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Effect of Covalent Attachment of Immunoglobulin Fragments on Liposomal Integrity†

Reinhard Bredehorst,*,‡ Frances S. Ligler,§ Anne W. Kusterbeck,‡,∥ Eddie L. Chang,∥ Bruce P. Gaber,∥ and Carl-Wilhelm Vogel‡

Departments of Biochemistry and Medicine and Vincent T. Lombardi Cancer Center, Georgetown University, Washington, D.C. 20007, Geo-Centers, Inc., Suitland, Maryland 20746, and Bio/Molecular Engineering Branch, Naval Research Laboratory, Washington, D.C. 20375

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ABSTRACT: Liposome stability during and after covalent coupling of Fab′ antibody fragments was investigated. Large unilamellar vesicles containing entrapped 5(6)-carboxyfluorescein (CF) as a marker for liposomal integrity were prepared by extrusion through polycarbonate membranes. *N*-[4-(*p*-Maleimidophenyl)-butyryl]phosphatidylethanolamine (MPB-PE) was employed as a liposomal anchor for the covalent coupling of Fab′ fragments. We observed that coupling of Fab′ fragments to liposomes containing 5 mol % MPB-PE caused a concentration-dependent increase in size and polydispersity of the liposomes. Dependent on the concentration of the MPB-PE anchor in the membrane and the concentration of Fab′ added, coupling was associated with the release of up to 95% of the entrapped CF. Rupture of the liposomes was identified as the primary mechanism of CF release during Fab′ coupling. Reduction of the MPB-PE concentration to 1 mol % resulted in liposomes that were stable during and after Fab′ coupling. The increased stability of these liposomes was due to the lower MPB-PE concentration and not to the lower number of attached Fab′ fragments. By proper adjustment of the experimental conditions for coupling, the number of Fab′ fragments attached to the 1 mol % MPB-PE liposomes could be increased without affecting the stability of the resulting liposomes. These stable liposomes, made by an extrusion method that avoids the use of organic solvents, detergents, or sonication, are therefore suitable for entrapment of labile compounds and can be used for immunotargeting or immunoassays.

Synthetic phospholipid bilayer vesicles (liposomes) with covalently attached proteins on their surface have been prepared by a number of investigators for a variety of purposes. Examples are liposomes coated with antibodies or their fragments for immunotargeting of entrapped drugs to cells [reviewed by Gregoriadis (1984)] or coated with protein antigens for use in liposome-based immunolytic assays (Ishimori et al., 1984; Bredehorst et al., 1985). Various coupling procedures have been reported with detailed analyses of coupling efficiency, preservation of antibody activity, and stability of the cross-link between antibody and liposome [reviewed by Gregoriadis (1984)]. However, very few studies have investigated the effect of covalent attachment of protein molecules on liposomes during and after the coupling reaction.

For this study, we have prepared large unilamellar liposomes under conditions that permit entrapment of molecules that are labile in the presence of organic solvents and detergents. As an anchor for the covalent attachment of Fab′ fragments through their free sulfhydryl group, these liposomes contained *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE)[1] (Martin & Papahadjopoulos, 1982). We have

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* Address correspondence to this author at the Department of Biochemistry.
‡ Georgetown University.
§ Geo-Centers, Inc.
∥ Naval Research Laboratory.

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studied the effect of the MPB-PE concentration in the liposomes and of the Fab′ concentration during the coupling reaction on liposomal integrity, size, and polydispersity. We report here that as Fab′ and MPB-PE concentration increases, liposomal integrity decreases, resulting in substantial release of entrapped 5(6)-carboxyfluorescein (CF) during the coupling reaction. Liposomes containing ≤2.5 mol % MPB-PE allowed coupling of high amounts of Fab′ fragments without impairing the liposomal integrity. Such liposomes remained stable for months. They are useful for immunotargeting of entrapped compounds or in immunoassays as carriers of reporter molecules.

Materials and Methods

Synthesis of MPB-PE. MPB-PE was prepared from succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) (Pierce, Rockford, IL) and dimyristoylphosphatidylethanolamine (DMPE) (Avanti Polar Lipids, Birmingham, AL) as described by Martin and Papahadjopoulos (1982).

