Articles

Stable Target-Sensitive Immunoliposomes†

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ABSTRACT: Interaction of immunoliposomes composed of dioleoylphosphatidylethanolamine (DOPE) (80%), dioleoylphosphatidic acid (DOPA) (20%), and a small amount of specific antibody with Herpes Simplex virus (HSV) were studied by detecting the immune-dependent lysis of liposomes. DOPA was used as the principal stabilizer of the immunoliposomes. Antibodies conjugated with *N*-glutarylphosphatidylethanolamine or oxidized GM_1 served as the target-specific ligands of immunoliposomes. These immunoliposomes (d = 160–180 nm) were stable for at least one month when stored at 4 °C. However, they undergo a rapid aggregation and lysis reaction in the presence of a membrane-bound target such as intact HSV virions. We have also employed epitope peptide-containing liposomes (target liposomes) to mimic the virus and showed that the immunoliposomes could be aggregated and lysed by the target liposomes in an antigen-dependent manner. Immunoliposome lysis could be accelerated by increasing the incubation temperature to 60–70 °C. No immunoliposome lysis was observed if the target liposomes were absent, indicating the prolonged stability of the immunoliposomes. Liposome lysis was always accompanied by liposome aggregation. However, the aggregation-induced liposome destabilization is unique to the H_{II} phase-forming lipids such as DOPE. DOPC-containing immunoliposomes did not lyse despite the fact that massive liposome aggregation had taken place.

The target-sensitive immunoliposomes have been developed in this laboratory to be a useful reagent for the targeted drug delivery and immunodiagnosis (Ho et al., 1987a,b). These liposomes composed of primarily an unsaturated PE such as DOPE[1] and PE prepared from the transphosphatidylated egg PC. Although the equilibrium phase of unsaturated PE at the physiological conditions is the inverted hexagonal (H_{II}) phase (Cullis & de Kruijff, 1979), target-sensitive immunoliposomes can be constructed by mixing PE with an appropriate amount of fatty acylated IgG (Huang et al., 1980; Ho et al., 1986). The acylated and hence amphipathic antibody molecules serve as a bilayer stabilizer for PE and, at the same time, endow the target specificity of the stabilized liposomes. Upon binding to a multivalent antigen such as a virion particle or a virus-infected cell, the immunoliposome undergoes a rapid and spontaneous destabilization and releases the entrapped contents (Ho et al., 1987b). Detailed kinetic and ultrastructural studies have revealed that the destabilization process involves membrane fusion and formation of the H_{II} phase (Ho et al., 1988).

Although the target-sensitive immunoliposomes are useful in many practical applications, we have found that the liposomes lack long-term stability for prolonged storage. This is because the acylated antibody is not a strong bilayer stabilizer. Furthermore, the stability of liposomes varies from one antibody to another, probably due to the difference in charge content of the antibody molecule. In order to overcome these problems, we have redesigned the immunoliposome by adding into the lipid composition a strong bilayer stabilizer which itself is not the targeting molecule. Amphipathic antibody is still present in the liposome formulation, but its principal role is for target binding and not as a stabilizer. We describe here the construction of the improved immunoliposomes and characterize the conditions which lead to the target-induced destabilization. We have used a monoclonal antibody against the glycoprotein gD of HSV (type 1) for the construction of immunoliposomes. Intact HSV virions and liposomes coated with the epitope peptide which the anti gD antibody recognizes are used as targets. These studies have shed light on the mechanism of bilayer destabilization of the newly designed target-sensitive immunoliposomes.

Materials and Methods

Materials. DOPE, DOPC, DOPA, and NGPE were purchased from Avanti Polar Lipids. Ganglioside GM_1 was purchased from Calbiochem.

Antibody Preparation. IgG_{2a} monoclonal antibody against HSV (type 1) antigen gD (clone 4.2) (hereafter called anti gD) was isolated from mouse ascites fluid. The antibody was originally isolated by Dr. Melvin Trousdale and characterized by Lathey et al. (1986). The IgG fraction was purified by protein A–Sepharose affinity chromatography followed by mono Q ion-exchange column. When necessary, antibody was radiolabeled with ^{125}I using iodobeads. Mouse IgG_{2a} monoclonal antibody (DX-4) against a peptide epitope of gp 120 of the human immunodeficiency virus was kindly provided by Dr. Dexi Liu of this lab. Rabbit polyclonal antibody against

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[1] Abbreviations: NGPE, *N*-glutarylphosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; HSV, Herpes Simplex virus; gD, glycoprotein D of HSV type 1; 23mer, first 23 N-terminal amino acids of gD; dp23mer; dipalmitoyl form of 23mer; PBS, phosphate-buffered saline; anti gD, monoclonal anti gD antibody (clone 4.2); EDTA, ethylenediaminetetraacetic acid.

