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Iodoacetylated and biotinylated liposomes: effect of spacer length on sulfhydryl ligand binding and avidin precipitability

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Because of the sustained interest in liposomes as immunogens and vehicles for drug delivery, the present investigation was designed to reevaluate the iodoacetyl group as a means of binding sulfhydryl-containing substances to liposomes in thioether linkage, and to develop an alternative method by which liposomes with bound ligand can be conveniently and rapidly separated from free ligand. For the purpose of the first goal, we synthesized a homologous series of dimyristoylphosphatidylethanolamine (DMPE) derivatives in which the iodoacetyl (IA) function was separated from the phospholipid amino group by either 0, 1, or 2 aminoethylthioacetyl (AETA) spacers. Results show that liposomes prepared with IA-DMPE can not bind ^{125}I-radiolabeled rabbit IgG which had been thiolated by reaction with *S*-acetylmercaptosuccinic anhydride. Significant IgG attachment was, however, obtained with liposomes containing either IA-AETA-DMPE or IA-$(AETA)_2$-DMPE, and the amount bound was directly related to spacer length. In contrast, spacer length had no effect on the covalent binding of a low molecular weight hapten, *N*-dinitrophenylcysteine. Other parameters (incubation time, IgG concentration, density of IA-$(AETA)_2$-DMPE, sulfhydryl inhibitors) were also examined. To achieve the second objective, biotinyl-$(AETA)_2$-DMPE was incorporated into the same liposomal bilayers that contained the iodoacetylated derivatives. Thus, liposomes with bound ligand could be readily precipitated by avidin, and washed free of unreacted IgG by low speed centrifugation. Comparative experiments with liposomes containing biotinyl-DMPE revealed that spacer length also had a pronounced effect on the avidin precipitability of liposomes in the presence of proteins that may be non-covalently absorbed or covalently bound to the model membrane surface.

Introduction

Numerous in vitro investigations have shown that antibodies or antibody fragments can direct liposomes with entrapped drugs to target cells that possess the corresponding antigens in their membranes. This phenomenon, which has potential therapeutic applications, requires prior incorporation or covalent linkage of the antibodies to the liposomal surface. Several methods have been devised for this purpose. The procedure, which was originally introduced by Leserman et al. [1,2] and Martin et al. [3], employed liposomes prepared with *N*-[3-(pyridyl-2-dithio)propionyl]phosphatidylethanolamine. This derivative (PDP-PE) readily reacts with sulfhydryl groups resulting in the binding of antibodies to the liposome bilayers via

Abbreviations: DMPE (or PE), dimyristoylphosphatidylethanolamine (or phosphatidylethanolamine); IA, iodoacetyl; AETA, aminoethylthioacetyl; DNP, 2,4-dinitrophenyl; NHS, *N*-hydroxysuccinimidyl; PDP, 3-(pyridyl-2-dithio)propionyl; MPB, 4-(*p*-maleimidophenyl)butyryl; SAMSA, *S*-acetylmercaptosuccinic anhydride.

disulfide bonds. However, the in vivo administration of such liposome-antibody conjugates may be compromised by the susceptibility of disulfide bonds to reductive cleavage by ubiquitous thiol compounds. Accordingly, Martin and Papahadjopoulos [4] synthesized *N*-[4-(*p*-maleimidophenyl)-butyryl]phosphatidylethanolamine. Liposomes prepared with this derivative (MPB-PE) effectively bound antibodies via thioether linkages formed by sulfhydryl addition across the maleimide double bond.

Because of the stability of thioether bonds, we considered using MPB-PE to make immunogenic liposomes with various sulfhydryl-containing haptens. The strategy would be analogous to the generation of liposomal immunogens carrying amino-containing haptens by reaction of the latter with *N*-hydroxysuccinimidyl esters anchored to the lipid bilayers [5,6]. This approach was, however, abandoned because of the possibility that an immune response might be elicited, not only against the hapten, but also toward the aromatic maleimidophenyl moiety present in the bridge portion of MPB-PE.

