Irreversible Coupling of Immunoglobulin Fragments to Preformed Vesicles

AN IMPROVED METHOD FOR LIPOSOME TARGETING*

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Rabbit Fab' antibody fragments were covalently coupled to preformed large unilamellar vesicles using a new sulfhydryl-reactive phospholipid derivative N-[4-(β-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE). A highly efficient reaction between the sulfhydryl group on each Fab' fragment and the maleimide moiety of MPB-PE molecules incorporated at a low concentration in vesicle bilayers led to the formation of a highly stable Fab'-vesicle linkage. Coupling ratios in excess of 250 μg of Fab'/μmol of vesicle phospholipid were reproducibly obtained without vesicle aggregation. Bound Fab' fragments did not elute from the vesicles in serum or in the presence of reducing agents (dithiothreitol or mercaptoethanol). Vesicles bearing Fab' fragments raised against specific human erythrocyte surface determinants bound selectively to human erythrocytes under physiological conditions (isotonic medium containing 50% human serum, pH 7.4) with minimal leakage of vesicle contents. Advantages of the present coupling method are discussed in relationship to our efforts to optimize the properties of liposomes as a carrier system.

In a previous report we introduced a method for the covalent attachment of antibody fragments to liposomes via disulfide bonds (1). Leserman et al. (2) have used a similar technique to cross-link thiolated IgG and protein A to SUV.

Although coupling reactions such as these are highly efficient, the product, a disulfide antibody-vesicle bond, is unstable under reducing conditions such as serum (1). Since it is likely that many clinical applications of "targeted" liposomes will require exposure to serum for considerable periods of time, we have devised an alternative coupling method which combines the advantages of our previous system with an essentially irreversible antibody-vesicle linkage.

MATERIALS AND METHODS

Synthesis of N-[4-(β-Maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE)—Transesterified egg PE (100 μmol, Avanti Polar Lipids) was dissolved in 5 ml of anhydrous methanol containing 100 μmol of freshly distilled triethylamine and 50 μg of succinimidyl 4-(β-maleimidophenyl)butyrate (Pierce). The reaction was carried out under an argon atmosphere at room temperature. Thin layer chromatography (6) of the mixture following 2 h revealed quantitative conversion of the PE (Fig. 1) to a faster running product (Rf 0.52, Silica Gel H, solvent: chloroform/methanol/water, 65:25:4). Methanol was removed under reduced pressure and the products redissolved in chloroform. The chloroform phase was extracted twice with 1% NaCl to remove unreacted succinimidyl 4-(β-maleimidophenyl)butyrate and water-soluble by-products. The MPB-PE was further purified by silica acid chromatography as described for PDP-PE (1). Following purification, thin layer chromatography indicated a single phosphatidylethanolamine, ninhydrin-negative spot. MPB-PE is stable for at least 4 months when stored at −50 °C as a chloroform solution sealed in glass ampules under argon.

Preparation of Vesicles—LUV were prepared by the reverse phase evaporation method of Szoka and Papahadjopoulos (3) with minor modifications. Briefly, 10 μmol of cholesterol, 9.5 μmol of PC, 0.5 μmol of MPB-PE, and a trace amount of [3H]DPPC (New England Nuclear) were dissolved in 1 ml of diethyl ether. Buffer I (20 mM citric acid, 35 mM disodium phosphate, 185 mM sucrose, and 1 mM EDTA, pH 4.5) was added (300 μl) and the two phases emulsified by sonication for 1 min at 25 °C in a bath type apparatus (Laboratory Supply Co., Hicksville, NY). Ether was removed under reduced pressure at room temperature and the resulting vesicle dispersion extruded (4) through 0.4-μ and 0.2-μ Unipore polycarbonate membranes (Bio-Rad). Entrapment efficiencies and rates of efflux were determined as described earlier (1) except that the vesicles were prepared in Buffer I (pH 5.0) containing 20 mM dithiothreitol was used for the reduction step. F(ab')2 fragments were radiolabeled with 125I to a specific activity of ~2 × 105 cpm/mg using the method of Bolton and Hunter (5) prior to reduction.

