Target-Sensitive Immunoliposomes: Preparation and Characterization†

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ABSTRACT: 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-immunoglobulin G (pIgG) to stabilize the bilayer phase of the unsaturated dioleoylphosphatidylethanolamine (PE) which by itself does not form stable liposomes. A mouse monoclonal IgG antibody to the glycoprotein D of Herpes simplex virus (HSV) and PE were used in this study. A minimal coupling stoichiometry of 2.2 palmitic acids 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 1.00×10^{-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. Dioleoylphosphatidylcholine 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.

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 by using a variety of fusogens such as Sendai virus (Okada, 1962; Nakanishi et al., 1985), synexin (Hong et al., 1979), and poly(ethylene glycol) (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 a pH below 6.5 and fuse with endosomes, this approach was successful for cytoplasmic delivery to antitumor drugs (Connor & Huang, 1985). 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)[1] 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 report, we have developed 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 for stabilization of 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. PE and 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.

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[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; TS, target sensitive; PE, dioleoylphosphatidylethanolamine; PC, dioleoylphosphatidylcholine; NHSP, *N*-hydroxysuccinimide ester of palmitic acid; DOC, deoxycholate; CE, hexadecylcholestanyl ether; HSV, Herpes simplex virus; MOI, multiplicity of infection; FCS, fetal calf serum; pIgG, palmitoyl-immunoglobulin G; pfu, plaque-forming unit(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; gD, glycoprotein D; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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Antibody. Mouse monoclonal IgG_{2a} antibody 4.2 against Herpes simplex virus (HSV) antigen gD (hereafter called IgG) was isolated from mouse ascites fluid and was kindly provided by Dr. Steve Norley. The antibody was originally isolated by Dr. Melvin Trousdale and characterized by Lathey et al. (1986). 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 radiolabeled with ^{125}I using chloramine T (Hunter & Greenwood, 1962) to a specific activity of 1×10^3 to 5×10^5 cpm/μg.

Derivatization of IgG with Palmitic Acid. Coupling of the *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 [^3H]NHSP or NHSP in PBS such that the final deoxycholate (DOC) concentration was 2%. The coupling was performed at 37 °C for 12 h. Palmitic acid, the hydrolyzed product of NHSP, in the reaction mixture was separated from derivatized IgG with a 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 ^{125}I-pIgG. Briefly, 4×10^6 pfu of HSV in 50 μL of PBS, pH 7.6, was incubated at 4 °C overnight in an Immulon Removawell (Dynatech Lab Inc.). After being washed, the Removawells were incubated with 50 μL of 5% goat serum in PBS for 1 h to block the nonspecific binding sites. The goat serum was then removed and replaced with ^{125}I-pIgG (0.1–10 μg/mL) in 40 μL of PBS containing 0.15% DOC. The incubation of pIgG was performed at 4 °C for 1 h. After being washed with PBS, pH 7.4, 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, and they were within ±4%. The dissociation constants, K_d, were determined by Scatchard analysis (Scatchard, 1949) based on least-squares fitted lines with correlation coefficients greater than 0.94.

Distribution of Palmitic Acid on pIgG. [^3H]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 mixtures of Fab and Fc fragments were separated on 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 ^3H radioactivity (Nicoli et al., 1974). After normalization with respect to the amount of protein and the molecular weight of each band, the distribution of [^3H]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-labeled protein A and detection with radioautography.

Liposome Preparation. Routinely, PE or PC (1–4 μmol) and a trace amount of hexadecyl[^3H]cholestanyl ether (CE) (Pool et al., 1982) [final specific activity $(4–11) \times 10^{13}$ cpm/mol of 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 microliters of PBS containing varying amounts of derivatized IgG and 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 5-min cycles with an intervening 30-min rest period at room temperature. The liposome suspension was then chromatographed on a Bio-Gel A-0.5M column to remove the untrapped calcein as well as DOC. When [^3H]DOC was used in a separate experiment to determine the efficiency of DOC removal by A-0.5M column chromatography, we found that less than 0.1% of the total DOC remained in the liposome fractions, indicating that the final DOC to PE molar ratio was less than 5×10^{-4}. The liposomes, eluted with PBS, pH 8.0, in the void volume, were detected by ^3H radioactivity. These fractions were pooled and stored at 4 °C. 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} = 490 nm and λ_{em} = 520 nm. Total calcein fluorescence was determined by the addition of DOC to a final concentration of 0.12%. The percent quenching was calculated by using the formula:

$$\% \text{ quenching} = (1 - F_0/F_t) \times 100$$

where F_0 and F_t are the fluorescence of the liposome samples before and after the addition of DOC, respectively. We found 50 mM liposome-entrapped calcein gave approximately 70% fluorescence quenching.

