuse and deliver liposomal contents into the cell (Pagano & Weinstein, 1978). Accumulated evidence indicates that only a minor fraction of cell-associated PC-based liposomes fuses with the cell membrane (reviewed by Poznansky & Juliano, 1984). Moreover, liposomes lacking recognition molecules (i.e. antibody) adsorb to the cells ineffectively. With recognition molecules, surface adsorption is improved and directed binding to selected target becomes feasible (for a recent review, see Connor et al., 1985). Nevertheless, even binding of liposomes to specific target

cells fails to ensure the delivery of their contents into the cell cytosol. Instead, following internalization via the endocytosis pathway, lysosomal degradation is the usual fate. Hence, most liposomal contents never reach the cytosol which is the site of biological action for most anti-tumor drugs.

Recently our laboratory, along with others, has developed a novel liposome that becomes unstable and fuses with endosomes upon acidification around an endosomal pH of approximately 5.0-6.5 (Huang & Liu, 1984; Duzgunes et al., 1985a; Nayar & Schroit, 1985; Ellens et al., 1984; Connor et al., 1984; Straubinger et al., 1985). As a consequence, most of the endocytosed liposomes deliver their contents into the cytoplasm before reaching the lysosomes (Connor & Huang, 1985a; Straubinger et al., 1985). This approach proved to be efficient in delivering antitumor drugs (Connor & Huang, 1986), DNA (Wang et al., 1987; Duzgunes et al., 1985b) as well as certain cytotoxins (Collins & Huang, 1987). This strategy only works well, however, with target cells with relatively high endocytotic activity. This activity may be lower in cells infected by viruses which shut down cellular metabolic activity (Ben-Porat & Kaplan, 1973), particularly endocytotic activity (Widnell et al., 1984; Wilcox et al., 1982). Under such conditions, liposome-mediated site-specific delivery of anti-viral drugs to the cell surface represents an alternative and perhaps more effective approach. To test this idea we have constructed liposomes that bear a targeting molecule that not only serves the purpose of binding to specific antigen, but also stabilizes the liposome. Following multivalent binding, such liposomes destabilize and rapidly release their

contents in high concentrations at the cell surface. In the case of nucleoside analogs, such drugs may be rapidly transported into the cytosol by an active transport mechanism.

Recently, we reported the preparation of such target-sensitive immunoliposomes which requires only a specific binding of antibody on liposomes to target cells expressing the specific surface antigen [hence, target sensitive (TS) immunoliposomes] (Ho et al., 1986a & 1986b). In this report, we explore the ability of TS liposomes to deliver cytotoxic drugs of nucleoside analogs into herpes simplex virus (HSV)-infected cells as a model of inhibition of virus replication. The results described here further support the hypothesis that site specific delivery of nucleoside analogs to the cell surface is an efficient and specific way for cytosolic delivery of transportable drugs.

Materials and Methods

Materials

Transphosphatidylated phosphatidylethanolamine (from egg phosphatidylcholine) (TPE), egg phosphatidylethanolamine (EPE), egg phosphatidylcholine (EPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). (Methyl-^{3}H)-thymidine ([^{3}H]dT) & [^{3}H]-acyclovir ([^{3}H]ACV) were from ICN Radiochemicals (Irvine, CA), and [5-^{3}H]-cytosine-β-D-arabinoside ([^{3}H]AraC) was from Amersham Co. (Arlington Heights, IL). AraC was from Calbiochem (San Diego, CA), ACV was from Burroughs Wellcome Co. (Research Triangle Parks, NC), dipyridamole was from Boehringer Ingelheim Ltd. (Indianapolis, IN), p-nitrothiobenzyl-

inosine [S-(p-Nitrobenzyl)-6-thioinosine] was from Aldrich Chemical Co. (Milwaukee, WIS). Other reagents were analytical grade.

Antibody Preparation

Isolation and purification of mouse monoclonal IgG_{2a} antibody D4.2 against HSV antigen gD were described previously (Ho et al., 1985a). When needed, the purified IgG was acylated with N-hydroxyl succinimide ester of palmitic acid in 20 fold molar excess over IgG. The acylated palmitoyl IgG (pIgG), in PBS (pH 8.0) containing 0.15% (w/v) deoxycholate (DOC) was separated from the free palmitic acid by Sephadex G75 column chromatography (Huang et al., 1982). This pIgG was previously shown to be the optimum for efficient membrane incorporation as well as the ability to stabilize the phosphatidylethanolamine (PE) bilayer (Ho et al., 1985a).

Liposome Preparation

Routinely, 5-10μmole of lipid was dried and evaporated free of solvent with a gentle stream of N_2 gas. The dry lipid was kept under vacuum for a minimum of 30 min. Then appropriate pIgG in 0.15% DOC was added to hydrate the lipid such that the final lipid to protein ratio was 4000:1 (mole/mole). This was followed by the addition of either AraC of ACV to be encapsulated. Finally, the volume was adjusted with phosphate buffered saline (PBS) to contain 1 mg/mL AraC or ACV, 20mM lipid, and 0.04% DOC. In order to estimate the trapping efficiency of these liposomes, a trace amount of [^{3}H]AraC or [^{3}H]ACV were added when it was necessary. For the liposome used in the target sensitivity assay, the specific activity of [^{3}H]AraC was 4.5×10^4cpm/μg. The

mixture was sonicated in a bath sonicator (Laboratory Supplies, Inc., Hicksville, NY) for two 5-min cycles with an intervening 30-min period as described previously (Ho et al., 1986a). The resulting translucent liposomes were purified chromatographed on a Bio-Gel A-0.5m column at a flow rate of 0.3mL/min to remove untrapped drugs as well as DOC. Liposome fractions, eluted in the void volume were pooled and extruded through 0.2 μm sterile filters (Gelman Sciences, Ann Arbor, MI) for sterilization. Analysis of a small fraction of extruded liposome suspension by Bio-Gel A-0.5m chromatography showed no apparent release of the drugs from liposomes due to extrusion. Assuming 90% recovery from the chromatography, the trapping efficiency of these liposomes was determined using a marker of [^{3}H]AraC or [^{3}H]ACV to be about 1.2μL per μmole of lipid. This was in agreement with our previous data on the average liposome size of 500Å (Ho et al., 1986; Enoch & Strittmatter, 1979). When needed, these liposomes were diluted with McCoys's medium containing 10% calf serum (CS).

Virus

HF strain of HSV-1 was propagated on Hep-2 cells. After 3 cycles of freeze-thawing virus-infected cells, the virus released was isolated from the supernatant after pelleting the cell debris at 1000g for 10 min. These virus stocks stored in -70^{o}C contained 1x10^{8}pfu/mL.

Infecting L929 Cells with HSV

L929 cells at 75-80% confluency in 96-well Coster plates were infected with 0.1 multiplicity of infection (MOI) in McCoy's medium with 2% CS for 1 hr. Then the infection medium was removed and

replaced with McCoy's medium containing 10% CS. For the target sensitivity assay (see below), L929 cells were grown in 35-mm six-well Linbro plates and a similar procedure was used to infect the L929 cell with various concentration of HSV, ranging from 0-10 MOI.