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1 10
Lys-Tyr-Ala-Leu-Ala-Asp-Ala-Ser-Leu-Lys

11 20
Met-Ala-Asp-Pro-Asn-Arg-Phe-Arg-Gly-Lys

21
Asp-Leu-Pro

FIGURE 1: First 23 amino acids of HSV gD antigen.

the HSV (immune IgG) and the normal rabbit IgG were kindly provided by Dr. B. T. Rouse (University of Tennessee, Knoxville).

Virus. HSV (type 1, strain KOS) was isolated from infected Vero cells as described (Norley et al., 1986). The infected cell suspension in PBS was subjected to three cycles of freeze–thaw. The virus in the supernatant was subsequently isolated from cell debris by centrifugation at 1000*g* for 10 min. Uninfected cell supernatant was also isolated in a similar manner as a control. Sendai virus isolated from the infected allantoid fluid of chick embryos was also used as a control.

Peptide. A synthetic peptide which mimics the first 23 amino acids of gD (23mer) and also the dipalmitoyl form of the 23mer (dp23mer) were synthesized by the solid-phase synthesis method and were kindly provided by K. Byrnestad of the Department of Microbiology, University of Tennessee, Knoxville. The amino acid composition of the 23mer is given in Figure 1. It contains the epitope to which anti gD binds. The dp23mer contained two palmitoyl residues attached to the α- and ϵ-amino groups of the N-terminal lysine residue. Both 23mer and dp23mer carried radioactive label due to the incorporation of [^{3}H]Leu (specific activity 1.3×10^3 cpm/μg).

Derivatization of Antibody with NGPE. Antibody was conjugated to NGPE as follows: Routinely, 0.067 μmol of NGPE was dissolved in 100 μL of MES buffer (5 mM MES, 0.15 M NaCl, pH 5.5) containing 10 μL of 0.1 M octyl glucoside. After the addition of 20 μL each of 0.25 M 1-ethyl-3-(3′-(dimethylamino)propyl)carbodiimide and 0.1 M *N*-hydroxysulfosuccinimide, the resulting mixture was incubated at room temperature for 10 min. The pH of the mixture was then adjusted to 7.5, and 6.7 nmol of IgG containing trace amounts of ^{125}I-labeled IgG in 50 mM sodium borate buffer, pH 7.6, was added immediately. The reaction mixture was incubated at 4 °C for 8–12 h with frequent mixing. The resulting IgG–NGPE conjugate was then dialyzed overnight against PBS, pH 7.8, to remove octyl glucoside and other excess reagents. The reaction mixture turned slightly turbid after dialysis. However, there was no precipitation of antibody–NGPE conjugate.

Derivatization of Antibody with GM_1. Anti gD was conjugated to oxidized GM_1 by reductive amination in the presence of $NaBH_3CN$. Reaction was carried out in 10 mM sodium borate buffer containing 60 mM NaCl, pH 8.2. Routinely, antibody and oxidized GM_1 (molar ratio 10:1) in borate buffer were mixed with a 10^3-fold excess of $NaBH_3CN$. The reaction mixture was incubated at 37 °C for 8 h before the excess reagent was removed by dialysis. Oxidation of GM_1 was carried out in the presence of sodium periodate according to the method of Lenten and Ashwell (1971). The oxidized GM_1 was separated from the native GM_1 by preparative thin-layer chromatography using the solvent system $CHCl_3/CH_3OH$/0.2% $CaCl_2$ (5:4:1).