The current investigation therefore was undertaken to reevaluate the iodoacetyl group as a means of attaching sulfhydryl-bearing ligands (proteins as well as haptens) to liposomes. Previously, Sinha and Karush [7] had shown that a fluorescent phospholipid, in which the amino group of phosphatidylethanolamine was substituted with *N*-α-iodoacetyl, *N*-ε-dansyllysine residues could alkylate the sulfhydryl group of a Bence-Jones monomeric protein, and that the modified hydrophobic protein could then readily associate with preformed liposomes. On the other hand, Wolff and Gregoriadis recently reported [8] (and we have been able to confirm) that preformed liposomes containing *N*-(iodoacetyl)phosphatidylethanolamine bound little, if any, thiolated protein in thioether linkage. They suggested, but were unable to show with the derivatives available, that protein binding may have been sterically hindered due to the proximity of the iodoacetyl function to the liposomal surface. In support of this suggestion, the present experiments demonstrate, however, that IgG can be covalently attached to liposomes prepared with dimyristoylphosphatidylethanolamine (DMPE) derivatives in which the iodoacetyl group (IA) is separated from the amino function of the phospholipid by one or more aminoethylthioacetyl (AETA) spacers (see Fig. 1). In contrast, spacer length is shown not to be a significant factor in the binding of a low molecular weight hapten (*N*-dinitrophenylcysteine) to liposomes that contained these iodoacetylated DMPE derivatives. A preliminary account of some of these experiments has been given [9].

Additionally, in the course of this investigation, a procedure was devised by which liposomes with bound ligand can be readily separated from free ligand. Previous methods employed for this purpose include chromatographic separation on Sephadex or Sepharose columns, ultracentrifugal sedimentation, density gradient flotation, and dialysis (for low-molecular weight substances). All of these procedures are time-consuming; with the exception of dialysis, they are also limited by the capacity or availability of equipment and hence not suitable for experiments that require examination of a large number of samples. For this reason, we developed a method based on precipitation of biotinylated liposomes by avidin, followed by washing at low speeds in a clinical centrifuge. Comparative studies with liposomes containing either biotinyl-DMPE or biotinyl-$(AETA)_2$-DMPE (Fig. 1) show that spacer length is also a critical factor in their avidin precipitability, particularly in the presence of proteins that may be noncovalently absorbed or covalently bound to liposomes.

Materials and Methods

Chemicals. The following were purchased from the suppliers indicated in parentheses: dimyristoylphosphatidylcholine, dimyristoylphosphatidylethanolamine (Avanti Polar Lipids; Birmingham, AL); Iodogen, *N*-hydroxysuccinimidylbiotin, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3′-dithiopropionate (Pierce Chemical Co.; Rockford, IL); cholesterol, cysteamine hydrochloride, dicetyl phosphate, 4,4′-dithiodipyridine, dithiothreitol, iodoacetic acid, iodoacetic anhydride, *S*-acetylmercaptosuccinic anhydride, *N*,*N*′-di(2,4-dinitrophenyl)cystine (Sigma Chemical Co.; St. Louis, MO); ^{125}I, [7-

^{3}H]cholesterol (New England Nuclear; Boston, MA).

Synthesis of derivatives. The reactions described below (Fig. 1), as well as the purity of the final products, were followed by chromatography on thin-layer plates (silica gel 60 F-254; 0.25 mm thickness; Brinkmann Instruments, Inc., Westbury, NY) in two solvent systems: chloroform/methanol/water (70 : 30 : 5, v/v) and chloroform/methanol/acetic acid (60 : 20 : 3, v/v). Compounds were detected by iodine vapor and phosphate (molybdenum blue) spray, and ninhydrin reagent when appropriate; they were quantitated by measurement of total phosphate [10].

IA-DMPE. Dimyristoylphosphatidylethanolamine (0.2 mmol) was dissolved in 50 ml of chloroform containing triethylamine (1 mmol) that had been distilled over ninhydrin to remove primary amine contaminants. Solid iodoacetic anhydride (0.6 mmol) was added to start the reaction. After incubation for 15 h at room temperature, the solvent was removed by rotary evaporation under reduced pressure at 40°C. The residue was dissolved in 300 ml of methanol/chloroform (2 : 1, v/v) and 80 ml of 0.075 M glycine in 2% KCl was added (glycine was employed to discharge excess iodoacetic anhydride). The chloroform phase, which resulted upon shaking with 100 ml each of chloroform and water, was isolated and taken to dryness. This residue was subjected to a second extraction as just described except that glycine was omitted. Chromatographic analysis of the final chloroform-soluble product revealed a single compound that gave a positive test for phosphate and no reaction with ninhydrin; the yield was 80% on the basis of phosphate content (average of four preparations).