Efficacy of Liposome in Inhibiting Virus Replication

HSV-infected L929 cells in 7-mm 96-well plates were used in these experiments and each experiment was performed in quadruplicate. At 3 hr post infection (PI), the growth medium of infected or uninfected cell was removed, and replaced with 180 µL of either liposome-entrapped or free drug AraC or ACV containing medium. In the experiments where nucleoside-transport inhibitors were used, the inhibitors were added at 30 min prior to the liposome addition. Inhibitors were present throughout the assay. For the control experiments, the infection medium was replaced with the fresh growth medium. At 6 hr PI, 1µCi of [^{3}H]dT was added in 20 µL for the cytotoxicity assay (see below). Then incubation continued at 37^{o}C in a CO_2 incubator. At 42 hr PI, the growth medium was removed and immediately titred for virus yield using the 50% tissue culture infective dose ($TCID_{50}$) assay using the Vero cells (Norley et al., 1986). The $TCID_{50}$ assay was done in quardruplicates and the results were expressed in average $\pm$ S.D.

L929 Cell Toxicity Assay

Cytotoxicity of the L929 cells was determined by labeling the cells at 6 hr PI with [^{3}H]dT. At 42 hr PI, the growth medium was removed and the cells were washed with cold PBS for a total of three times. Then the DNA was precipitated with 5% trichloroacetic acid

(TCA) at 4°C. After two cycles of 2 hr incubation with fresh TCA, the precipitates at the bottom of the plates were washed one more time with 200μL of fresh TCA. Finally, the precipitates were redissolved and dequenched with 1N NaOH overnight. The solubilized TCA precipitates were counted for tritium radioactivity. Data presented were the average ± S.D of the quadruplicates.

Target Sensitivity of Immunoliposomes Composed of TPE

L929 cells infected with various MOI in six-well plates were used in these experiments. Quadruplicates samples were used for each MOI. At 6 hr PI, the growth medium was removed and replaced with 1mL of 0.88μg [^{3}H]AraC (specific activity = 4.5×10^4 cpm/μg) in McCoy's medium containing 10% CS. After a 30 min incubation in a CO_2 incubator at 37°C, the supernatant was removed and the amount of [^{3}H]AraC released into the medium was determined. This was done by separating the free and liposome associated AraC in the supernatant collected. Total amount of [^{3}H]AraC recovered in the cell supernatant ranged from 64 to 90 percent of the amount added. Three hundred μL of cell supernatant was loaded on a 1mL spun column containing Sephadex G25 (superfine). The recovery of liposome entrapped AraC from the G-25 column using the control liposomes was 28 percent. After correcting for recovery of liposomes from the G-25 spun-column, the percent of total AraC was determined as follows:

$$\% \text{ of Total} = [\ 1 - \text{AraC}_r/\text{AraC}_t\ ] \times 100$$

where $AraC_r$ was the corrected value of liposome associated [^{3}H]AraC recovered in the void volume, and $AraC_t$ was the total [^{3}H]AraC which included free and liposome associated AraC. These experiments were done in quadruplicate and data presented as the average $\pm$ S.D.

Results

We have previously shown that target-sensitive immunoliposomes directed to glycoprotein D (gD) of HSV can specifically recognize, bind, and become unstable upon binding to the HSV-infected L929 cells (Ho et al., 1986a). Although these TS immunoliposomes, composed of dioleoylphosphatidylethanolamine (DOPE), were stable at room temperature in PBS, they were relatively unstable at 37°C in the presence of serum and divalent cations containing growth media. Hence, we chose to use transphosphatidylated PE (TPE) which contains a higher degree of saturation of fatty acyl chains. These immunoliposomes composed of TPE were stable in the absence of target membrane and in 10% serum containing tissue culture medium at 37°C over the time span of our experiments (5-10% destabilization in 48 hr).

Target Sensitive Immunoliposomes Enhanced the Potency of Antiviral Drugs While Reducing the Non-Specific Cytotoxicity to the Cell Population

When cells are infected with HSV at a low multiplicity of infection (MOI), only a small fraction of infected cells produce virus that subsequently infects neighboring cells. Additionally, kinetic studies have shown that the virus antigen gD appears at the infected cell

surface about 3 hr after infection (Johnson & Spear, 1984), but the release of progeny virions is not detectable until about 8 hr post infection (PI) (Ben-Porat & Kaplan, 1973). Therefore, we chose to react virus infected L929 cells with the TS immunoliposomes, directed to gD, at 3 hr PI. Since these liposomes were stable in the absence of antigen binding, they were not removed from the incubation medium during these experiments. At 42 hr PI, the supernatant of the L929 cells was collected and titred for virus yield. The toxicity of the drug on the general cell population was monitored by [^{3}H]dT incorporation since both AraC and ACV inhibit DNA synthesis. With the low MOI used in these experiments, only a small fraction of cells would be infected with virus and hence, the [^{3}H]dT incorporation assay was mainly a measurement of uninfected cell DNA synthesis.

Figure 28 compares the effect of the free and TS immunoliposome encapsulated AraC on HSV-infected L929 cells. Encapsulation of the non-viral-selective drug, AraC, reduced virus production with a 50% effective dose (ED_{50}) of 1.8ng/mL. At least a 1000 fold higher amount of free AraC was required to achieve the same effect (Fig. 28B). While increasing the drug potency, the TS immunoliposomes also reduced the cytotoxicity of AraC to the cell population. Thus the 50% cytotoxicity (CD_{50}) of free AraC was 0.3ng/mL while the liposome entrapped AraC was at least 4 orders of magnitude less toxic (Fig. 28A). Control non-targeted liposomes composed of egg phosphatidylcholine (EPC) showed little effect on virus production but this formulation also provided a similar reduction in cell cytotoxicity. Additionally, TS immunoliposomes without AraC had no effect on virus reduction, indicating that