Sucrose Density Gradient Centrifugation. Analytical centrifugation of linear 5–20% sucrose gradients was done as described previously (Huang et al., 1980). Briefly, 5 mL of a linear 5–20% sucrose gradient in PBS, pH 7.4, was overlayed on 0.5 mL of a 65% sucrose cushion. Liposome samples were loaded in 200 μL and centrifuged in an SW50.1 rotor at 200000*g* for 5 h at 4 °C. The gradients were fractionated from the bottom with a peristaltic pump.

Infecting L929 Cells with Viruses. L929 cells were grown as monolayer cultures in 35-mm six-well Linbro plates (Flow Inc.) using McCoy's medium containing 10% fetal calf serum (FCS). Cells were infected with HSV or Sendai virus with MOI = 10 in McCoy's with 2% FCS in 1 mL. After 1 h, the infection media was removed and replaced with normal culture media. Incubation was continued at 37 °C for 5 more h 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 35-mm plates were cooled to 4 °C and were incubated in 5% goat serum containing McCoy's medium for 1 h to block the nonspecific binding sites. Subsequently, immunoliposomes containing ^{125}I-pIgG and [^3H]CE in 1 mL were added at various concentrations, and incubation was continued for 2 h 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 2 mL of PBS. The cells were solubilized with 0.5 mL of 1% Triton X-100 at room temperature for 2 h, followed by an additional 0.5 mL of Triton wash. The solubilized cells were counted for both ^3H and ^{125}I radioactivity. Measurements were done in duplicate, and they were within ±3%. K_d values were determined from the slope of the Scatchard plot based on least-squares fitted lines with correlation coefficients of 0.95 for

Table I: 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.

^{3}H-lipid binding and 0.90 for ^{125}I-Ab binding, respectively.

Cell-Induced Lysis of Immunoliposomes. The infected and normal L929 cells were scraped with a rubber policeman and washed 3 times with PBS. Two microliters of liposomes with entrapped calcein was added to the Linbro Titertek plates containing 20 μL 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 2 mL 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

$$\% \text{ release} = [(F - F_0)/(F_t - F_0)] \times 100$$

where F_0 and F are the calcein fluorescence of the sample before and after the interaction with the cells, respectively. 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 ±10%.

Electron Microscopy. Immunoliposomes (0.42 μmol/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 (41 μM lipid) with entrapped calcein were added to PBS containing 1 mM EGTA, 1 mM 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 ±0.5 °C.

Results

Derivatization of Anti-HSV-gD-IgG with Palmitic Acid. We first examined the degree of palmitic acid coupling to 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 I, increasing the 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 pIgG, we used papain to cleave a pIgG preparation with a 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 32.5K and 26.5K were detected. They were identified in Western blots (Burnette, 1981) as Fc and Fab, respectively. By counting the radioactivity of [^{3}H]palmitic acid, the distribution of palmitic acid on Fab and Fc was determined to be approximately 30% and 70%, respectively.

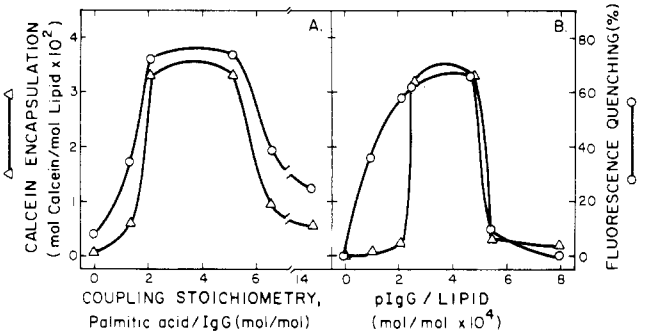

FIGURE 1: Stabilization of PE liposome bilayer by palmitoyl-anti-gD-IgG. Calcein encapsulation (Δ) and fluorescence quenching (O) were measured with various coupling stoichiometries, palmitic acid/IgG (A), or with various pIgG to PE ratios (B).