Coupling of Anti-hRBC Fab' Fragments to Vesicles—Freshly prepared vesicles (1–2 μmol of phospholipid in deoxygenated Buffer I) were mixed with Fab' fragments (final concentration 0.5–4.0 mg/ml). The pH was adjusted to 6.5 with 1 N NaOH and the reaction mixture stirred under a stream of argon for up to 12 h at room temperature (Fig. 2). Vesicles were separated from unconjugated antibody fragments using either gel filtration (1) or flotation in polymer gradients (6). Protein and phospholipid concentrations were calculated from the specific activities of 125I-Fab' fragments and [3H]DPPC, respectively.

Hemagglutination and Cell Binding—These assays were as before (1) except Buffer I was used throughout and incubation times were extended to 2 h for cell-binding experiments.

Acylating Activity—The maleimide content of MPB-PE was estimated by reaction with excess 2-mercaptoethanol and subsequent titration with 5,5'-dithiobis(2-nitrobenzoic acid) (7).

RESULTS

Coupling of Fab' Fragments to MPB-PE Vesicles—Vesicles (PC-cholesterol-MPB-PE, 9.5:10:0.5) prepared by the re-
verse phase evaporation method and extruded through 0.2-μm unipore membranes entrapped about 15% of the original aqueous volume (4.73 μL/μmol of phospholipid). Sucrose efflux was less than 0.5%/h in Buffer I at 25 °C and less than 3%/h in 50% serum. The half-life of the maleimide was greater than 4 h in Buffer I at pH 4.5-6.5.

Fab' fragments prepared as described under "Materials and Methods" contained an average of 0.85 —SH groups/molecule. The half-life of the —SH was at least 4-5 h in Buffer I at pH values less than 6.5.

MPB-PE-containing vesicles (1.4 μmol/ml) were reacted with freshly reduced Fab' fragments (0.5-4.0 mg/ml) for 8 h at 25 °C. When such mixtures were chromatographed on Sephadex G-200, 20-30% of the Fab' co-eluted with vesicles in the void volume. The Fab' remained with vesicles during rechromatography, indicating a stable association. When exposed to a 1:32 dilution of goat anti-rabbit IgG serum (Flow Laboratories), greater than 95% of both the 125I-Fab' and [3H]DPPC labels co-precipitated, suggesting a rather homogeneous lipid to protein ratio (1). Nonspecific binding of Fab' to control vesicles (PC-cholesterol, 1:1) was less than 4 μg/μmol of phospholipid at Fab' concentrations below 5 mg/ml.

We found a linear relationship between the amount of Fab' coupled to vesicles (in 8 h) and the initial Fab' concentration. For antibody concentrations of 0.5, 2.0, and 4.0 mg/ml, we obtained coupling ratios of 70 ± 15, 330 ± 20, and 584 ± 40 μg of Fab'/μmol of vesicle phospholipid, respectively. Some aggregation of vesicles occurred at Fab' concentrations above 4 mg/ml.

The time course of a typical coupling reaction is shown in Fig. 3. Under these conditions, approximately 340 μg of the Fab' was coupled to vesicles in 8 h. This value corresponds to greater than 3000 Fab' molecules/each vesicle (0.2 μm diameter). For comparison, we have included in Fig. 3 the time course of Fab' coupling to PDP-PE vesicles. At equivalent protein and lipid concentrations, the reaction of Fab' with the maleimide-PE at pH 6.5 was more efficient than with the pyridyl-dithio derivative at pH 8.0 (the optimal pH for this reaction) (1).

The thioether bond formed between Fab' and MPB-PE vesicles was quite stable. No coupled Fab' was lost from vesicles during incubation for 24 h in dithiothreitol (50 mM, pH 7.5) or human serum (50%, pH 7.4). In contrast, during the same time period, 92% of coupled Fab' eluted from PDP-PE vesicles in dithiothreitol (50 mM, pH 8.0) and 62% in serum (50%).