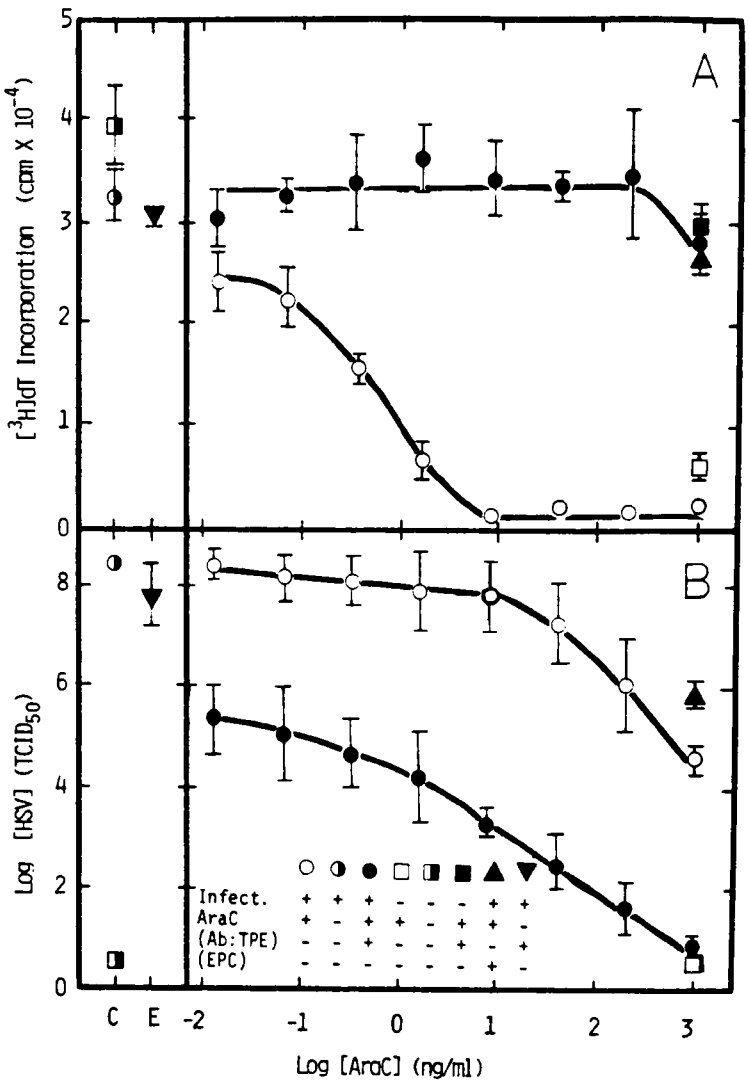

Figure 28. Effects of Free and Liposome Encapsulated AraC on HSV-Infected L929 Cells. To uninfected (□, ◨, ■) or HSV-infected (○, ◑, ●, ▲, ▼) L929 cells, AraC at indicated concentration was added as free from (□, ○), TS immunoliposome-encapsulated form (■, ●) or non-targeted EPC-liposome-encapsulated form (△). At 42 hr PI, cellular cytotoxicity was measured by [^{3}H]dT incorporation (A), and the yield of progeny virus was determined by $TCID_{50}$ assay (B). The effects of TS immunoliposome without AraC, but contained the same amount of liposomes to that of 1 μg/mL AraC (▼) was also analyzed. In the control experiments, no drug was added to either infected (◑) or uninfected (◨) cells.

the immunoliposome-mediated antiviral activity was due to delivery of the cytotoxic drug. This result further indicated that neutralization of virus by immunoliposome bound antibody was not a major factor in contributing to the therapeutic effect. No apparent decrease in [^{3}H]dT incorporation by uninfected control cells occurred in the presence of AraC-entrapped TS immunoliposomes providing further evidence for the stability and specificity of these liposomes (Fig. 28).

To address the question of whether the liposome-mediated delivery system described is useful for other drugs, parallel experiments were done with another nucleoside analog, acyclovir (ACV), a potent and commonly used anti-HSV drug (Swallow & Kampfner, 1985; Balfour, Jr., 1984). As shown in figure 29, a similar but less dramatic effect was observed with TS immunoliposomes containing ACV in comparison to free drug. The ED_{50} was 1.1μg/mL and 125 ng/mL and the CD_{50} was 12.5ng/mL and 1.4μg/mL for free and TS immunoliposome-entrapped ACV, respectively. Additionally, nontargeted EPC liposomes containing ACV on either uninfected or infected cells, showed neither antiviral nor toxic effect.

Comparison of Liposome Composition

To establish the optimum lipid composition, various formulations of liposomes were tested for their efficacy at inhibiting virus replication. Among the liposomes tested, the AraC-entrapped immunoliposome composed of TPE (Ab:TPE) was the most effective at inhibiting virus replication with no apparent cytotoxicity to virus-infected L929 cells (Fig. 30B). Replacing the TPE with EPE which contained a lesser degree

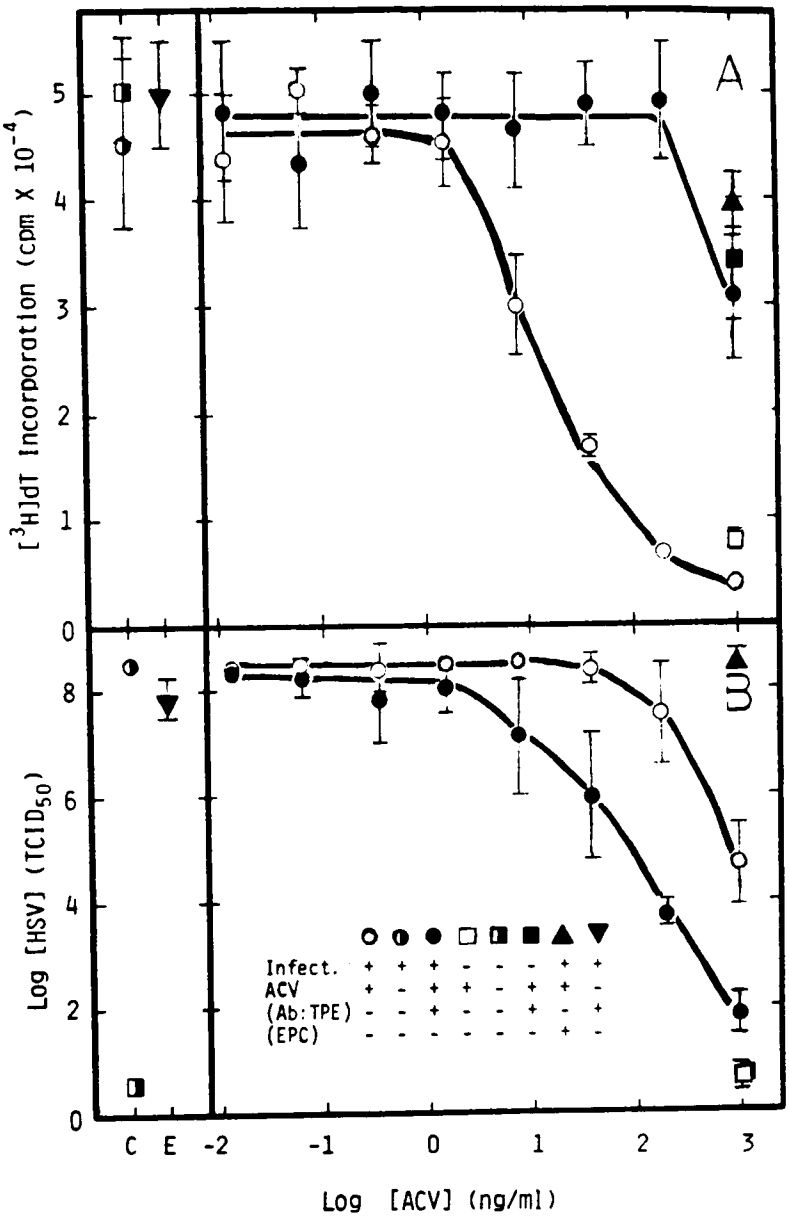

Figure 29. Effects of Free and Liposome Encapsulated ACV on HSV-Infected L929 Cells. Experimental conditions and all the symbols used for this figure are the same as that of Fig. 28.