Immunoliposome Preparation. In routine experiments, 1.6 μmol of DOPE and 0.4 μmol of DOPA were mixed with a trace amount of hexadecyl [^{3}H]cholestanyl ether and dried under a stream of N_2 and vacuum desiccated for no less than 2 h. The lipid mixture was then hydrated with 200 μL of PBS containing 300 μg (0.1 mol %) of antibody–NGPE or antibody–GM_1 conjugates and 50 mM calcein. The mixture was incubated at 4 °C for 12–24 h with occasional mixing, followed by sonication in a bath sonicator for 5 min. One additional sonication was performed after a 6–8-h incubation at 4 °C. Unentrapped calcein was removed by chromatography on a Bio-Gel A 15M column. The fractions were measured for radioactivity and calcein fluorescence in the presence and absence of 0.15% deoxycholate or Triton X-100. Immunoliposomes eluted in the void volume fractions contained approximately 0.06 mol % antibody conjugate, and the calcein fluorescence quenching was about 75–80% (see below).

Target Liposome Preparation. Small unilamellar vesicles were prepared by a sonication method. Routinely, 1.8 μmol of DOPC was mixed with 0.2 μmol of dp23mer in $CHCl_3/CH_3OH$ (1:1) and the mixture was dried under a stream of N_2 and vacuum desiccated for no less than 2 h. The lipid mixture was then hydrated with 200 μL of PBS at room temperature with occasional mixing. The mixture was vortexed and subsequently sonicated in a bath sonicator for two 5-min cycles with an intervening rest period of 2–3 h. The resulting liposome suspension was fractionated using a Bio-Gel A 1.5M column to separate the unincorporated dp23mer. The DOPC/dp23mer liposomes (hereafter called target liposomes) eluted in the void volume fractions showed about 30% incorporation of the dp23mer.

Fluorescence Quenching Measurements. Fluorescence of liposomes was measured using a Perkin-Elmer LS5 spectrofluorometer with λ_{ex} = 490 nm and λ_{em} = 520 nm. Lipid concentration in the cuvette was 1 μM in PBS containing 1 mM EDTA. Percent fluorescence quenching was calculated from 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 0.15% Triton X-100, respectively.

Target-Induced Release of Calcein from Immunoliposomes. Immunoliposomes (300–500 μM) containing entrapped calcein were incubated at room temperature with HSV (2 mg/mL) for various time intervals (0–90 min). Alternatively, the same amount of immunoliposomes was incubated with the target liposomes (same concentration as the immunoliposomes) at room temperature for 5 min and then heated for 3 min at a different temperature. The reaction mixture was then diluted with 2 mL of PBS containing 1 mM EDTA for the fluorescence measurement as described above. The percentage of calcein release was calculated using the formula

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

where F_0 and F are the calcein fluorescence before and after the addition of the target, respectively, and F_t is the total fluorescence after addition of Triton X-100.

Light-Scattering Measurements of Liposome Aggregation. Liposome aggregation was measured by monitoring the light scattering at 90° using a Perkin-Elmer LS5 spectrofluorometer with $\lambda_{ex} = \lambda_{em}$ = 660 nm and a slit width of 5 nm. Immunoliposomes were mixed with the target liposomes in the presence or absence of free 23mer or anti gD. Light scattering was measured after 5 min of incubation at room temperature.

RESULTS

Antigen Binding Capacity of the Derivatized Antibodies. Enzyme-linked immunosorbent assay (ELISA) performed