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Time course of coupling of Fab' to MPB-PE and PDP-PE-containing vesicles. Freshly reduced Fab' fragments (2 mg/ml) were mixed with MPB-PE vesicles (1.4 μmol, PC/cholesterol/MPB-PE, 9.5:100.5) or PDP-PE vesicles (1.4 μmol, PC/cholesterol/PDP-PE, 9.5:100.5) and reacted for 8 h at the indicated pH. The amount of Fab' conjugated to vesicles at each time point was calculated following separation of vesicles from unreacted antibody fragments by discontinuous dextran gradients (6).

Fig. 1. Synthesis of N-[4-(p-maleimidophenyl)butyryl]phosphatidylethanolamine.

PHOSPHATIDYLETHANOLAMINE

\[ R_1-C-(CH_2)_3-N-R_2 \]

\[ R_3-C-(CH_2)_3-N-R_4 \]

N-SUCCINIMIDYL 4-(P-MALEIMIDOPHENYL) BUTYRATE

\[ R_1-C-(CH_2)_3-N-S-(P-MALEIMIDOPHENYL) BUTYRATE \]

\[ \text{PHOSPHATIDYLETHANOLAMINE} \]

\[ \text{N-SUCCINIMIDYL 4-(P-MALEIMIDOPHENYL) BUTYRATE} \]

\[ \text{FIG. 2. Covalent coupling of Fab' fragments to MPB-PE vesicles.} \]

**Fig. 2.** Covalent coupling of Fab' fragments to MPB-PE vesicles. Fab' fragments are prepared by pepsin digestion of the IgG fraction of whole rabbit serum. Fab' monomers are generated from these by reduction with dithiothreitol at low pH. Immediately following the removal of dithiothreitol, Fab' fragments are mixed with MPB-PE-containing vesicles and the pH adjusted to 6.5. Addition of the Fab'—SH to the double bond of the maleimide moiety of MPB-PE molecules present in vesicle membranes results in a stable thioether cross-linkage.

![Antigen Combining Sites](http://www.jbc.org/)

**FIG. 2.** Antigen Combining Sites

IgG

\[ R_1-C-(CH_2)_3-N-R_2 \]

\[ R_3-C-(CH_2)_3-N-R_4 \]

\[ \text{MPB-PE Vesicle} \]

\[ \text{Antigen Combining Sites} \]

\[ \text{Pepsin} \]

\[ \text{DTT} \]

\[ \text{2 Fab'} \]

\[ \text{Fab'—Vesicle} \]

\[ \text{Fig. 2. Covalent coupling of Fab' fragments to MPB-PE vesicles.} \]

\[ \text{Fab'}, \text{ dimers are prepared by pepsin digestion of the IgG fraction of whole rabbit serum.} \]

\[ \text{Fab' monomers are generated from these by reduction with dithiothreitol at low pH. Immediately following the removal of dithiothreitol, Fab' fragments are mixed with MPB-PE-containing vesicles and the pH adjusted to 6.5. Addition of the Fab'—SH to the double bond of the maleimide moiety of MPB-PE molecules present in vesicle membranes results in a stable thioether cross-linkage.} \]
Binding of anti-hRBC Fab'-bearing vesicles to human and sheep erythrocytes is shown in Fig. 4. Virtually quantitative binding was observed to hRBC at vesicle concentrations up to 65 nmol of phospholipid/1.6 × 10^6 hRBC. This saturation value corresponds to about 4900 vesicles (0.2 μm diameter) per cell (assuming 1.2 × 10^12 vesicles/μmol of phospholipid). Binding to control cells (sRBC) at this vesicle concentration was less than 5%. Binding of MPB-PE vesicles alone and vesicles bearing an equivalent amount of nonspecific Fab’ (290 μg of Fab’/μmol of phospholipid) did not exceed 2% for both cell types (not shown). Bound vesicles retained virtually all (>80%) of their aqueous contents (14C-labeled sucrose) during cell-binding experiments.