AETA-DMPE. IA-DMPE (0.2 mmol) was dissolved in 40 ml of a 1 : 1 volume mixture of chloroform and methanol that contained redis-

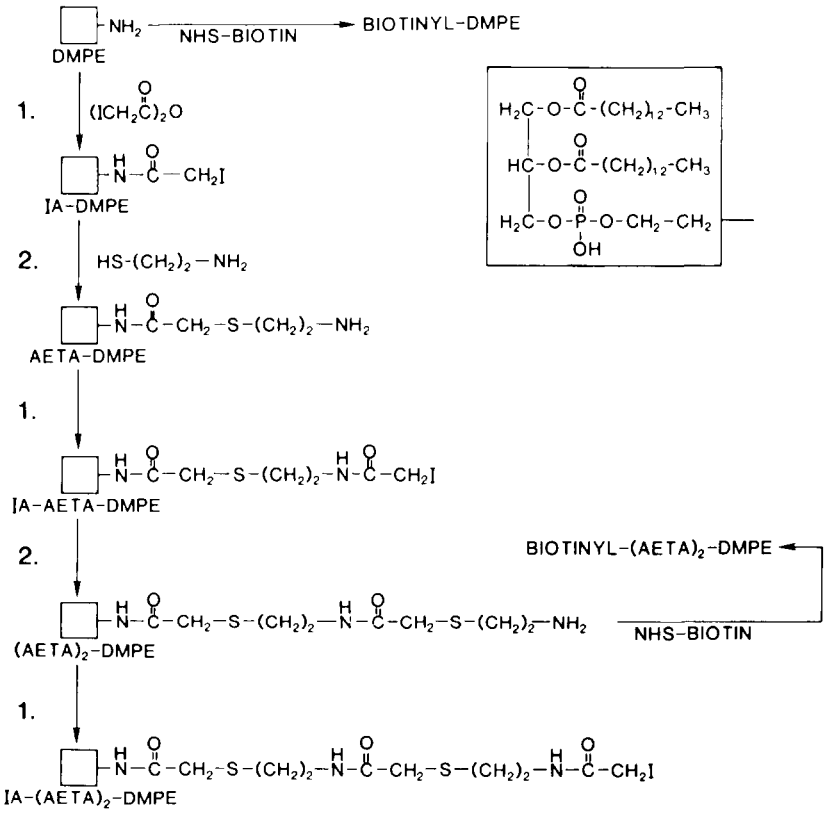

Fig. 1. Reaction sequence used to synthesize iodoacetylated (IA) and biotinylated dimyristoylphosphatidylethanolamine (DMPE) derivatives with different numbers of aminoethylthioacetyl (AETA) spacers.

tilled triethylamine (4 mmol). After the addition of solid cysteamine hydrochloride (1 mmol), the reaction mixture was stirred at room temperature for 15 h. At this time, thin-layer chromatography indicated complete disappearance of IA-DMPE and formation of a new phospholipid that possessed a primary amino group as detected by ninhydrin spray. This compound was obtained in chromatographically pure form by the same extraction procedure (glycine omitted) used to isolate IA-DMPE; the yield was 70% (average of four preparations).

IA-AETA-DMPE and IA-$(AETA)_2$DMPE. These derivatives were made and isolated by procedures identical to those used to prepare IA-DMPE, except that DMPE was replaced by either AETA-DMPE (see above) or $(AETA)_2$-DMPE (see below).

$(AETA)_2$-DMPE. This intermediate was synthesized by the same method employed to make AETA-DMPE except that IA-AETA-DMPE was substituted for IA-DMPE.

Biotinyl-DMPE and biotinyl-$(AETA)_2$-DMPE. *N*-Hydroxysuccinimidylbiotin (0.2 mmol) was dissolved in 50 ml of methanol/chloroform (1 : 1, v/v) containing triethylamine (0.5 mmol), and either DMPE (0.1 mmol) or $(AETA)_2$-DMPE (0.1 mmol). After 15 h incubation at room temperature, 25 ml of methanol was added to the reaction mixture to produce a 2 : 1 methanol : chloroform ratio. The biotinylated derivative was extracted from this solution by the procedure used to isolate IA-DMPE (see above) except that proportionately smaller volumes of the solvents were employed and glycine was omitted from the 2% KCl solution. To obtain these compounds in pure form, the chloroform-soluble material was further subjected to preparative plate chromatography as described elsewhere [6]. Overall yield (two preparations of each derivative) averaged 50%.