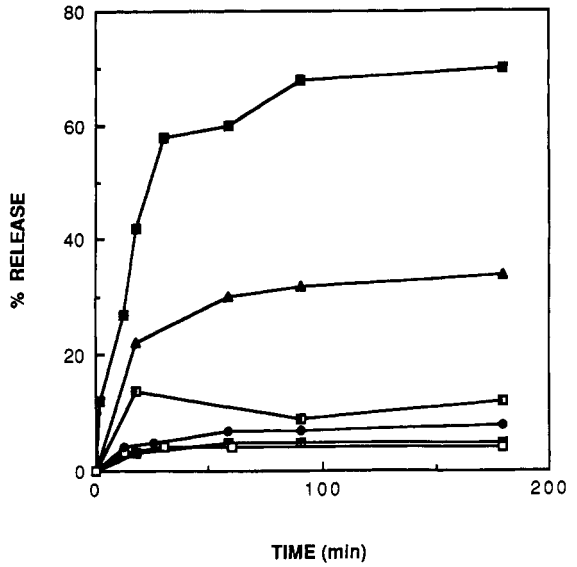

FIGURE 2: Virus-induced lysis of the stabilized DOPE/DOPA immunoliposomes. Immunoliposomes (500 μM) bearing anti gD–NGPE (■, ▲, ⊞, ◧) or anti-gD GM_1 (●) were incubated with Herpes Simplex virus (2 mg of viral protein/mL) in the presence (▲) or absence of excess anti gD (■, ●), Sendai virus (◧) or uninfected Vero cell supernatant (⊞). At various time intervals, the sample was diluted in PBS/EDTA buffer and the percent of dye release was determined. Control liposomes containing DOPE/DOPA but no antibody were also tested for lysis by HSV (□).

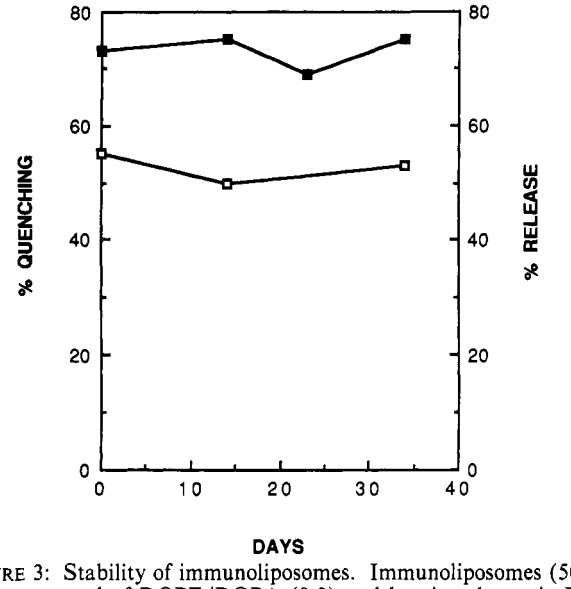

FIGURE 3: Stability of immunoliposomes. Immunoliposomes (500 μM) composed of DOPE/DOPA (8:2) and bearing the anti gD–NGPE were stored at 4 °C for the indicated period of time. The percent of fluorescence quenching of the entrapped calcein (■) and the HSV-induced lysis (□) were determined at different times.

using virus-coated plates and derivatized antibodies showed similar binding patterns to that of the underivatized antibody (data not shown). Furthermore, if the derivatized antibody was preincubated with excess HSV before incubation with the virus-coated plates, there was a 90% inhibition of binding. This result confirms that the derivatized antibodies (either with NGPE or GM_1) maintained their specificity for target antigen.

Virus-Induced Immunoliposome Lysis. Immunoliposomes composed of DOPE/DOPA (8:2) and either anti gD–NGPE or anti gD–GM_1 conjugate were incubated at room temperature with HSV. Figure 2 shows the lysis of immunoliposomes in the presence of virus as observed by the release of the entrapped fluorescent marker calcein. Although immunoliposomes bearing anti gD–NGPE could be lysed by incubation with virus in a time-dependent manner, immunoliposomes containing anti gD–GM_1 were not lysable up to 3 h of incubation time. Control liposomes of the same lipid composition but containing no antibody were also not lysable by HSV. The lysis of immunoliposomes containing anti gD–NGPE could be partially inhibited by the addition of excess (100-fold more than the liposomal antibody) free antibody. Liposome lysis appeared to be specific because Sendai virus at the same protein concentration induced little calcein release and because the supernatant of uninfected Vero cell lysate also did not lyse the immunoliposomes (Figure 2).

Immunoliposome Stability. The immunoliposomes composed of DOPE/DOPA (8:2) and containing anti gD–NGPE could be stored at 4 °C in PBS for a prolonged period of time (Figure 3). The percent of fluorescence quenching of the entrapped calcein stayed about 75%, indicating no leakage of calcein during this time period. Furthermore, immunoliposomes could still be lysed by HSV even after 34 days of storage. The average liposome diameter also stayed approximately the same (160–180 nm). These results indicate that the immunoliposomes bearing anti gD–NGPE are stable over a prolonged period of time.