Preparation of Fab′ Fragments. Fab′ fragments were generated from nonimmune sheep $F(ab')_2$ fragments (Cappel Laboratories, Malvern, PA). $F(ab')_2$ (10 mg) in 1.0 mL of 100 mM NaCl, 35 mM sodium phosphate, 20 mM citric acid, and 1 mM EDTA, pH 5.0, was reduced with 20 mM (final concentration) dithiothreitol (DTT) for 90 min at room temperature under nitrogen atmosphere. DTT was removed by gel chromatography on Sephadex G-25 (Pharmacia, Piscataway, NJ) preequilibrated with deoxygenated 100 mM NaCl, 35 mM sodium phosphate, 20 mM citric acid, and 1 mM EDTA, pH 6.5 (buffer A). Fab′ fragments appearing in the void volume were used immediately for covalent attachment to preformed liposomes.

Radiolabeling of Immunoglobulin Fragments. $F(ab')_2$ fragments were radiolabeled with $Na^{125}I$ (Amersham, Arlington Heights, IL) by use of immobilized chloramine T (Iodo-Beads, Pierce). Stock solutions of radiolabeled $F(ab')_2$ had a specific radioactivity of $(0.5–2) \times 10^6$ cpm/μg. Radiolabeled Fab′ fragments were prepared from ^{125}I-labeled $F(ab')_2$ fragments as described above.

Preparation of Large Unilamellar MPB-PE Liposomes. The extrusion procedure described by Olson et al. (1979) was applied with minor modifications to prepare large unilamellar liposomes. Phospholipid mixtures composed of dimyristoylphosphatidylcholine (DMPC) (Avanti Polar Lipids), cholesterol (Avanti Polar Lipids), dicetyl phosphate (DCP) (Sigma, St. Louis, MO), and varying quantities of MPB–PE (molar ratio 5:4:1:0.1–0.5) were dried to a thin film by using a rotary evaporator, and the residual solvent was removed overnight in a vacuum chamber at room temperature. The lipids were resuspended in 20 mM Tris, 150 mM NaCl, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$, pH 8.0 (buffer B), containing 60–100 mM CF (Sigma) by gentle agitation. The resulting multilamellar liposome mixture was then extruded by argon pressure through polycarbonate membranes (Nucleopore, Pleasanton, CA) with decreasing pore sizes (0.6-, 0.6-, 0.4-, 0.4-, 0.2-, and 0.2-μm pore size). Vesicles formed by this procedure had a lamellarity of 1.5 as determined by the method of Nordlund et al. (1982). Nonencapsulated CF was removed by gel chromatography on Sephadex G-50 (Pharmacia). The liposomes were used immediately for coupling of Fab′ fragments.

Coupling of Fab′ Fragments to MPB-PE Liposomes. Freshly prepared liposomes in buffer A (final concentration 0.3–5.0 μmol of lipid/mL) were mixed with freshly reduced Fab′ fragments in buffer A (final concentration 0.03–2.0 mg/mL) and incubated at room temperature with stirring under a stream of argon. Liposomes were separated from unconjugated Fab′ fragments by column chromatography at 4 °C on Sepharose 4B or Sephadex G-150 (Pharmacia) preequilibrated with buffer A. The number of Fab′ fragments bound per liposome was calculated from the specific activity of ^{125}I-labeled Fab′ or determined by the procedure of Lowry et al. (1951) in the presence of sodium deoxycholate (Sigma) as described by Heath et al. (1981). The number of Fab′ molecules per liposome was calculated by assuming a molecular weight of 50 000 for each Fab′ monomer and 2.4×10^{-18} mol of lipid per liposome (calculated for unilamellar liposomes with a diameter of 2500 Å). When ^{125}I-labeled Fab′ fragments were used for coupling, background radioactivity was determined by control experiments with corresponding amounts of ^{125}I-labeled $F(ab')_2$ fragments.

Size Determination of Liposomes by Photon Correlation Spectroscopy. The procedure and instrumentation used were as described by Chang et al. (1982) with slight modifications. A Spectra-Physics He–Ne laser (Santa Clara, CA) was used as the coherent light source. The beam was focused down to 50 μm inside the center of a rectangular cuvette containing the vesicle suspension. The light scattered at 90° was collected onto a photomultiplier tube and fed into a Langley–Ford CM-64 correlator (Amherst, MA). The average size of the liposomes was determined by the cumulant method (Koppel, 1972), which also gave a polydispersity measure defined as the ratio of the half-width at half-height to the average value of the Gaussian size distribution.