Biotinyl-S-S-DMPE. This derivative was synthesized by the procedure described in the preceding section, except that the reaction with DMPE was carried out in 50 ml of chloroform/methanol/water (1 : 2 : 0.8, by vol.) due to the limited solubility of sulfosuccinimidyl 2-(biotinamido)ethyl-1,3′-dithiopropionate in the absence of water. Following incubation, the chloroform phase, which was obtained by shaking with 13.1 ml each of chloroform and water, was taken to dryness. The residue was dissolved in a minimum volume of chloroform/methanol (1 : 1, v/v), and the desired product was subsequently isolated in chromatographically pure form (20% yield) on preparative plates.

Iodination of IgG. Lyophilized rabbit IgG (Calbiochem-Behring Diagnostics; San Diego, CA) was reconstituted to a concentration of 10 mg/ml with Dulbecco's phosphate-buffered saline, pH 7.4, lacking Ca^{2+} and Mg^{2+}. Any insoluble material, which appeared after overnight dialysis against cold phosphate-buffered saline, was removed by centrifugation for 30 min at $14000 \times g$. Radiolabeling was performed essentially by the procedure described by Manthei et al. [11]. Briefly, the following solutions were added sequentially to a 12×75 mm glass tube whose bottom had been precoated with 10 μg of Iodogen: 60 μl of phosphate-buffered saline, 15 μl (approx. 100 μg) of dialyzed IgG, and 30 μl (approx. 300 μCi) of ^{125}I in phosphate-buffered saline. After incubation for 15 min at room temperature, 33 μl of 1 M potassium iodide was added to displace any ^{125}I that was not covalently attached to the protein. The iodinated IgG was subsequently isolated (> 99% free of unbound ^{125}I) by centrifugal passage of the reaction mixture through a small packed column of an anion exchange resin (approx. 1 g of AG 1-X8 (chloride form); Bio-Rad; Richmond, CA).

Thiolation of IgG. 300 μl of *S*-acetylmercaptosuccinic anhydride (SAMSA; 132 mM in dimethylformamide) was stirred with 15 ml of dialyzed rabbit IgG (2 mg IgG/ml of 0.1 M sodium phosphate buffer (pH 7)) to which a trace of ^{125}I-labeled IgG had been added (36 μl, initially containing approx. $8 \cdot 10^6$ cpm). After incubation for 30 min at room temperature, excess SAMSA was removed by overnight dialysis against 2 liter of cold phosphate buffer supplemented with 2 mM EDTA (ethylenediaminetetraacetate). The IgG was then dialyzed against 2 liter of a buffer containing 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 145 mM NaCl, 2 mM EDTA (pH 7.4) and finally concentrated 4–5-fold by vacuum dialysis against a fresh 2 liter change of this buffer.

Under these conditions, IgG preparations were obtained with a ratio of 5 mol blocked sulfhydryl

group per mol of protein (M_r 150000). The sulfhydryl content was determined by the following two-step procedure. First, the protective acetyl group was removed by incubation (30 min at room temperature) of the IgG (appropriately diluted, if necessary, with the Hepes-NaCl-EDTA buffer) with 0.1 M hydroxylamine (final concentration) in a volume of 50 μl. The concentration of free sulfhydryl was then assayed by minor modification of the spectrophotometric procedure described by Grassetti and Murray [12]. Briefly, this involved the addition of 940 μl Hepes-NaCl-EDTA buffer to the above tubes, followed by 10 μl of 4 mM 4,4′-dithiodipyridine, and measurement of the absorbance at 324 nm after 30 minutes; standard curves were constructed with cysteamine hydrochloride.

Liposome preparation. Unless otherwise noted, liposomes were generated from dried lipid films composed of the following materials in the mol ratios indicated: dimyristoylphosphatidylcholine, 2: cholesterol, 1.5; dicetyl phosphate, 0.2; biotinyl-(AETA)$_2$-DMPE, 0.04; and IA-(AETA)$_2$-DMPE, 0.2. In some experiments, the same amount of either IA-DMPE or IA-AETA-DMPE was substituted for IA-(AETA)$_2$-DMPE to produce liposomes that contained 5 mol% of iodoacetylated derivative. Similarly, biotinyl-DMPE was occasionally used in place of biotinyl-(AETA)$_2$-DMPE at the equivalent density of 1 mol%. The films were dispersed by vortexing in sufficient Hepes-NaCl-EDTA buffer, and then sonicated (10 min at 22°C), to give a 10 mM DMPC suspension.