Immunoliposomes Bearing Polyclonal anti HSV Antibody. Previous studies of the target-sensitive immunoliposomes have used monoclonal antibodies as a recognition ligand (Ho et al., 1987a,b). One of the unanswered questions is whether polyclonal antibody can also be used to construct these liposomes. A purified IgG fraction from the serum of a rabbit immunized with HSV was derivatized with NGPE, and the conjugate was used for the construction of the target-sensitive immunoliposomes. It should be noted that the principal antibody activity of the immune IgG is against the gD antigen of HSV (B. T. Rouse, personal communication). Table I shows the data of an experiment in which the virus-induced lysis of these immunoliposomes is compared with that of the immunoliposomes containing monoclonal anti gD–NGPE. As can be seen from the column of "specific release", immunoliposomes containing polyclonal antibody could be lysed by HSV to a similar extent as immunoliposomes containing monoclonal antibody. Control immunoliposomes containing either a polyclonal normal IgG or an irrelevant monoclonal antibody (DX-4) were not lysed by the virus. Furthermore, the lysis of the immunoliposomes

Table I: Virus-Induced Lysis of Immunoliposomes[a]

antibody attached to immunoliposome[b]	pretreatment	% release	% specific release[c]
none	none	19.0 ± 4	0
normal IgG	none	28.0 ± 4	8.0 ± 3
	anti gD	26.8 ± 3	7.2 ± 2
	23mer	28.6 ± 5	9.5 ± 3
immune IgG	none	68.2 ± 6	47.0 ± 6
	anti gD	41.8 ± 4	21.0 ± 4
	23mer	60.3 ± 3	40.0 ± 4
anti gD	none	73.6 ± 3	54.2 ± 5
	anti gD	39.3 ± 4	20.0 ± 3
	23mer	41.5 ± 3	22.6 ± 3
DX-4	none	28.0 ± 4	7.0 ± 3
	anti gD	28.6 ± 3	8.0 ± 2
	23mer	25.8 ± 4	6.5 ± 3

[a] Immunoliposomes were incubated with the virus in the presence or absence of 23mer or excess anti gD for 5 min at room temperature and then heated to 65 °C for an additional 3 min. The percent of calcein release is shown as mean ± sd (n = 5). [b] Immunoliposomes were prepared by the sonication method. The lipid composition was DOPE/DOPA (8:2) with 0.1 mol % antibody–NGPE. Both normal IgG and immune IgG were rabbit polyclonal IgG. Anti gD and DX-4 were mouse monoclonal IgG. [c] The percent of release was corrected for the nonspecific lysis of the antibody-free liposomes by the virus.

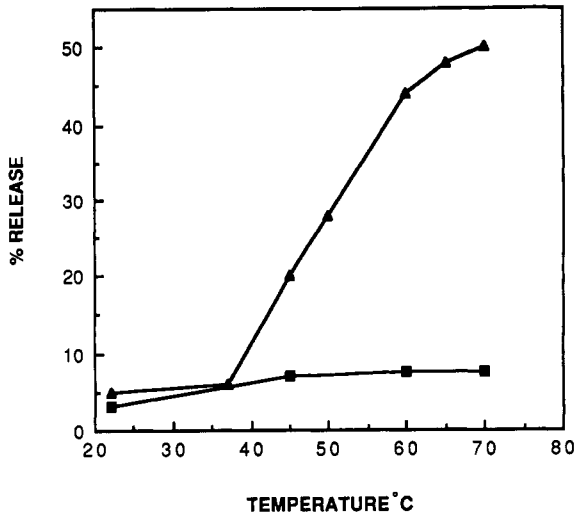

FIGURE 4: Effect of incubation temperature on the calcein release from immunoliposomes. Target liposomes (5 mol % dp23mer in DOPC) were mixed with immunoliposomes bearing anti gD–NGPE (▲) or DX-4–NGPE (■) at room temperature for 5 min. The resulting reaction mixture was heated to different temperatures for 3 min.