Fluorescence Measurements. For fluorescence measurements 20 μL of liposomes (160 nmol of lipid) was diluted into 1.5 mL of buffer B. CF was excited at 490 nm and emission was read at 518 nm (Spex Fluorolog fluorometer, Metuchen, NJ). The total liposome-encapsulated CF was determined by lysing the liposomes with 2% (w/v, final concentration) CHAPSO (Sigma). The self-quench (Q) of liposome-entrapped CF was calculated from the equation

$$Q = \frac{(1 - \text{fluorescence of intact liposomes}) \times 100}{(\text{fluorescence of lysed liposomes})}$$

The fluorescence of lysed liposomes was corrected by 2% for addition of CHAPSO. The fluorescence of CF was found not to be affected by the presence of Fab′ fragments up to concentrations of 2 mg/mL.

Other Methods. Phospholipid concentrations were determined according to the procedure of Bartlett (1959). Protein was measured by the method of Lowry et al. (1951) or by the method of Bradford (1976). The maleimide content of MPB-PE was estimated by reaction with excess 2-mercaptoethanol and subsequent titration with 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) (Abbott & Schachter, 1976).

Results

Effect of Fab′ Coupling on Liposomal Size and Integrity. We prepared unilamellar liposomes consisting of DMPC, cholesterol, DCP, and the sulfhydryl-reactive phospholipid derivative MPB-PE (molar ratio 5:4:1:0.5) by sequential extrusion of multilamellar liposomes through polycarbonate membranes. The liposomes contained entrapped CF as a fluorescent marker for liposomal integrity. The liposome

[1] Abbreviations: CF, 5(6)-carboxyfluorescein; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DCP, dicetyl phosphate; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DTT, dithiothreitol; MPB-PE, *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

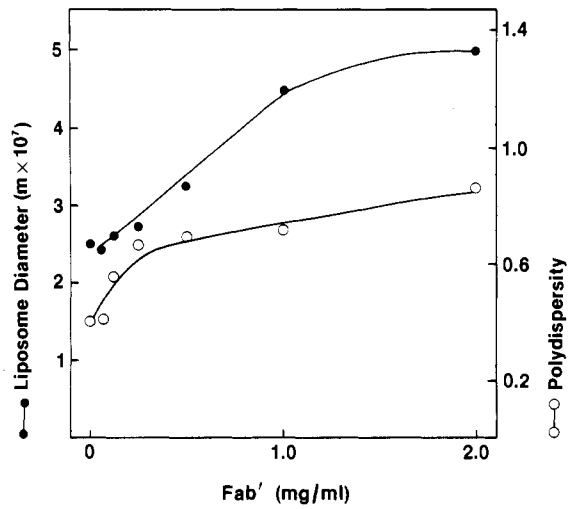

FIGURE 1: Effect of coupling of Fab' fragments on size and polydispersity of liposomes. Liposomes containing 5 mol % MPB-PE were incubated with increasing amounts of Fab' fragments at a total lipid concentration of 1.5 μmol of lipid/mL. After 20 h at room temperature, free Fab' fragments were removed by gel filtration on Sepharose 4B, and the size (●) and polydispersity (○) of the Fab'-coated liposomes were determined by using photon correlation spectroscopy as described under Materials and Methods.