Stabilization of PE Liposome Bilayer by Palmitoyl Anti-HSV-gD-IgG. Formation of stable liposomes was monitored by their ability to encapsulate a 50 mM 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 50 mM calcein with various pIgG to PE ratios for each pIgG preparation. A wide range of coupling stoichiometries (0–14.6) was used. After being chromatographed to remove the untrapped dye, liposome formation was detected by analyzing the total amount of calcein encapsulated per mole of PE. To demonstrate the encapsulation of calcein, quenching of calcein fluorescence was also determined (Figure 1). As shown in Figure 1A, we found the optimal coupling stoichiometry of palmitic acid to IgG was 2.2–5.1, as evidenced by the highest amount of total calcein encapsulated per mole of lipid. In this particular experiment, the pIgG to lipid ratio was kept constant at 2.5×10^{-4}. Similar coupling stoichiometry optima was also found with a pIgG to lipid ratio of 4.7×10^{-4}.

We further analyzed these PE immunoliposomes by sucrose gradient centifugation. A typical gradient profile is shown in Figure 2. With a palmitic acid to IgG coupling stoichiometry of 2.2–5.1, practically no underivatized IgG was detected. In addition, the pIgG was all incorporated into the PE immunoliposome composed of 14000 pIgG/PE, 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 that the liposomes enriched with more pIgG migrated further into the gradient than the ones with fewer pIgG. For the gradient shown in Figure 2, the pIgG/PE ratio in the population (fractions 13–17) was calculated to be $(1.2–4.3) \times 10^{-4}$. 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 approxi-

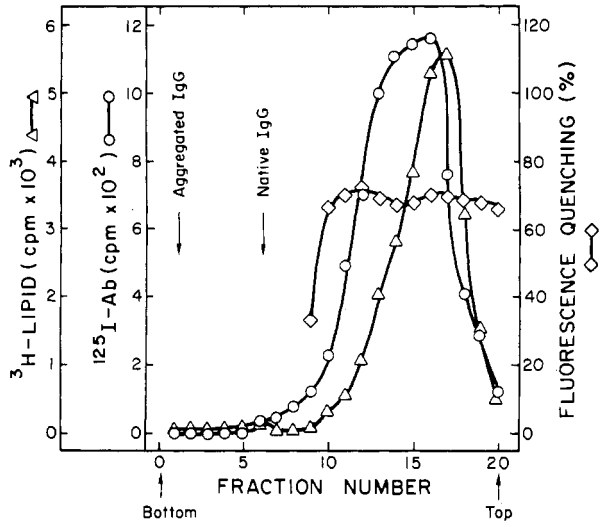

FIGURE 2: Sucrose gradient centrifugation analysis of antibody-stabilized PE liposomes. PE immunoliposomes were fractionated with 5–20% linear sucrose gradient centrifugation as described under Materials and Methods. Each collected fraction was analyzed for ^{3}H-lipid (△), ^{125}I-IgG (○), and fluorescence quenching (◇). The arrows indicate where self-aggregated pIgG and native IgG sediment.

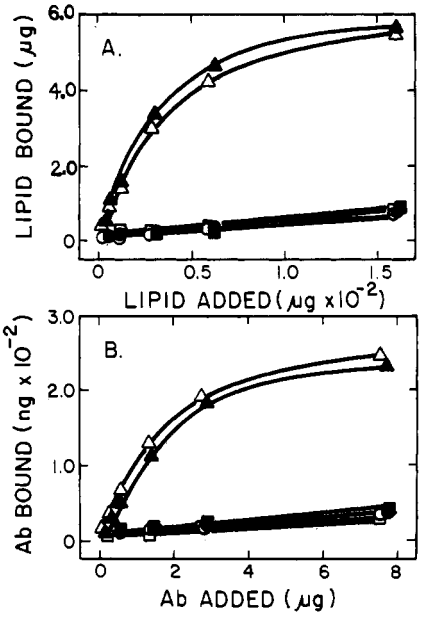

FIGURE 3: Binding immunoliposomes to L cells. Uninfected (○, ●) and HSV-infected (△, ▲, □, ■) L cells were incubated with varying amounts of PE liposomes (○, △, □) and PC liposomes (●, ▲, ■). The amount of lipid (A) and IgG (B) bound to the cell was plotted against the amount of lipid and IgG added in the form of immunoliposomes. Twenty micrograms of free anti-HSV-gD-IgG was used to block PE (□) and PC (■) immunoliposome binding to the HSV-infected L cells.

mately 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.