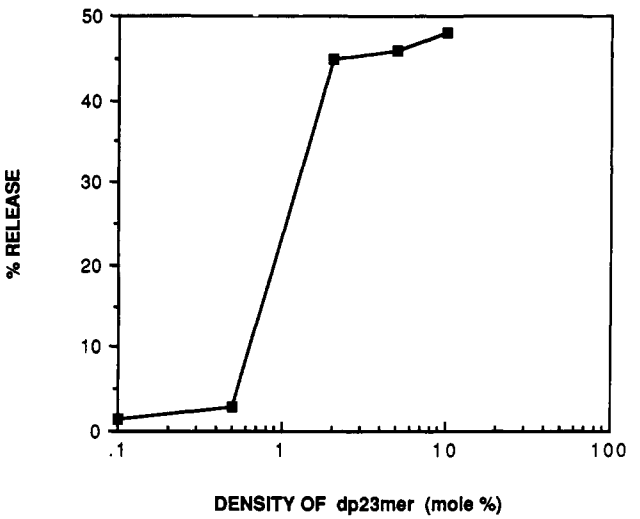

FIGURE 5: Effect of epitope density of the target liposomes on the immunoliposome lysis. Sonicated liposomes containing various amounts of dp23mer peptide were incubated with immunoliposomes containing anti gD–NGPE at room temperature for 5 min followed by incubation at 65 °C for 3 min.

containing specific antibody, either polyclonal or monoclonal, could be partially inhibited by free monoclonal anti gD or by a synthetic peptide (23mer) which contains the epitope of the monoclonal anti gD, indicating the immunospecific nature of the liposome lysis.

Temperature Dependence of the Immunoliposome Lysis. The experiment described in Table I was done by mixing immunoliposomes with HSV at room temperature for 5 min, followed by an incubation at 65 °C for 3 min. Aggregation of the liposomes and virus was instantaneous, and upon heating at 65 °C the aggregated liposomes lysed and released the entrapped calcein within a short time period. Although significant dye release was observed for immunoliposomes containing specific antibody, it was clear that liposomes containing no antibody also released a smaller amount of the entrapped dye (Table I). Such a nonspecific lysis of liposomes is probably caused by the thermally denatured viral proteins. Thus, although the incubation at an elevated temperature could greatly accelerate the speed of immunoliposome lysis, nonspecific lysis of the immunoliposomes presents a relatively high background for the measurement.

In order to study the temperature dependence of the target-induced lysis of immunoliposomes, we have decided to use a model membrane vesicle as a target. The 23mer which contains the anti gD epitope was incorporated by a simple sonication protocol into DOPC vesicles via the two palmitic acid chains attached to the N-terminal lysine residue. The dp23mer vesicles (target liposomes) are approximately 170–200 nm in diameter and can be used as a target for lysing the immunoliposomes (Figure 4). Furthermore, the DOPE/DOPA (8:2) liposomes containing control antibody (DX-4) did not lyse when they were incubated with the target liposomes even at 65 °C (Figure 4), probably because of the thermal stability of the peptide. Figure 4 also shows the temperature dependence of the immunoliposome lysis induced by the target liposomes. A significant level of lysis for a short 3-min incubation did not occur until the incubation temperature was above 40 °C. If the data in Figure 4 are plotted in an Arrhenius plot (log of release vs reciprocal temperatures in Kelvin), a straight line with a correlation coefficient of 0.97 was obtained (not shown). The activation energy of the lytic process was calculated from the slope of the line and had a value of 13 kcal/mol.

Table II: Target Liposome-Induced Aggregation and Lysis of Immunoliposomes[a]

target	pretreatment	% aggregation[b]	% lysis[c]
buffer	none	50	12 ± 6
23mer	none	52	11 ± 5
DOPC liposome	none	100	10 ± 5
dp23mer liposome[d]	none	250	50 ± 8
dp23mer liposome	23mer	115	15 ± 6
dp23mer liposome	anti gD	125	18 ± 4
dp23mer liposome[e]	none	260	16 ± 6

[a] The immunoliposomes contained DOPE/DOPA (8:2) and 0.1 mol % anti gD–NGPE. [b] Liposome aggregation was measured by light scattering. The percent of aggregation = (light scattering of the sample/light scattering of DOPC liposomes) × 100. [c] Immunoliposomes were incubated with the target for 5 min at room temperature and then heated to 65 °C for 3 min. The percent of lysis was then measured and shown as mean ± sd (n = 5). [d] Liposomes consisted of DOPC and 5 mol % dp23mer. [e] The immunoliposomes used in this case contained DOPC and 0.1 mol % anti gD–NGPE.