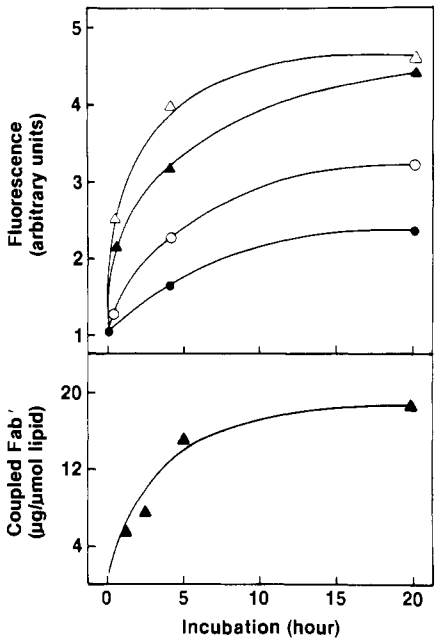

FIGURE 2: Release of entrapped CF during coupling of Fab' fragments to MPB-PE liposomes. (Upper panel) Liposomes containing 5 mol % MPB-PE were incubated at room temperature with four different amounts of Fab' fragments [final concentrations: 2.0 mg/mL (△); 0.5 mg/mL (▲); 0.125 mg/mL (○); 0.063 mg /mL (●)] at a total lipid concentration of 1.5 μmol of lipid/mL. At time intervals as indicated, the fluorescence of the reaction mixtures was measured. Arbitrary fluorescence units measured after 20 h of incubation correspond to 42.0% (2.0 mg of Fab'/mL), 39.5% (0.5 mg of Fab'/mL), 25.3% (0.125 mg of Fab'/mL), and 16.1% (0.0625 mg of Fab'/mL) of total fluorescence observed after detergent treatment. (Lower panel) Liposomes containing 5 mol % MPB-PE were incubated with 0.5 mg/mL ^{125}I-labeled Fab' fragments under conditions as described above. At time intervals as indicated, free ^{125}I-Fab' fragments were removed by gel filtration on Sepharose 4B, and the amount of coupled ^{125}I-Fab' fragments was determined.

preparations were homogeneous with a mean diameter of approximately 2500 Å as determined by photon correlation spectroscopy (Figure 1).

As shown in Figure 1, the mean diameter of Fab'-coated liposomes increased as a function of initial Fab' concentration

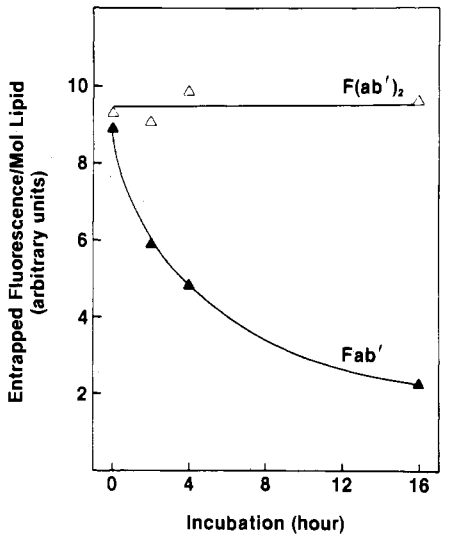

FIGURE 3: Demonstration that Fab' binding is responsible for release of CF. Liposomes containing 5 mol % MPB-PE were incubated at a total lipid concentration of 1.5 μmol of lipid/mL at room temperature with freshly reduced Fab' fragments (final concentration: 0.5 mg/mL) or with nonreduced $F(ab')_2$ fragments (final concentration: 0.5 mg/mL). At time intervals as indicated, free Fab' or $F(ab')_2$ fragments and released CF were removed by gel filtration on Sepharose 4B, and the remaining entrapped fluorescence per mole of lipid was determined.

from 2500 to 5000 Å. This increase in size was associated with an increase in the size heterogeneity of the liposomes (polydispersity).

The upper panel of Figure 2 demonstrates that the coupling of Fab' fragments to the liposomes caused a substantial loss of entrapped CF. Depending on the Fab' concentration in the reaction mixture, an increasing fluorescent signal was measured during the coupling reaction. The loss of entrapped CF predominantly occurred during the first 10 h of incubation. This loss of CF was paralleled by the binding of ^{125}I-labeled Fab' (Figure 2, lower panel), suggesting that the actual binding reaction of Fab' to the liposomes is responsible for the release of the entrapped CF. In order to confirm the causal relation of Fab' binding and CF release, liposomes were incubated with $F(ab')_2$ fragments that contain no free sulfhydryl group and, therefore, are unable to bind to the MPB-PE-containing liposomes. As shown in Figure 3, $F(ab')_2$ fragments did not affect the integrity of the liposomes while the same concentration of Fab' fragments caused a substantial release of CF.