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 1B, the optimal pIgG to PE ratio was found to be (2.5–4.7) × 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 a pIgG to PE ratio less than 2.5 × 10^{-4}, a sharp decrease was detected in the calcein encapsulation without a 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. A pIgG to PE ratios greater than 4.7 × 10^{-4}, the excess pIgG molecules were self-aggregated as revealed by sucrose gradient centrifugation (data not shown).

Our results indicate that a minimum coupling stoichiometry of 2.2 palmitic acids per IgG was required for the pIgG to stabilize the PE liposomes (Figure 1A). 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 500 ± 130 Å 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 immunolipsomes, we infected the mouse fibroblast L929 cells with HSV and measured the binding of the immunoliposomes at 6-h postinfection 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, we detected the binding of immunoliposomes to the cells by counting ^{3}H and ^{125}I radioactivity. With increasing concentrations of liposomes added, increasing amounts of both PC and PE immunoliposomes were bound specifically to the HSV-infected cells (Figure 3). 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 3-fold excess of the highest concentration of liposome-associated pIgG used.

In addition, we analyzed the affinity of these immunoliposomes using Scatchard analysis (Scatchard, 1949). We found that the apparent dissociation constants, K_d, for pIgG and lipid were 1.00 × 10^{-8} and 4.02 × 10^{-5} M, respectively. The K_d of pIgG incorporated in the immunoliposomes (1.00 × 10^{-8} M) was very similar to the K_d of free pIgG (1.17 × 10^{-8} M, Table I), indicating that significant denaturation of the antibody did 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., 1984). By comparison of the apparent K_d values for pIgG and lipid, the pIgG to lipid ratio of the cell-bound liposomes was determined to be 1/4020 (mol/mol). 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 liposomes 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 4, 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

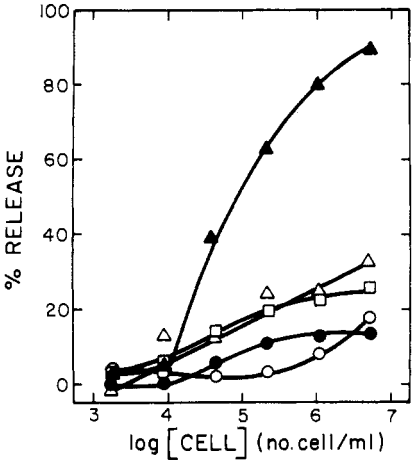

FIGURE 4: Cell-induced lysis of immunoliposomes. To uninfected (○, ●), HSV-infected (△, ▲, ○), or Sendai virus infected (□) L cells in suspension was added 0.58 nmol of calcein-encapsulted PE (△, ▲, ○, □) or PC (●) immunoliposome. To inhibit the lysis of PE immunoliposomes by HSV-infected L cells, 1.8 nmol of PC immunoliposomes containing no calcein was also added (△).

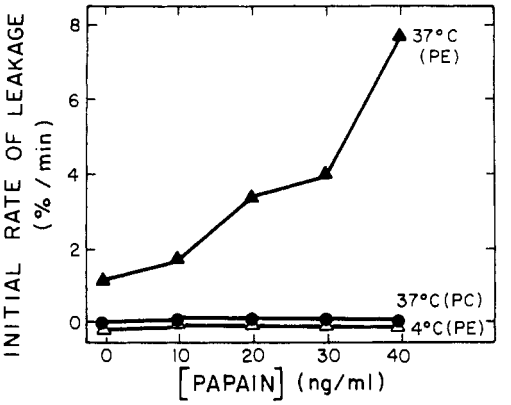

FIGURE 5: Papain-induced lysis of immunoliposomes. Various concentrations of papain were added to PE (△, ▲) and PC (●) immunoliposomes at 37 °C (▲, ●) and 4 °C (△), and the initial rate of calcein leakage was measured.