Epitope Density Dependence of the Target-Induced Immunoliposome Lysis. Using the dp23mer vesicles as a model target, we could address the question of how does the lysis of immunoliposomes depend on the epitope density of the target. Target liposomes containing various amounts of dp23mer were prepared and used as a target for the immunoliposomes containing anti gD–NGPE. Target liposomes and immunoliposomes were mixed and incubated for 5 min at room temperature before heating to 65 °C for 3 min. Data in Figure 5 indicate that there is a threshold epitope density of approximately 1 mol % of dp23mer below which no target-induced immunoliposome lysis was observed. Above 1 mol %, maximum level of liposome lysis occurred.

Aggregation of Immunoliposomes Induced by the Target. The target-induced immunoliposome lysis was generally accompanied by liposome aggregation. This was observed visually as an increase of liposome turbidity. To quantitate the degree of liposome aggregation, we have measured 90°-light scattering and correlated to the degree of target-induced immunoliposome lysis (Table II). Immunoliposomes containing anti gD–NGPE were mixed with dp23mer target liposomes and incubated for 5 min at room temperature. An aliquot was diluted with PBS for the measurement of 90°-light scattering, with the remainder being heated at 65 °C for 3 min and measured for dye release. Since the target liposomes them-

selves contributed to the light scattering, the target-induced increase in light scattering was compared to the light scattering of the sample in which sonicated DOPC vesicles without dp23mer was added. As can be seen from Table II, a large increase in light scattering was observed for the sample containing a mixture of immunoliposomes and dp23mer target liposomes. The increase in liposome aggregation was accompanied by a significant dye release from the immunoliposomes. Both aggregation and dye release were inhibited by the presence of excess free 23mer which bound to the anti gD on the immunoliposomes. Free anti gD antibody had a similar inhibitory effect. Free 23mer peptide did not induce either immunoliposome aggregation or release of the entrapped dye. Interestingly, immunoliposomes composed of DOPC, instead of DOPE/DOPA (8:2), aggregated well with the target liposomes, but there was no significant amount of dye release from these immunoliposomes. These observations indicate that immunoliposome lysis is always accompanied by liposome aggregation, but liposome aggregation does not necessarily lead to liposome lysis.

Discussion

We describe here the construction and lysis of immunoliposomes composed of DOPE and DOPA in the presence of target antigen on the viral membrane or on the liposome surface. One of the major differences between the immunoliposomes used here and the liposomes employed by Ho et al. (1986) is that the new liposomes are very stable upon storage (Figure 3). The control liposomes containing no antibody were also stable for more than 3 months as evidenced by a high degree of fluorescence quenching of the entrapped calcein (data not shown). Negatively charged lipids, such as DOPA, are excellent bilayer stabilizers for DOPE (Collins et al., 1989) but not the amphipathic antibody (Ho et al., 1986; Huang & Kennel, 1979). The present design of the target-sensitive immunoliposome takes advantage of using a separate strong stabilizer which greatly improves liposome stability. Antibody is present on the liposomal membrane merely for the purpose of specific recognition of the target, and not as a stabilizer of the DOPE bilayer.

When virus was added to immunoliposomes at room temperature, there was a time-dependent lysis of immunoliposomes as observed by the release of entrapped calcein (Figure 2). The stabilization of immunoliposome was immunospecific as it was inhibitable by free antibody and as it did not occur with a nontarget antigen, i.e., the Sendai virus. It is interesting to note that the anchor of the derivatized antibody seems to play an important role. Anti gD–GM_1 immunoliposomes were not lysable compared to the anti gD–NGPE immunoliposomes. GM_1 itself is a good stabilizer of DOPE in the bilayer phase due to the presence of the negatively charged sialic acid residues (Pinnaduwage & Huang, 1988). The antibody–GM_1 conjugate may exert more bilayer stabilization activity to the DOPE liposomes such that the resulting immunoliposomes are too stable to be lysed when they bind to the target virus.