Mechanism of CF Release during Coupling of Fab' Fragments. CF release during covalent attachment of Fab' fragments to liposomes may be due to leakage of CF from all liposomes and/or complete release of CF from a limited number of ruptured liposomes. By measuring the degree of self-quenching of CF within a population of liposomes, together with the amount of dye entrapped per liposome, we can differentiate between these two possible release pathways (Weinstein et al., 1981; Klausner et al., 1981). Furthermore, by the same measurements we can determine whether swelling of the liposomes during the coupling reaction plays a significant role for the observed fluorescence enhancement. At high concentrations, CF fluorescence is self-quenched due to dye–dye interaction (Blumenthal et al., 1977). Thus, if the dye is entrapped in liposomes at a concentration of 60 mM, the fluorescence intensity is only 10% of that obtained if the dye is released from the liposomes and diluted into the surrounding buffer. Leakage of CF from liposomes during the coupling reaction would decrease the intraliposomal CF concentration, resulting in a lower self-quench. Swelling of the liposomes would also decrease the intraliposomal CF concentration, but

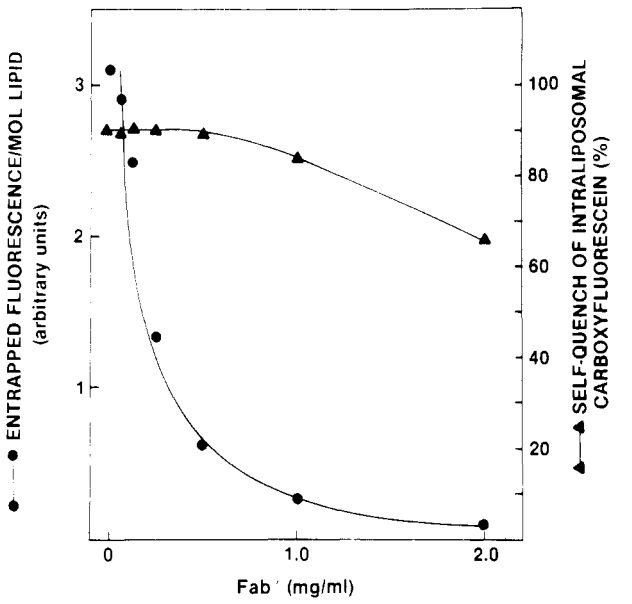

FIGURE 4: Distinction between leakage and rupture during coupling of Fab′ fragments. Liposomes containing 5 mol % MPB-PE were coated with increasing amounts of Fab′ fragments by incubation for 20 h at room temperature at a lipid concentration of 1.5 μmol/mL. After isolation of the liposomes by gel filtration on Sepharose 4B, the percent self-quench of the CF in the intact liposomes (▲) and the fluorescence per mole of lipid after detergent lysis of the liposomes (●) were determined.

in contrast to leakage, swelling should not affect the amount of dye entrapped per liposome. On the other hand, complete release of CF from a limited number of liposomes due to rupture would not change the self-quench of CF within the remaining intact liposomes, but would result in a decreased mean amount of dye entrapped per liposome.

Liposomes containing 5 mol % MPB-PE and encapsulating 60 mM CF were incubated with increasing amounts of Fab′ fragments and subsequently separated from nonbound Fab′ fragments and released CF by using gel filtration on Sepharose 4B. The lipid content of the purified liposomes and their fluorescence before and after detergent lysis were determined. As shown in Figure 4, the self-quench of CF within the collected liposomes remained unchanged after incubation with up to 0.5 mg/mL Fab′, while the amount of entrapped CF per mole of lipid was dramatically reduced (by approximately 80%). These results indicate that at Fab′ concentrations of 0.5 mg/mL and below the release of entrapped CF is due to rupture of a limited number of vesicles. At Fab′ concentrations above 0.5 mg/mL, a lower self-quench was observed. For example, at 2.0 mg/mL Fab′ the self-quench of CF was reduced to 66%, which corresponds to an intraliposomal CF concentration of approximately 15 mM, one-fourth of the initial CF concentration. These observations indicate that, at higher Fab′ concentrations, leakage of entrapped CF and possibly swelling of the liposomes occur in addition to rupture.