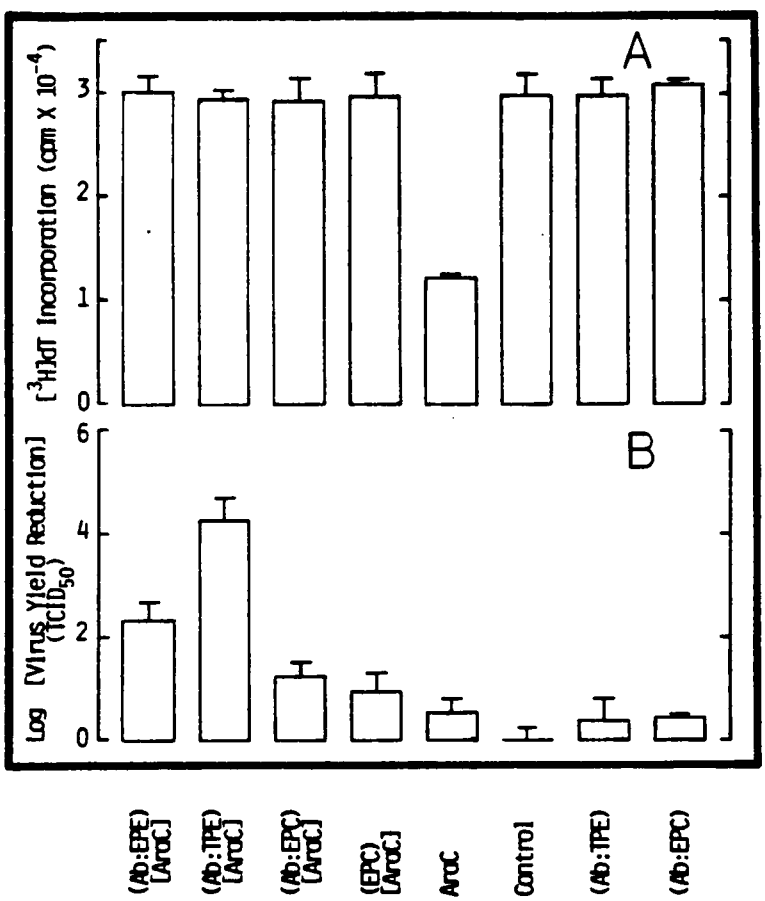

Figure 30. Effects of Liposome Composition on HSV-Infected L929 Cells. HSV- infected L929 cells were treated with various formulation of liposome encapsulated AraC. Cellular cytotoxicity was measured by [^{3}H]dT incorporation (A), and the reduction in virus yield at 42 hr PI was measured by $TCID_{50}$ assay (B). Parentheses indicate the liposome composition, and the bracketed AraC indicates the liposome encapsulated drug. Free AraC, without bracket, as well as no treatment-control was also carried out along with liposome without drugs, (Ab:TPE) & (Ab:EPC).

of saturation of fatty acyl chain, led to about one order of magnitude reduction in therapeutic effect. Although the immunoliposomes composed of EPC (Ab:EPC) (which were target insensitive) were slightly more effective than the free AraC, the degree of enhancement in drug potency was about one and a half orders of magnitude lower than that of TPE immunoliposomes. Non-targeted-EPC (EPC) liposomes had little effect on virus yield but this formulation abolished the cytotoxicity (Fig. 30A) for uninfected cells. Free AraC introduced at the same concentration of 10 ng/mL was cytotoxic to infected (Fig. 30A) and uninfected cells (data not shown) while it had only a marginal effect on virus replication (Fig. 30B). Accordingly, AraC-entrapped TS immunoliposomes composed of pIgG and TPE were the most effective formulation for inhibiting virus replication with no apparent cytotoxicity to the host cell population.

Target Sensitivity of Immunoliposomes Composed of TPE

In order to determine whether immunoliposomes composed of pIgG and TPE were target sensitive, the L929 cells were infected with various MOI of HSV and the extent of liposome destabilization in tissue culture medium at 37^{o}C was measured by [^{3}H]AraC released from the immunoliposome composed of TPE. The amount of AraC release was MOI dependent (Fig. 31). Additionally, under the same conditions, uninfected cells did not cause a detectable [^{3}H]AraC release (Fig. 31). Taken together, these data indicate that immunoliposomes composed of TPE maintained the property of target-induced self-destabilization upon binding to the virus-infected cells.

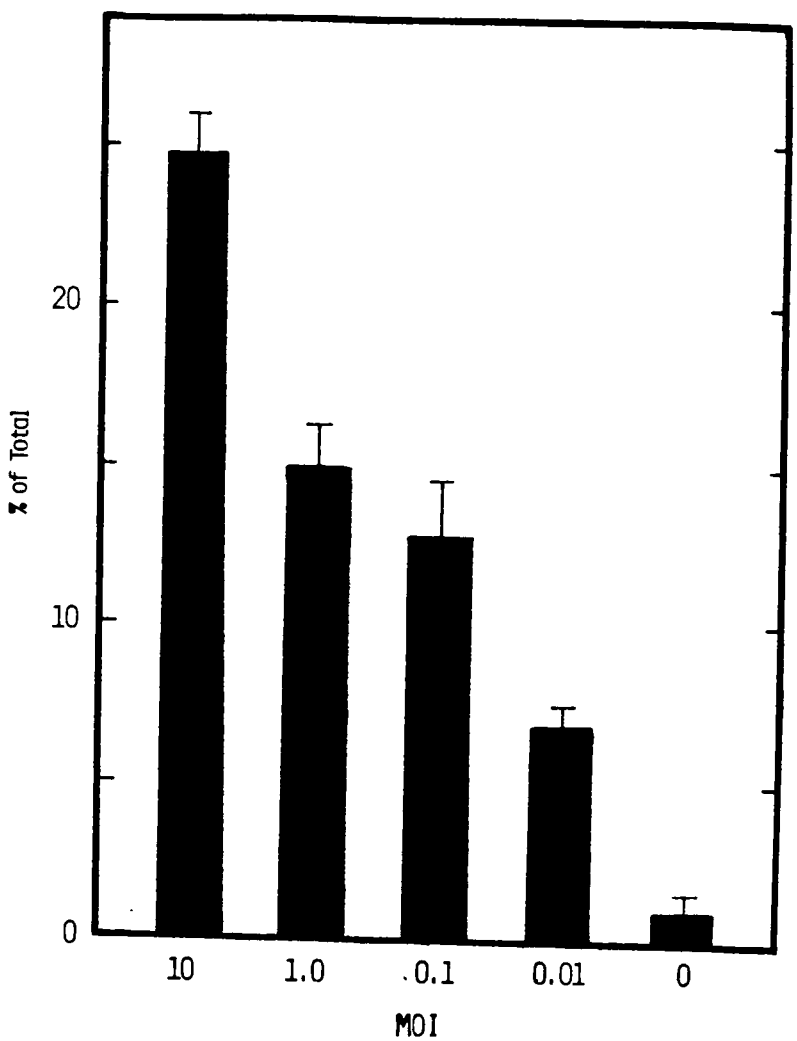

Figure 31. Target Sensitivity of Immunoliposomes Composed of TPE. L929 cells were infected with HSV at indicated multiplicity of infection (MOI) and 0.88 µg/mL of TPE-immunoliposomes were added at 6hr PI. Percent AraC release was determined on the tissue culture supernatant recovered as described in the materials and methods.