Serum had no effect on the extent of binding of anti-hRBC Fab’ vesicles to hRBC nor did it induce leakage of vesicle contents. However, 50% serum reduced somewhat the nonspecific binding of such vesicles to hRBC (at 100 nmol of phospholipid, nonspecific binding drops from 5% in Buffer 1 to less than 2% in 50% serum, Fig. 4).

Anti-hRBC Fab’ vesicles (340 μg of anti-hRBC Fab’/μmol of phospholipid) agglutinated hRBC but not sRBC. The hemagglutination titer for a 100-nmol suspension of such vesicles was 1024 for hRBC and less than 1 for sRBC. Vesicles bearing nonspecific Fab’ (290 μg of Fab’/μmol of phospholipid) exhibited no hemagglutination activity for either cell type.

**DISCUSSION**

Growing interest in the use of “antibody-targeted” liposomes as drug carriers (8) has led to the need for efficient methods of covalently coupling immunoglobulins to vesicle lipids (1, 2, 9, 10). We report here a new conjugation procedure which provides improved coupling efficiencies and leads to the formation of a stable antibody-vesicle linkage. MPB-PE is easily prepared from commercially available reagents and is stable for many months in organic solvents at −50 °C. The extended spacer arm between the phospholipid head group and the maleimide moiety (Fig. 1) reduces the possibility of steric constraints at the bilayer interface and thereby ensures favorable thiol reactivity (11). The solubility properties of this molecule are similar to PE and it can easily be mixed with other phospholipids during liposome formation. The maleimide moiety retains alkylating activity for many hours in aqueous solution (pH 4.5–6.5).

LUV, prepared by the reverse phase evaporation method, were chosen because of their favorable “carrier” properties. Vesicles of fairly homogeneous size (in this case, 0.2 μm) are obtained by extrusion through unipore membranes without loss of material (4, 12). Entrapment efficiencies are good and internal volumes close to theoretical values for single layered vesicles. Large macromolecules such as proteins and nucleic acids can be encapsulated in vesicles by this procedure (13). Inclusion of cholesterol in vesicle membranes ensures their stability in serum (14). For instance, vesicles that contain 50 mol % of cholesterol retain at least 50% of their entrapped sucrose following incubation for 16 h in 50% serum. MPB-PE (up to 10 mol %) and other reactive lipid derivatives (such as PDP-PE) can be included into REV with no adverse effects on the bilayer structure (1).

In practice, it should be possible to link any thiol-containing protein ligand to MPB-PE-containing vesicles. We use rabbit Fab’ fragments because these can easily be prepared containing a single, highly reactive thiol group in the hinge region of each molecule (1). Coupling via this thiol reduces the possibility of vesicle aggregation and results in a favorable orientation of each Fab’ on the vesicle surface (1). In addition, the absence of a Fc region eliminates the possibilities of Fc-mediated binding and complement activation and reduces the likelihood of anti-idiotypic antibody production in vivo.

The advantages of using Fab’-bearing LUV for targeting are apparent from the cell-binding results presented here. Anti-hRBC Fab’-vesicles bind specifically to hRBC up to a saturating multiplicity of about 5000 vesicles/cell. This number of vesicles represents a combined internal volume of 1.8 × 10^-8 μl equivalent to about 20% of the total volume of each target erythrocyte. Serum does not cause elution of conjugated Fab’ from vesicles nor does it interfere with binding of liposomes to cells. In fact, serum actually reduces nonspecific interactions (Fig. 4).

In the present study, we have focused on optimization of the carrier properties and targeting potential of liposomes. The question of cytoplasmic delivery of liposome contents is currently being investigated using mouse monoclonal antibodies as ligands and actively metabolizing cells as targets.

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