Effect of Relative Concentration of the MPB-PE Anchor in Liposomes on Stability during and after Coupling of Fab′ Fragments. At 5 mol % MPB-PE, the fluorescent signal of the reaction mixture increased up to 3.5-fold during coupling of Fab′ fragments (Figure 5, top panel). Decreasing the molar concentration from 5 mol % MPB-PE resulted in a dramatic increase of the stability of the liposomes. At 2.5 mol % MPB-PE, less than 10% CF release was observed (Figure 5, center panel). At 1 mol % MPB-PE, no release of CF could be detected during coupling even at high Fab′ concentrations (Figure 5, bottom panel). When the incubation period for the coupling of Fab′ fragments was extended from 5 (data in Figure 5) to 20 h, the stability of the 1 mol % MPB-PE liposomes remained unaffected.

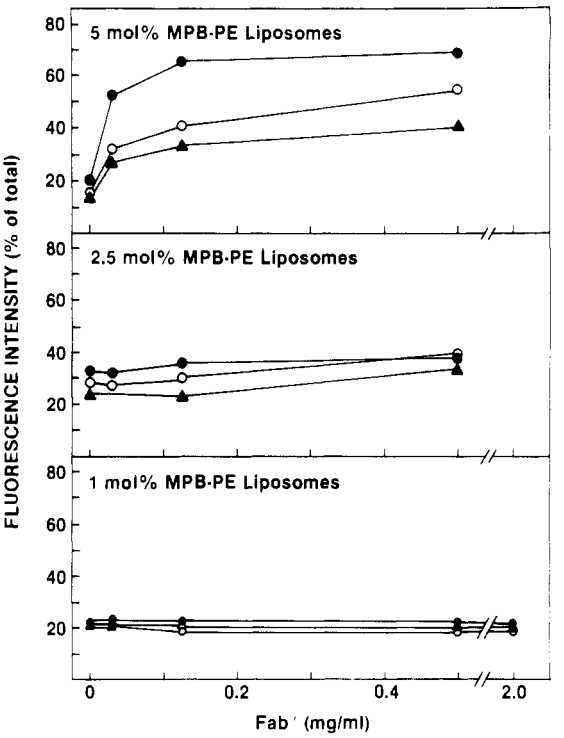

FIGURE 5: Effect of relative MPB-PE concentration and total lipid concentration on integrity of liposomes during Fab′ coupling. Liposomes containing 5 mol % MPB-PE (top panel), 2.5 mol % MPB-PE (center panel), and 1 mol % MPB-PE (bottom panel) were prepared as described under Materials and Methods. The liposomes were incubated with increasing amounts of Fab′ fragments for 5 h at room temperature at different lipid concentrations [0.33 μmol/mL (●), 1.66 μmol/mL (○), 5.0 μmol/mL (▲)]. Subsequently, the fluorescence of the samples was measured. It is expressed as percent of total fluorescence observed after detergent treatment.

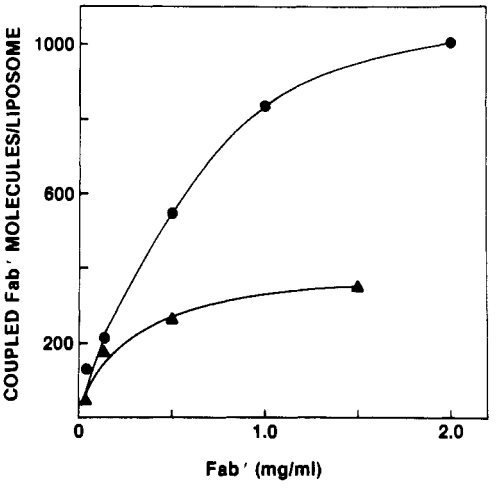

FIGURE 6: Effect of relative MPB-PE concentration on binding of Fab′ fragments to liposomes. Liposomes containing 5 mol % MPB-PE (●) or 1 mol % MPB-PE (▲) were incubated for 20 h at room temperature with increasing amounts of ^{125}I-labeled Fab′ fragments at a total lipid concentration of 1.66 μmol/mL. After removal of free ^{125}I-Fab′ by gel filtration on Sepharose 4B, the number of coupled ^{125}I-Fab′ molecules per liposome was determined.