Effects of Nucleoside Transport Inhibitors on TS Immunoliposome Mediated Therapeutic Effect

We predicted that surface deposition of TS immunoliposomes was followed by transport of liposome-released drugs into the cytosol via cellular nucleoside transporters. To test this hypothesis, the effect on virus replication of two nucleoside-transport inhibitors, p-nitrothiobenzylinosine and dipyridamole were investigated. p-Nitrothiobenzylinosine is a competitive inhibitor of the nucleoside transport system with a K_i of 1 nM whereas dipyridamole is a noncompetitive inhibitor of the nucleoside transporter with a K_i of 3 μM (Schwenk et al., 1984; Plagemann et al., 1978). As shown in Table 8, the addition of either nucleoside transport inhibitor repressed the therapeutic effect of AraC-encapsulated TS immunoliposomes. A small reduction in virus replication was also noted in the presence of the nucleoside-transport inhibitors. Additionally, the abrogation of therapeutic effect by p-nitrothiobenzylinosine and dipyridamole was concentration dependent. Taken together, these data clearly indicate that TS immunoliposome-mediated cytosolic delivery of AraC, introduced at the surface of infected cells, was mediated by nucleoside transporters.

Table 8

Repression of Target-Sensitive Immunoliposome Mediated Therapeutic Effects by Nucleoside Transport Inhibitors

Inhibitor	Concentration	Log [HSV][a]		
		Liposome[b]		
		With	Without	Difference
None	-	1.4±0.3	7.2±0.5	5.8
p-Nitrothio-benzylinosine	100.0nM	3.8±0.4	4.6±0.2	0.8
	12.5nM	2.8±0.8	5.1±0.9	2.3
Dipyridamole	11.1μM	3.4±0.4	4.8±0.3	1.4
	1.4μM	3.7±0.3	5.3±0.3	1.6

[a]Virus yield from the supernatant of HSV-infected cells at 42 hr PI was determined using the $TCID_{50}$ assay as described in the materials and methods. These data were the mean ± S.D. of the quardruplicates.

[b]HSV-infected cells were treated with or without 0.1 μg/mL of AraC encapsulated in the TS immunoliposomes.

Discussion

The goal of this study was to explore the potential use of target sensitive (TS) immunoliposomes for specific delivery of cytotoxic nucleoside analogs to cells that were endocytotically deficient. Our results demonstrate that these immunoliposomes, which destabilize upon binding to the target cells, effectively inhibit virus replication and as a bonus markedly minimize nonspecific cell cytotoxicity. This significant enhancement of antiviral potency by the TS immunoliposome resulted from a combination of target cell-induced release of drug at the cell surface along with a rapid uptake by the cellular nucleoside

transport system. Previous theoretical calculations using a fluorescent dye, carboxyfluorescein, indicated that a site-specific delivery of drugs at the cell surface would be inefficient for cytosolic delivery. This conclusion was based on the idea that a faster rate of diffusion would overpower the slower rate of uptake (Blumenthal et al., 1982). Since there was no cellular transporter for carboxyfluorescein, the major route of entry would be the inefficient diffusion across the lipid membrane. Our data clearly demonstrate that inhibition of the nucleoside transporter repressed the liposome mediated therapeutic effect. Therefore, an effective site-specific delivery system probably requires not only a site-specific delivery of drugs at high concentration, but also an active membrane-bound transporter that would pass the released drug across the cell membrane. This hypothesis is also consistent with the previous observation using a heat-sensitive dipalmitoyl-PC-immunoliposome delivery system. Upon heating to release the liposome-entrapped [^{3}H]uridine, a rapid cellular uptake of released uridine was shown to be mediated by the nucleoside transport system (Sullivan & Huang, 1986).

The possible mechanism of destabilization of TS immunoliposomes has been discussed previously (Ho et al., 1986a & b). As shown in figure 32, we envisioned that the TS immunoliposomes bind specifically to target cells and upon binding, the liposomes self-destabilize and in so doing release encapsulated anti-viral drugs at the cell surface. Thus the binding results in the removal of the pIgG membrane stabilizer, from a critical region of the PE bilayers (contact capping) eventually permitting phase separation of the PE which cannot by itself

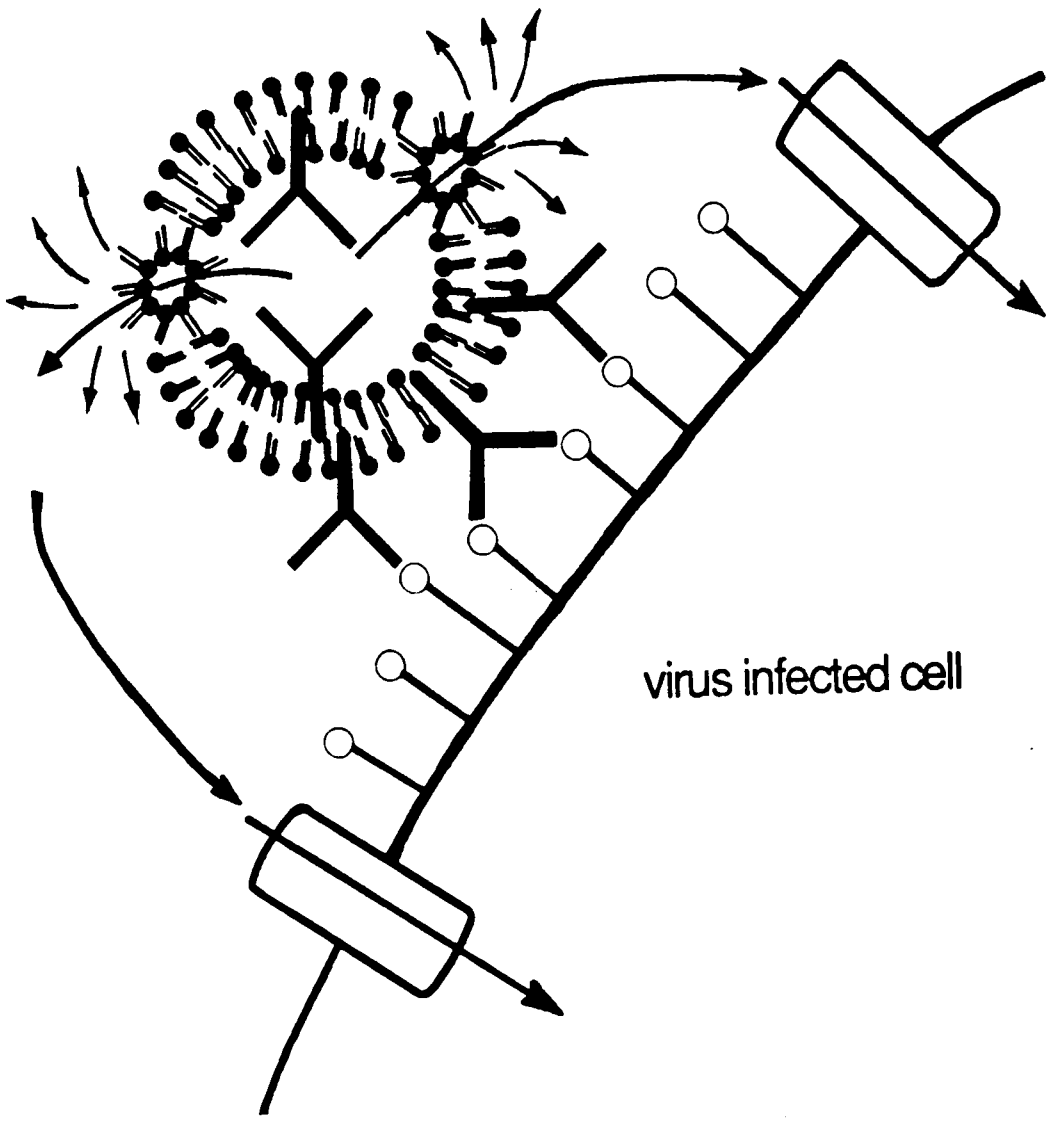

Figure 32. Schematic Presentation of the Preferred Mechanism of Target-Sensitive Immunoliposome Mediated Site-Specific Cytosolic Delivery of Transportable Drugs.