The 23mer peptide-containing liposomes were useful as the target liposomes because we could alter several experimental parameters of the target antigen on the liposome membrane. Figure 5 shows that the lysis of immunoliposomes increases with increasing surface density of the peptide on the liposome surface. We have varied the number of peptide molecules on the liposome membrane from 0 to 6600 molecules per vesicle (0–10 mol %). No lysis occurred at low density of antigen (dp23mer) on the liposome (0–300 molecules/vesicle). Saturation was observed around 1300 molecules per vesicle. These data suggest that multivalent binding between the liposomal bound antibody and the gD antigen (or the 23mer peptide) on either the virus envelope membrane or the liposome surface is necessary for destabilization. A threshold phenomenon such as the one shown in Figure 5 has been reported for other multivalent binding systems. For example, binding of antigen-expressing cells to a glass surface containing immobilized antibody molecules requires an above-the-threshold surface density of the antibody (Huang, 1985). This is because the stable multivalent binding can be established only when a sufficient number of the antigen/antibody bonds are formed to overcome the repulsive force between the two surfaces (Bell et al., 1984). That a multivalent binding between immunoliposomes and target liposomes is required for the destabilization is also supported by the fact that soluble 23mer peptide alone could not induce the destabilization of immunoliposomes (Table II).

One of the consequences of multivalent binding between two populations of particles is the aggregation of particles. Aggregation of immunoliposomes with target liposomes correlated very well with the lysis of immunoliposomes (Table II). Conditions (excess free antigen or free antibody) which inhibited liposome aggregation also led to low levels of immunoliposome lysis, indicating that liposome aggregation is a necessary step for the liposome lysis. This is not surprising in view of the observations that bilayer-to-H_{II} phase transition of PE requires a close bilayer contact between the aggregated liposomes (Ellens et al., 1984; Siegel, 1984). We have demonstrated that the rate of bilayer destabilization induced by wheat germ agglutinin of DOPE liposomes stabilized with glycophorin A is linearly proportional to the square of the liposome concentration, indicating a second-order reaction kinetics (Hu et al., 1986). In the work of Ho et al., the target-induced destabilization of immunoliposomes stabilized by palmitoyl antibody was also found to be dependent on the contact between the neighboring liposomes bound to the same target membrane (Ho et al., 1986). A similar situation must exist in the present immunoliposome system. This proposed mechanism of liposome destabilization is further supported by the observation that immunoliposome lysis did not occur with liposomes composed of DOPC, despite the fact that massive liposome aggregation had taken place (Table II). Contact-dependent bilayer destabilization is unique to the H_{II} phase forming lipids such as DOPE (Ho et al., 1986; Pinnaduwage & Huang, 1988; Hu et al., 1986). PC bilayers do not undergo a bilayer-to-nonbilayer transition even with close apposition to each other. This is because the interfacial repulsive hydration force of a PC bilayer is much greater than that of a PE bilayer (Sen & Hui, 1988; Cevc & March, 1985; Newman & Huang, 1975).

It was possible to study the temperature dependence of immunoliposome lysis by using a model target membrane (Figure 4). In the absence of a specific target, immunoliposomes are very stable even when they are incubated at elevated temperatures. This is quite impressive in view of the fact that the incubation temperature could be as high as 60 °C above the bilayer-to-H_{II} phase transition temperature (10–12 °C) of pure DOPE (Allen et al., 1990; Gruner et al., 1985). However, the immunoliposomes undergo a rapid destabilization reaction at the elevated temperature if they are aggregated in the presence of target membrane. The activation energy of the process is estimated to be 13 kcal/mol. Such a relatively large activation energy has made it unlikely that the bilayer-to-H_{II} phase transition, i.e., the final step of the destabilization process, is the rate-limiting step. This is because the bilayer-to-H_{II} phase transition of DOPE only involves a small

amount of enthalpy change (approximately 290 ± 40 cal/mol for pure PE) (Epand, 1985). Although the activation energy is not always proportional to the equilibrium energy changes, the rate-limiting step is likely to be the close apposition of immunoliposomes which has to overcome the large repulsive hydration force between the liposomes (approximately 10–100 kcal/mol for pure PE) (Rand & Parsegian, 1989).

In summary, we have improved the stability of the target-sensitive immunoliposomes by adding a strong bilayer stabilizer to the lipid composition. The resulting immunoliposomes can be stored for a prolonged period of time without loss of the entrapped calcein or the ability to be lysed by the target. Target-induced lysis can be accelerated by incubation at an elevated temperature. This improved design of the target-sensitive immunoliposomes should find its useful applications in the target-specific drug delivery and in immunodiagnosis.

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