As shown in Figure 6, reduction of the MPB-PE concentration caused a corresponding decrease of the number of Fab′ molecules bound per liposome. Dependent on the initial Fab′ concentration, 1 mol % MPB-PE liposomes bound 30–50% of the Fab′ molecules that could be coupled to 5 mol % MPB-PE liposomes. However, the binding of Fab′ molecules to 1 mol

Table I: Effect of Lipid Concentration on Coupling of Fab' Molecules[a]

liposome concn (μmol of lipid/mL)	coupled Fab' molecules/liposome: 1 mol % MPB-PE liposomes	5 mol % MPB-PE liposomes
0.33	607	1378
1.66	251	543
5.0	99	149

[a] ^{125}I-Labeled Fab' fragments (final concentration 0.5 mg/mL) were coupled to 1 and 5 mol % MPB-PE liposomes at final lipid concentrations as indicated for 20 h at room temperature. Noncoupled Fab' fragments were removed by column chromatography on Sepharose 4B, and the number of coupled Fab' molecules per liposome was determined.

Table II: Stability of MPB-PE-Containing Liposomes with and without Covalently Attached Fab' Fragments[a]

liposomes	coated with Fab' fragments	% fluorescence, days at 4 °C: 0	14	28
5 mol % MPB-PE	+	13.9	33.5	33.0
	−	18.8	40.5	44.5
2.5 mol % MPB-PE	+	10.9	13.7	17.0
	−	12.1	12.7	12.0
1 mol % MPB-PE	+	10.6	12.3	13.3
	−	12.2	14.8	11.7

[a] Fluorescence was determined as 100 × (fluorescence of liposome suspension)/(fluorescence of liposome suspension after detergent lysis).

% MPB-PE liposomes could be improved by increasing the initial Fab' concentration (Figure 6) or by decreasing the liposome concentration in the coupling reaction mixture (Table I). For example, by a 15-fold decrease of the liposomal lipid concentration, binding of Fab' molecules to 1 mol % MPB-PE liposomes could be increased from ~100 to ~600 Fab' molecules per liposome. This number (~600) is similar to that obtained with 5 mol % MPB-PE liposomes at 1.66 μmol of lipid/mL (Table I).

These data additionally demonstrate that the stability of the liposomes is primarily a consequence of the relative concentration of the MPB-PE anchor and not of the number of coupled Fab' molecules. While 1 mol % MPB-PE liposomes remained stable during coupling of 600 Fab' molecules (Figure 5, bottom panel), 5 mol % MPB-PE liposomes released approximately half of the entrapped CF during the binding of this number of Fab' molecules (Figure 5, top panel). However, while the stability of 1 mol % MPB-PE liposomes is independent of the number of Fab' molecules bound (Figure 5, bottom panel), 5 mol % MPB-PE liposomes exhibited a greater instability with increasing numbers of coupled Fab' molecules (Figure 5, top panel).

Liposomes containing lower percentages of MPB-PE were also found to be more stable during storage at 4 °C after covalent attachment of Fab' molecules. Table II demonstrates that substantial amounts of entrapped CF were released from 5 mol % MPB-PE liposomes over a period of 1 month, while 1 mol % MPB-PE liposomes were found to be rather stable upon storage. As is also evident from Table II, the fragility of the 5 mol % MPB-PE liposomes upon storage is due to the MPB-PE anchor and not due to the different amounts of attached Fab' molecules per liposome since noncoated 5 mol % MPB-PE liposomes were also unstable upon storage.

Discussion

This study demonstrates that covalent coupling of immunoglobulin fragments to preformed liposomes can have major effects on liposomal size, polydispersity, and integrity. At a high molar ratio of the anchor lipid for covalent attachment of Fab' fragments (5 mol % MPB-PE) in the liposomal bilayer and a high concentration of the protein to be coupled (2.0 mg/mL Fab' fragments), up to 95% of liposome-entrapped molecules (CF) were released during the coupling reaction. Fluorescence measurements of intraliposomal CF concentrations revealed liposome rupture as the mechanism of CF release at low Fab' concentrations while leakage and possibly swelling were identified as additional mechanisms at higher Fab' concentrations.

The coupling of Fab' fragments to the 5 mol % MPB-PE liposomes caused an increase of their mean diameter. This increase, of up to 100% at higher Fab' concentrations, cannot simply be explained by the attachment of Fab' molecules and may be due to liposome aggregation and/or fusion. An increase of the diameter due to fusion of ruptured liposomes would be consistent with the observed instability of the liposomes during Fab' coupling and the simultaneous release of entrapped CF by rupture and leakage.