maintain a stable bilayer (Gruner et al., 1985; Cullis & DeKruijff, 1979). Hence, the target bound TS immunoliposomes probably destabilize and rapidly release liposome-entrapped drugs. Target cell-induced destabilization may also require collision between cell-associated PE immunoliposomes (Ellens et al., 1984; Hu et al., 1986). This hypothesis is consistent with our findings that the cell induced release of [^{3}H]AraC from the TS immunoliposomes composed of TPE was MOI dependent (Fig. 31). Although in the absence of any stabilizer and divalent cations, TPE liposomes could maintain bilayer structure at 37^{o}C (Boggs et al., 1981), divalent cations such as Ca^{+2} and Mg^{+2}, present in the tissue culture medium, destabilized the TPE bilayer. Moreover, the presence of divalent cation Ca^{+2} and Mg^{+2} may lower the H_{II} phase transition temperature of TPE immunoliposome PE bilayers, a measure of bilayer stability (Connor & Huang, 1985b; Duzgunes et al., 1985; Ellens et al., 1985). A combination of these destabilizing factors in tissue culture medium probably required the stabilizer pIgG to counter-balance these forces which resulted in preserving the target sensitivity of the TPE immunoliposomes to self-destabilize upon binding to the target cells.

As a result of target-induced destabilization, the released drug concentration at the cell surface will be high promoting rapid up-take by an functional transport system. For instance, the rate of uptake by nucleoside transporters ranges from 13-60 pmole/μL cell H_2O/sec, and the nucleosides are retained intracellularly following phosphorylation (Plagemann et al., 1980). The phosphorylated anti-viral drugs would inhibit the DNA synthesis pathway [for a review, see Swallow & Kampfner

(1985)]. As the liposome-released drugs diffuse away from the affected cells, the final concentration dilutes rapidly to a concentration at which no cytotoxicity was observed. Since this whole process would be specific only to the virus infected cells, the normal cells which do not express HSV antigen gD, would not be affected. Consequently, this strategy is expected to kill only the cells that are virus infected while sparing the uninfected cells in the population.

At present, the possibility that liposomes enter by cellular endocytosis could not be dismissed. Since endocytosis inhibitors such as cytochalasin B and weak bases also interfere with the functional activity of nucleoside transporters (Plagemann et al., 1980), experiments to discount endocytotic delivery by means of inhibitors can not be interpreted conclusively. However, the fraction of drugs delivered via this process was probably minute due to a large decrease in the cellular endocytotic activity that occurs following virus infection (Widnell et al., 1984; Wilcox et al., 1982). Additionally, the uninfected cell without target antigen adsorbs only a small fraction of TS immunoliposomes which could be endocytosed subsequently. Endocytosed AraC entrapped liposomes were, however, ineffective since they would be eventually transported into lysosomes without cytosolic delivery (Huang et al., 1984). It has been proposed that AraC is readily degraded in lysosomes to become biologically inactive (Rustum et al., 1981; Huang et al., 1984).

Another possible mechanism of TS immunoliposome-mediated drug delivery may involve fusion between liposome and the target cell membranes. The antigen binding induced, destabilized PE bilayer may fuse

with the virus infected cell on contact. These TS immunoliposomes were able to fuse with HSV virions as an intermediate state that led to the final non-bilayer, H_{II} equilibrium phase (Ho et al., 1987; Chapter VI). $Hexagonal_{II}$ phase has been proposed to be involved as a fusion intermediate (Siegel, 1986). Although entry of HSV into the cell has been suggested to involve fusion (Johnson et al., 1984), whether the HSV-infected cell can fuse with target sensitive immunoliposomes remains to be seen.

Although the exact mechanism of TS immunoliposome mediated drug delivery remains to be further characterized, the target selective inhibition of virus replication could be improved by using a non-specific cytotoxic drug with high potency. The specificity of TS immunoliposomes to release the drug at the target site minimizes the unwanted cytotoxicity to other cells in the population. A rapid and efficient cellular uptake mechanism at the cell surface is an essential requirement for this design to be effective. In our design, we took advantage of the nucleoside transport system of the cell. Other transport systems, such as folate and glucose transporters may also serve the same purpose (Plagemann et al., 1980). While the in vivo stability of these liposomes remains to be investigated, the TS immunoliposomes containing nucleoside analogs should be useful in topical treatment of herpetic lesions. Theoretically, these liposomes should also be efficient in the reduction of virus replication and such experiments are under our current investigation.

In summary, we have demonstrated the potential use of TS immunoliposomes for delivery of cytotoxic nucleoside analogs into virus-

infected cells to inhibit virus replication *in vitro*. The principle described here should also be applicable to engineer target sensitive liposomes for a wide variety of target cells such as bacterial or virus infected cells, as well as tumorogenic cells for which specific antigens and antibodies are available.

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CHAPTER IX

CONCLUSIONS AND FUTURE PROSPECTS

Conclusions

We have successfully engineered target sensitive liposomes and demonstrated their utility in a site-specific drug delivery system as well as in constructing immunoassays. Target specific release of liposomal contents was shown by target (virus or cell) induced release of liposome entrapped markers as well as cytotoxic drugs. The principles of target specific release of encapsulated marker from TS immunoliposomes were applied to design immunoassays.

Detailed molecular events following the binding of TS immunoliposomes to targets, i.e. virus or cell, remain to be elucidated further. Accumulated data, including those from kinetic and ultrastructural studies (Chapter VI), indicates that destabilization of TS immunoliposomes involves the binding to multivalent antigens. This binding causes lateral phase separation of bilayer stabilizers; either lipid or transmembrane proteins, producing a fusion intermediate that eventually leads to the reversion of the PE bilayer to a H_{II} phase. Kinetic studies of calcein released from the TS immunoliposomes indicates that the initial binding of TS immunoliposomes may induce a partial calcein leakage immediately followed by a fast fusion event that results in less leakage. Finally, collision among virus-bound liposomes within the same fusion intermediates that have 14-41 liposomes attached to each virus, causes the total release of the calcein. This hypothesis

is consistent with the observations both from kinetic studies of calcein release and from ultrastructural studies. The exact rate constant, as well as, the extent of each event contributing the content release, however, are difficult to measure. We are limited technically and experimentally at present from obtaining such molecular details. For example, the event of target binding induced release of calcein from TS immunoliposomes has to be separated from the subsequent events such as fusion and bilayer collapse that may also contribute to calcein release. In addition, a much more sensitive H_{II} phase detection technique would be required to detect the rate of H_{II} phase formation under our experimental conditions. Current techniques of H_{II} phase detection such as NMR or X-ray diffraction require a minimum concentration of 20mM lipid which is at least 2000 fold higher than the lipid (hence the virus) concentration used in our kinetic studies.