Precipitation of liposomes. The basic procedure for precipitation of biotinylated liposomes with avidin was the following. Tubes (12 × 75 mm) initially contained 40 μl of liposomes and sufficient Hepes-NaCl-EDTA buffer (plus any other additions as indicated in the figure legends) to give a total volume of 2.5 ml. The liposomes were precipitated upon incubation for 30 min at 40°C with 20 μl of egg white avidin (3.5 mg/ml buffer; Sigma). After centrifugation at room temperature for 10 min at 1000 × *g* (Beckman TJ-6 centrifuge), the liposomal pellet was dispersed by vortexing in 2.5 ml of Hepes-NaCl-EDTA buffer. The liposomal precipitate, which reappeared upon incubation as above without further avidin addition, was again collected by centrifugation; it was then subjected to another cycle of resuspension, incubation, and centrifugation. The washed precipitate was finally dispersed in 1 ml of Hepes-NaCl-EDTA buffer, and aliquots analyzed for phosphate content [10]. Results are expressed as percent of input liposomal DMPC recovered in the precipitate.

IgG binding to liposomes. As a consequence of the experiments described below, the following protocol was developed to measure IgG binding to liposomes. Appropriate amounts (usually 200 μg) of SAMSA-modified, iodinated IgG were treated for 30 min at room temperature with 0.01 M hydroxylamine in Hepes-NaCl-EDTA buffer (total volume, 100 μl; 12 × 75 mm tubes). 40 μl of liposome preparation and 60 μl of Hepes-NaCl-EDTA buffer were then added, and the tubes incubated with gentle rocking for the desired time (usually 16 h). The reaction was terminated with 50 μl of 50 mM iodoacetic acid in Hepes-NaCl-EDTA buffer. Each tube then received 2 ml of Hepes-NaCl-EDTA buffer and 200 μl of bovine serum albumin (10 mg per ml buffer), which was added to minimize the non-covalent absorption of IgG. The liposomes were subsequently precipitated with avidin as described in the preceding section, and the final dispersion of twice-washed liposomes was assayed for both radioactivity in a gamma counter and phosphate content. Results are expressed as μg of IgG bound (calculated from the specific radioactivity of the IgG preparation) per μmol of DMPC.

DNP-hapten binding to liposomes. Reaction tubes contained 270 μl of Hepes-NaCl-EDTA buffer, 80 μl of liposomes, and either 50 μl of 20 mM *N*,*N*′-diDNP-cystine or 50 μl of 32 mM *N*-DNP-cysteine. The latter was generated 30 min prior to use by the addition of 16 μmol of solid dithiothreitol per ml of 20 mM *N*,*N*′-diDNP-cystine. After incubation at room temperature for 17 h, free hapten was removed by prolonged dialysis of the reaction mixture, and the amount of bound hapten was determined spectrophotometrically (356 nm). Details of this procedure have been presented elsewhere [5,6]. Results are expressed as nmol of liposomal bound DNP per μmol of DMPC.

Miscellaneous. IgG concentration was calculated from the absorbance of solutions at 280 nm

using 15.5 as the extinction coefficient of a 1% (w/v) solution. Trinitrophenylated sheep erythrocytes were prepared by the method previously cited [13]. These hapten-substituted cells were used to determine the agglutination titer of rabbit IgG anti-DNP antibodies (Gateway Immunosera Co.; St. Louis, MO) after thiolation and as target cells for liposomal delivery (see legend to Fig. 6). The Fig. 6 legend also describes the procedure by which liposomes with bound anti-DNP antibodies were isolated by means of the cleavable derivative, biotinyl-S-S-DMPE.