The molecular event that causes the increase in liposomal diameter and release of entrapped CF during the coupling reaction is the binding of the Fab' molecules to the liposomes. $F(ab')_2$, which cannot covalently bind to the liposomes, had no effect on liposomal integrity (Figure 3). Furthermore, the Fab' fragments bound to the liposomes with a time course identical with that for release of the entrapped fluorescent dye (Figure 2).

It is well-known that the lipid composition plays a significant role for the stability of liposomes [reviewed by Szoka and Papahadjopoulos (1980) and Gregoriadis (1984)]. Cholesterol, when present at 30–50 mol %, has been shown to stabilize liposomes (Finkelstein & Weissmann, 1979; Kirby et al., 1980; Damen et al., 1981). Liposomes used in this study contained 40 mol % cholesterol. However, despite the stabilizing effect of cholesterol, higher concentrations of the anchor molecule MPB-PE (>2.5 mol %) for the covalent attachment of protein to the liposomes dramatically affected the stability of the liposomes. MPB-PE caused instability of liposomes upon storage even without coupled Fab' fragments as well as during the coupling reaction. Dependent on the Fab' concentration during coupling, liposomes containing 5 mol % MPB-PE released up to 95% of the entrapped CF. In contrast, liposomes containing 1 mol % MPB-PE exhibited no CF release during coupling. The increased stability of the 1 mol % MPB-PE liposomes during coupling was found to be primarily due to the lower concentration of the MPB-PE and not the lower number of Fab' molecules bound to the liposomes.

The MPB-PE anchor has been used by other investigators for the preparation of liposomes from different lipid mixtures (Martin & Papahadjopoulos, 1982; Derksen & Scherphof, 1985). Using lipid mixtures containing 2.5 mol % MPB-PE, these investigators produced stable liposomes, but no data were presented on the liposomal stability during the coupling of proteins. Although Derksen and Scherphof (1985) demonstrated an increased protein coupling at 5 mol % MPB-PE concentrations, they decided for practical reasons to routinely use 2.5 mol % MPB-PE in their experiments. Similarly, Martin and Papahadjopoulos (1982) used 2.5 mol % MPB-PE in the liposomes, although they report that up to 10 mol % MPB-PE has no adverse effects on the bilayer structure.

It is currently unknown how different lipid compositions will affect the stability of MPB-PE liposomes during coupling of proteins. Similarly, the effect of different lipid anchors for covalent attachment of protein on liposomal integrity during

the coupling reaction remains to be determined. Furthermore, different procedures for the preparation of large unilamellar liposomes may have an effect on the stability of the resulting liposomes. For the preparation of liposomes used in this study, we followed the procedure of Olson et al. (1979), which avoids the use of organic solvents, detergents, and sonication. Therefore, this method offers a significant advantage over other methods since it does not expose the molecules to be entrapped to harsh conditions and allows liposomal entrapment of labile compounds such as proteins (Gaber et al., 1983; Thompson & Gaber, 1985).

In this study, we prepared liposomes that were stable during covalent attachment of Fab′ fragments and during subsequent storage by reducing the relative MPB-PE concentration to 1 mol %. The reduction of the relative concentration of MPB-PE caused a corresponding reduction of the number of Fab′ molecules coupled per liposome. The number of attached Fab′ molecules per 1 mol % MPB-PE liposome was, depending on the initial Fab′ concentration, 30–50% of that achieved with 5 mol % MPB-PE liposomes. However, this reduction of the number of attached Fab′ molecules per liposome could be reversed by increasing the ratio of the Fab′ concentration vs. the lipid concentration in the coupling reaction mixture without impairing liposomal integrity during the coupling reaction and during subsequent storage.

In conclusion, large unilamellar liposomes containing MPB-PE can be prepared from multilamellar liposomes by the sequential extrusion method, a method known to allow entrapment of molecules into the liposomes under mild conditions. We have defined the conditions under which these liposomes are stable during coupling of Fab′ fragments to their surface. Such stable Fab′-derivatized liposomes may be useful as carriers of molecules for immunotargeting and immunoassays.

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