Although we have begun to unravel the molecular events involved in interactions of TS liposomes with targets, there are yet a number of questions to be answered about this system. Such questions will be raised and some of the potential solutions will be discussed in the last section of this chapter. Nevertheless, we have successfully constructed and refined a biodegradable drug carrier (or liposome) which can self-destruct upon reaching its destination. We have further demonstrated its utility as both a site specific drug carrier and as a principle reagent for designing an homogeneous assay for immune detection.

Future Prospects

Immunoliposome Assays

Over the last decade, significant progress has been made in the area of liposome-based assays for immune-detection. Although the sensitivity and practicality of ILA may vary from one application to another, homogeneous assays will certainly be preferable over the heterogeneous assays for most application since they eliminate time consuming washing procedures. A membrane lytic type assay is advantageous over agglutination assays in terms of sensitivity, because of signal amplification resulting from the use of enzymes as reporter molecules. Future developments are likely to be directed toward membrane lytic assays even though the simplicity of liposome agglutination is appealing for certain application (Kung et al., 1985).

A major problem in liposome lytic assays using exogeneous lytic molecules (i.e. complement components) is their instability. By employing target-sensitive immunoliposomes, the ILA has eliminated the use of lytic molecules. Accordingly, the assays are much simpler (no extra washing steps), and more stable (no labile lytic molecules). Finally, flexibility in choosing the type of antigen and antibody used as a stabilizer allows modification of assays without extensive redesign of liposome formulations.

There is certainly room for improvement of homogeneous ILA with respect to the target-sensitive immunoliposomes themselves. PE could be replaced with other lipids which favor hexagonal phase formation, or with surfactants (which exhibit a bilayer to hexagonal phase transition

at low concentration) in order to improve the sensitivity of the assays. Furthermore, lipase resistant lipids, such as those which are ether-linked rather than ester-linked, may be used to extend the shelf life of liposomes.

Another approach in improving ILA design is to use a double liposome assay to amplify the detection signal. The theory behind this is that destabilization of target-sensitive immunoliposomes can be brought about by proteolytic removal of the essential Fab domain of stabilizer palmitoyl-IgG on the surface of the immunoliposomes. We have shown that enzymatic degradation of the stabilizer efficiently renders a target-sensitive immunoliposome unstable (Ho et al., 1986a and Fig. 13). Similarly, Hu et al. (1986) have shown that tryptic digestion of glycophorin on the glycophorin-stabilized PE-liposomes resulted in liposome destabilization and release of liposomal contents. Based on this principle, one might be able to design a coupled liposome assay as presented in figure 33. The first liposome in the assay mixture could be a target-sensitive type, or another type such as a complement sensitive liposome. The enzyme encapsulated in the first liposome would be the one which attacks the stabilizer of the second liposome and causes the second liposome to release its contents. The second liposome would carry large numbers of reporter molecules. When the first liposome comes in contact with the analyte, it releases the encapsulated enzymes which degrade the stabilizer of the second liposome, causing the release of the signal generating molecules (Fig. 33). In theory, the first liposome can carry a large number of enzyme molecules which can catalytically destabilize many secondary liposomes

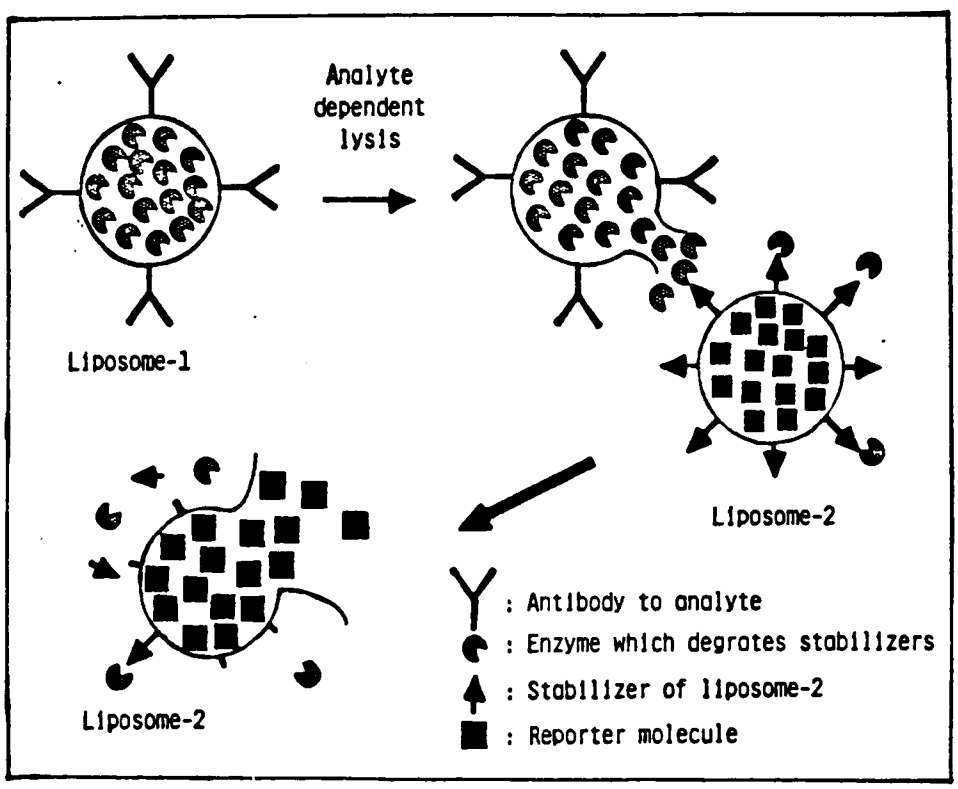

Figure 33. Schematic Representation of A Coupled Liposome Assay. Liposome-1 containing the recognition receptor for analyte encapsulates an enzyme which degrades the stabilizers of liposome-2. When an appropriate analyte is introduced, the analyte-mediated release of the enzyme from liposome-1 will degrade the stabilizers on a large number of liposome-2 containing the reporter molecules. Thus, the enzyme mediated lysis of liposome-2 amplifies the analyte induced signal.

and generate a large amplification of the signal. In this way, one can expect to improve the sensitivity of the assay at least two to three orders of magnitude. Another obvious advantage of the design is that it allows the common use of second liposome for all ILA's based on the same principle of design. Since the stabilizer of a PE liposome can be a lipid, protein, glycoprotein or glycolipid (Ho & Huang, 1985 a & b; Hu et al., 1986; Nayar & Schroit, 1985; Cullis & DeKruijff, 1979; Ellens et al., 1984; Tsao & Huang, 1986; Connor et al, 1984; Taraschi et al., 1982), the enzyme to be encapsulated in the first liposome can be chosen from a long list of stable, and inexpensive enzymes.

An important factor which remains to be considered is the stability of liposomes in serological fluids. Application of ILA to detect serological analytes requires liposomes remain stable in plasma or serum. This is particularly important for the design of homogeneous ILA since liposomes are directly in contact with serum components. In heterogenous ILA which does not require direct contact of serum with liposomes, however, stability of liposomes in serum will not be a problem.