Results and Discussion

Precipitation of liposomes

In this investigation, a precipitation procedure was used to recover liposomes with bound ligand (IgG antibody) from mixtures containing unreacted free ligand. Avidin, which has four high-affinity binding sites for biotin, was initially chosen as the precipitant in view of the finding by Bayer et al. [14] that it can readily combine with biotinylphosphatidylethanolamine. They therefore exploited this derivative, in conjunction with ferritin-avidin conjugates, to visualize the fate of a liposomal lipid constituent after interaction with membranes of various cells. Low concentrations of avidin (14 μg/ml) could, indeed, precipitate more than 60% of liposomal phospholipid when the liposomes were prepared with 1 mol% biotinyl-DMPE (Fig. 2). Precipitation of these liposomes was, however, inhibited by non-covalently absorbed proteins. Thus, a significantly higher avidin concentration (42 μg/ml) was required to produce equivalent precipitation in the presence of 80 μg IgG/ml (Fig. 2A), and no liposomes were precipitated by avidin (up to 167 μg/ml) in the presence of 800 μg bovine serum albumin/ml (Fig. 2B).

These results indicated that biotinylphosphatidylethanolamine could not be employed for our purpose, and that it may also have been less than ideal for the original purpose (see above) if endogenous membrane protein had similarly prevented access of ferritin-avidin conjugates to biotinylated phospholipid which had been incorporated into intracellular membranes. Biotinyl-$(AETA)_2$-DMPE was therefore synthesized to increase the

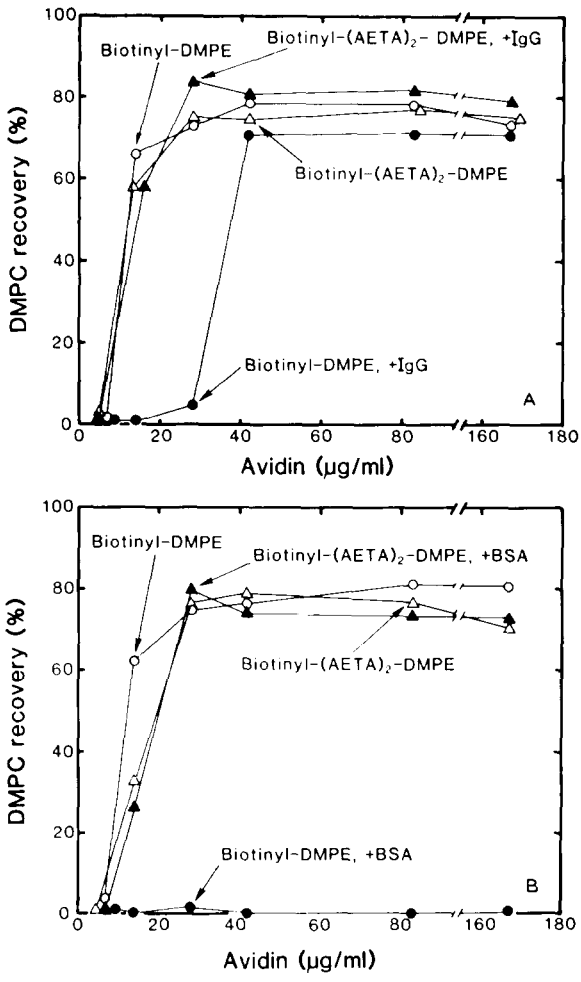

Fig. 2. Effect of spacer length on the avidin precipitability of biotinylated liposomes in the absence and presence of rabbit IgG (80 μg/ml; A) or bovine serum albumin (800 μg/ml; B). Precipitation was determined by the basic procedure described in Materials and Methods except that varying amounts of avidin were added as indicated on the abscissa.

distance between the biotinyl groups and the liposomal surface. The validity of this approach was demonstrated by the fact that neither IgG (Fig. 2A) nor bovine serum albumin (Fig. 2B) inhibited the avidin precipitability of liposomes containing 1 mol% of this derivative. Accordingly, biotinyl-$(AETA)_2$-DMPE was routinely incorporated into liposomes in subsequent experiments. In this regard, it should be noted that a long chain analog of *N*-hydroxysuccinimidylbiotin is now commercially available (Pierce), which should facilitate

synthesis of a biotinylated-phosphatidylethanolamine derivative having properties comparable to biotinyl-$(AETA)_2$-DMPE.