HSV-infected cell-induced calcein release could be blocked by incubating PE immunoliposomes with a 3-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 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 (Figure 3A,B). 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 was 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.

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 concentrations of papain added, an elevating rate of leakage up to 8% per minute at 37 °C was observed with PE immunoliposomes (Figure 5). 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. The 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)], with gangliosides such as GD_{1a} (Tsao & Huang, 1986), with fatty acids and derivatives such as oleic acid (Düzgünes et al., 1985; Huang & Liu, 1985), cholesteryl hemisuccinate (Ellens et al., 1984), and palmitoylhomocysteine (Connor et al., 1984), and finally with proteins such as glycophorin A (Taraschi et al., 1982; Ho & Huang, 1985). The present report 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 (Figure 4), 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 (Figure 6). 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 (Figure 5), clearly demonstrates that an intact Fab domain is essential for stabilization of PE bilayers. Since the hinge region is available for 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 I and Figure 3). Our model of stabilization is also consistent with the physical principles involved in membrane organization. According to the "hydration" theory (Marcvelja & Radic, 1976), PE, which has a relatively low head-group charge density, 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 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 a higher degree of hydration, due to the hydrophilic Fab portion at the bilayer surface, and the intrinsic molecular conformation of pIgG that gives the average

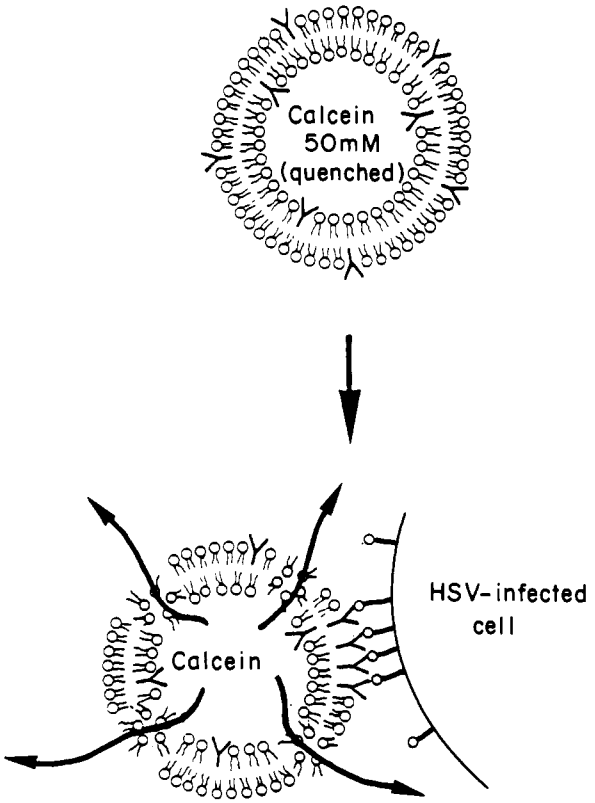

FIGURE 6: Schematic representation of the specific lysis of target-sensitive immunoliposomes. Note that the molecules are not drawn to scale.

dynamic shape of a truncated cone. In addition, membrane-bound pIgG may serve as a repulsion force against PE bilayer contact, thereby preventing the already formed PE bilayer from reverting to the hexagonal phase. It is also interesting to note that only five pIgG molecules were required to stabilize a 500-Å 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 provides a higher degree of hydration to the bilayer surface. Hence, the pIgG molecules 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 (Figure 4). 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/s (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 the dissociation constant of liposome-bound pIgG due to multiple immune complex formation (Table I and Figure 3), the possibility of multivalent binding cannot be ruled out at present. It is likely that appreciable multivalent binding is canceled by the steric factors which increase the dissociation constant for all the interactions between cells and membrane-bound pIgG. 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 conditions.

Regardless of the nature of multivalent binding, the resulting lateral phase separation may decrease the effective concentration of pIgG in the bulk lipid bilayer which destabilizes the PE immunoliposomes. 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 by collision either between adjacent liposomes bound to the same cell or between liposomes on separate cells. Although the kinetics of this process remain to be investigated, the overall destabilization rate of the TS liposomes is likely dependent on the 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 analogues such as fluorodeoxyuridine, iododeoxyuridine, acylclovir, 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). 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 analogues 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.

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