Initial studies using phosphatidylcholine (PC)-based liposomes showed that both serum and plasma, induce a rapid leakage of the liposome encapsulated marker within a few minutes of incubation (Scherphof et al., 1978; Kirby et al., 1980; Allen & Cleland, 1980; Mayhew et al., 1979). High density lipoprotein (Scherphof et al., 1980; Tall et al., 1983; Senior et al., 1983), albumin (Zborowski et al., 1977; Kimelberg, 1976; Hamilton & Cistola, 1986), and other serum proteins (Juliano & Lin, 1980; Scherphof et al., 1980) have been identified as serum

components which destabilize liposomes. Further evaluation reveals that liposome stability in serum can be improved by addition of cholesterol (Kirby et al., 1980; Allen & Cleland, 1980; Mayhew et al., 1979; Damen et al., 1981) and/or gangliosides (Allen et al., 1985). The importance of membrane fluidity has also been noted (Ponpipom et al., 1984). Liposome stability in serum can be significantly improved by using sphingomyelin:cholesterol (1:1, mol/mol) instead of PC in liposomes (Senior & Gregoriadis, 1984). On the other hand, antigen stabilized PE liposomes without cholesterol or gangliosides are relatively stable in mouse serum (Ho & Huang, unpublished data). In addition, preliminary studies in our laboratory indicate that target-sensitive, antibody stabilized PE immunoliposomes are also quite stable in human serum. Therefore, liposomes stable in serum can be designed by selecting appropriate lipid compositions. Another approach is to improve ILA sensitivity such that 10^3 to 10^6 fold diluted serological samples can still be used. Since dilution of serum will also dilute the interfering serum components, the serum interference to ILA can be minimized.

In closing, we like to consider the current status of ILA by comparing it with other established immunoassays. It is evident from table 1 that, at present, none of the ILA have been expanded to a commercial scale although their sensitivities are comparable with commercially used immunoassays. ILA for HSV requires a significantly shorter time than the conventional plaque assay and eliminates the washing processes. Similarly, for rheumatoid factor detection, the sensitivity of ILA is about ten-fold higher than the clinical latex-

agglutination assay. Thus, most ILA provides a better overall performance than the existing assays in addition to avoiding the use of radioisotopes. A lack of clinical ILA application to date has been partly due to the instability of reagents such as the complement components used in earlier designs. Recent advances in design (such as the one based on the target-sensitive immunoliposomes described in Chapter II) have removed some of the instability problems. With further improvement in sensitivity and stability, the commercial application of the ILA is realistic in the near future.

Target Sensitive Immunoliposomes as Drug Carriers

Although therapeutic application of the TS immunoliposomes was demonstrated in the previous chapter to be very effective *in vitro*, their application *in vivo* remains to be investigated. In order for liposomes with target sensitive design to be useful, such a liposome has to survive in the biological fluids. Both the integrity of membrane components (i.e. targeting molecule must remain associated with the lipid in order to retain their proper function), and the ability to retain the drug or molecules within the liposomes must be maintained. Our initial studies indicate that human serum does not induce significant leakage of contents from TS immunoliposome over a 20 min incubation time; however, their functional activity in serum remains to be explored. Our preliminary studies of TS immunoliposomes in diluted (1/20, v/v) serum showed that these liposomes are still target sensitive. Additional studies will be required to evaluate systematically the effect of serum or other biological fluids on liposome integrity.

An additional factor for consideration is the accessibility of the TS immunoliposome to the target organ of interest. One of the most popular routes of liposome administration is IV injection. It is however, a very ineffective route of administration due to the difficulty in transporting liposomes out of the vascular space because the vascular endothelial cells maintain by tight junctions. However, targeting in blood is possible at present, provided the liposome can reach its target before being removed by the reticuloendothelial system. The feasibility of this approach has recently been shown by encapsulating plasmid DNA in pH-sensitive liposomes bearing anti-$H2K^k$-IgG. Upon IP injection of these liposomes into nude mice bearing circulating RDM4 lymphoma cells, the liposomes bound specifically to the target cells, delivered the DNA that coded for a functional protein product (Wang and Huang, 1987). This study additionally showed that liposomes remained in the circulation for only a very brief period of time and were rapidly cleared away. Hence, IV injected liposomes have to be able to reach their target rapidly, and deliver their contents (in the case of water soluble drugs or molecules) before being cleared from the circulation.

At present no liposome design with the capability of escaping the endothelial lining of the vascular walls is available. However, as we begin to learn more about trafficking of monocytes (which require to cross in and out of the vascular endothelial lining), we may be able to design such liposome . Particularly, studies on the thymocytes trafficking indicated there are different sets of homing receptors for B and T lymphocytes on endothelial wall (Jalkanen et al., 1986;

Weissman, 1986). T lymphocytes bind and cross endothelial cells in the peripheral lymph nodes, while B lymphocytes dock and cross the endothelia of Peyer's patches (also known as high endothelial venules, HEV) (Stevens et al., 1982). It is not known whether the homing molecules themselves on these lymphocytes are sufficient to allow the crossing of the cells. Molecular processes required for trafficking of endothelial lining by other monocytes such as macrophages, is not known at present. Until elucidation of such trafficking processes, transport of particles greater than 50Å, such as a liposome, across the vascular wall is not feasible at present.

While the goal of extravascular liposome targeting may seem distant, the effectiveness of TS immunoliposomes in vitro as a drug carrier can be adapted immediately for topical applications. With proper modification and optimization, anti-viral drug encapsulated in TS immunoliposomes directed against HSV should be useful to treat cutaneous herpetic lesions as well as herpetic keratitis. Other types of topical treatment could also be designed accordingly.

Where Do We Go From Here?

We must study the stability of TS immunoliposomes in biological fluids. A wide range of naturally occurring or synthetic lipids capable of assuming a H_{II} phase must also be sought for use in ILA. Since successful modifications would be dependent on understanding the role of stabilization of the PE bilayer by membrane stabilizers, additional effort must be undertaken to study the principle underlying the molecular mechanism of stabilization and destabilization of TS

immunoliposomes. Also, the functional activity of the target in eliciting the liposome destabilization and release of encapsulated contents in the appropriate microenvironment must be evaluated for specific applications. As we can see, successful targeting of TS immunoliposomes requires not only liposome stability before reaching the target, but also the functional activity of the liposomes in the presence of serum proteins which can nonspecifically adsorb to the surface of liposomes. Additional effort must be placed on optimizing the ability of liposomes to reach target organ (and cells) in vivo following their administration. At present, even the smallest liposome of 200Å can not penetrate the tight junctions of the endothelial cell lining vessel walls; hence, liposome targeting via IV route to organs other than cells in blood circulation is not feasible. As we begin to understand the process of transcytosis of particles across endothelial cells [see Mostov and Simister (1985) for a review] and homing and transport of B and T lymphocytes across the endothelia walls (Jalkanen et al., 1986), we will be able to design liposomes that are capable of crossing endothelial barriers. The answers to these questions undoubtedly will have great impact on liposome targeting technology, particularly in designing liposomes capable of going in and out of blood circulation to encounter such things as infectious diseases as well as metastasizing tumors.

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