Thiolation of IgG

In the present study, *S*-acetylmercaptosuccinic anhydride (SAMSA) was used to introduce protected suflhydryl groups into IgG. While these experiments were in progress, Derksen and Scherphof [15] reported that succinimidyl-*S*-acetylthioacetate (SATA) could be similarly employed, and demonstrated the attachment of SATA-modified bovine γ-globulin to liposomes sensitized with MPB-PE. Duncan et al. [16] originally introduced succinimidyl-*S*-acetylthioacetate as a replacement for SAMSA because the latter alters the electrostatic properties of proteins as a consequence of the loss of one positive, and gain of one negative, charge for each sulfhydryl group that is inserted. However, such change does not affect the immunological activity of IgG (at least not the anti-dinitrophenyl antibodies used in some of the current experiments). Thus, the ability of these antibodies to agglutinate trinitrophenylated sheep erythrocytes was completely maintained under conditions in which increasing numbers of sulfhydryl groups were introduced by reaction with increasing concentrations of SAMSA (results not shown). Although a maximum of approx. 25 mol sulfhydryl/mol IgG can be inserted by reaction with excess SAMSA (40 mM), subsequent experiments were performed with IgG that contained significantly lower amounts of thiol residues (approx. 5 mol sulfhydryl/mol IgG obtained by incubation in the presence of 2.6 mM SAMSA). This was done to favor the binding of more IgG molecules to the liposomal surface via fewer thioether bonds as opposed to the binding of fewer molecules via a greater number of bonds.

Binding of IgG to liposomes

The main reason for using SAMSA rather than *N*-succinimidyl-3-(pyridyl-2-dithio)propionate (SPDP), which has been frequently employed in earlier studies [1,2], is the following (see also the extensive discussion in Ref. 15). Exposure of sulfhydryl residues in proteins modified with SPDP requires incubation with an excess of another thiol such as dithiothreitol; the latter then must be removed because its presence would competitively inhibit conjugation to liposomes. Such competition is, however, unlikely to occur with hydroxylamine, which is used to deblock thiol groups in proteins that have been derivatized with SAMSA. Thus, in mixtures containing SAMSA-modified IgG and liposomes sensitized with IA-$(AETA)_2$-DMPE, covalent binding of the protein is dependent on the addition, and can be carried out in the presence, of hydroxylamine (Fig. 3A). This time-course experiment further shows that the non-co-

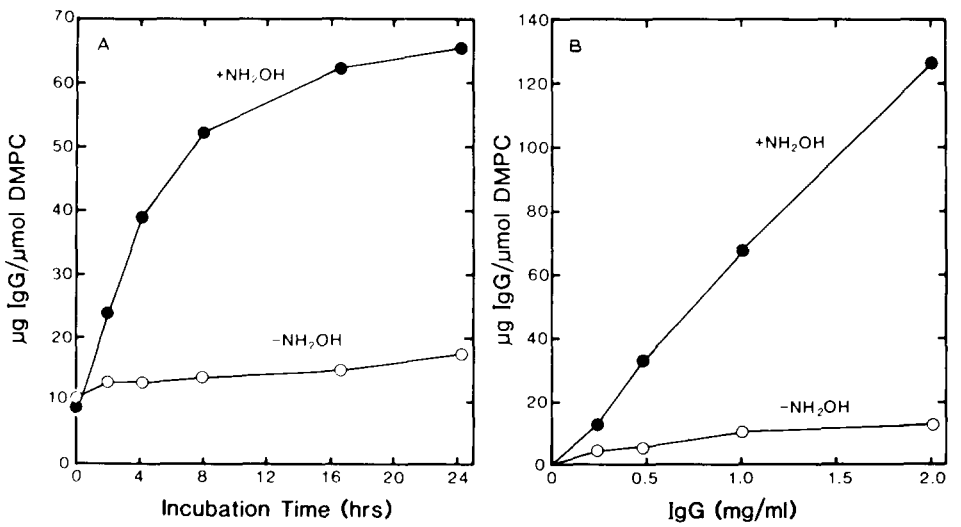

Fig. 3. Effect of incubation time (A) and IgG concentration (B) on IgG binding to liposomes. (n.b., in the standard protocol, the reaction mixture was incubated for 16 h and the IgG concentration was 1 mg/ml.)

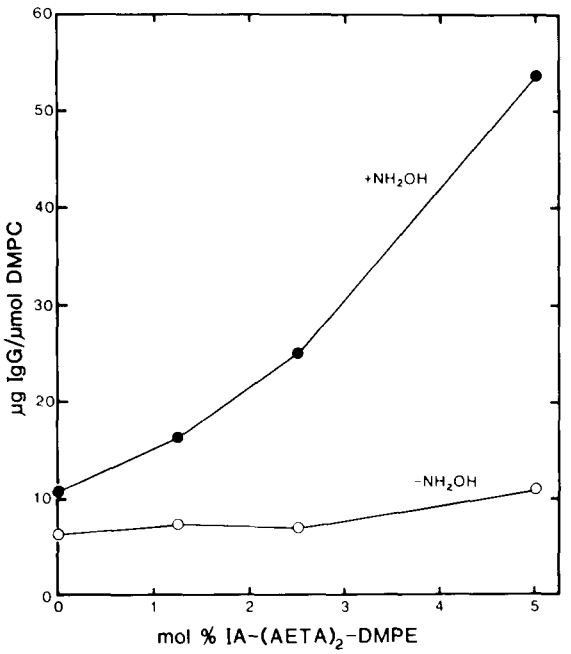

Fig. 4. Binding of IgG to liposomes prepared with different densities of IA-$(AETA)_2$-DMPE. (n.b., all liposomes contained 1 mol% biotinyl-$(AETA)_2$-DMPE; 5 mol% IA-$(AETA)_2$-DMPE is incorporated in the standard liposome preparation.)

valent absorption of IgG by liposomes is essentially instantaneous; the gradual increase observed over 24 h may, in fact, reflect covalent attachment due to the slow hydrolysis (hydroxylamine-independent) of thioacetyl residues. Subsequent experiments (not presented) demonstrated that hydroxylamine-dependent binding could be completely inhibited by incubation of the treated IgG with 12.5 mM iodoacetic acid for 20 min before the addition of liposomes, or by similar incubation of the liposomes with 12.5 mM cysteamine before their addition to hydroxylamine-treated IgG. These results indicate that binding does indeed occur via the formation of thioether bonds between IgG and liposomes.

Although the hydroxylamine-dependent binding was 70% completed in 8 h (Fig. 3A), we have, for convenience, generally allowed the reaction to proceed for 16 h (overnight incubation). Under these conditions, covalent binding was a function of both IgG concentration (Fig. 3B) and the density of IA-$(AETA)_2$-DMPE incorporated in the liposomes Fig. 4). It should be noted that, under the experimental conditions employed, saturation with either IgG or IA-$(AETA)_2$-DMPE was not attained.

Effect of spacer length

Most importantly, IgG attachment is dependent on the number of AETA residues separating the iodoacetyl group from the amino group of DMPE (Fig. 5A). Covalent IgG binding to liposomes sensitized with IA-DMPE was not observed in agreement with the results of Wolff and Gregoriadis [8], who had previously examined the interaction of liposomes, which contained this derivative, and either human serum albumin or IgG that had been substituted with SPDP (see Introduction). Liposomes prepared with 5 mol% IA-AETA-DMPE bound a measurable amount of IgG, although significantly less than the quantity covalently attached to liposomes sensitized with the same density of IA-$(AETA)_2$-DMPE. In fact, the latter may not represent the maximum amount of IgG which can be bound by alkylation, since liposomes containing the next higher homolog (IA-$(AETA)_3$-DMPE) have not yet been examined. Similarly, the possibility exists that liposomal antibody binding by formation of disulfide bonds or sulfhydryl addition across the maleimido double bond could be further increased with derivatives, which possess longer bridges than present in PDP-PE [1–3] or MPB-PE [4], respectively.

In striking contrast to IgG binding, the covalent association of a low-molecular weight hapten to liposomes via thioether linkages is not influenced by spacer length. As shown in Fig. 5B, essentially the same amount of *N*-DNP-cysteine was bound regardless of whether the liposomes were prepared with IA-DMPE, IA-(AETA)-DMPE, or IA-$(AETA)_2$-DMPE; the involvement of thioether bonds is indicated by the fact that none of the liposomes bound significant quantities of *N,N'*-diDNP-cystine. However, although hapten binding is not affected by spacer length, previous studies [13,5] have shown that liposomes differ markedly in their immunogenicity depending on the distance between the determinant and the bilayer surface. On this basis, we expect that liposomes, which have bound *N*-DNP-cysteinyl groups via IA-$(AETA)_2$-DMPE instead of IA-DMPE, will elicit a higher anti-DNP antibody response, and experiments to test this are now

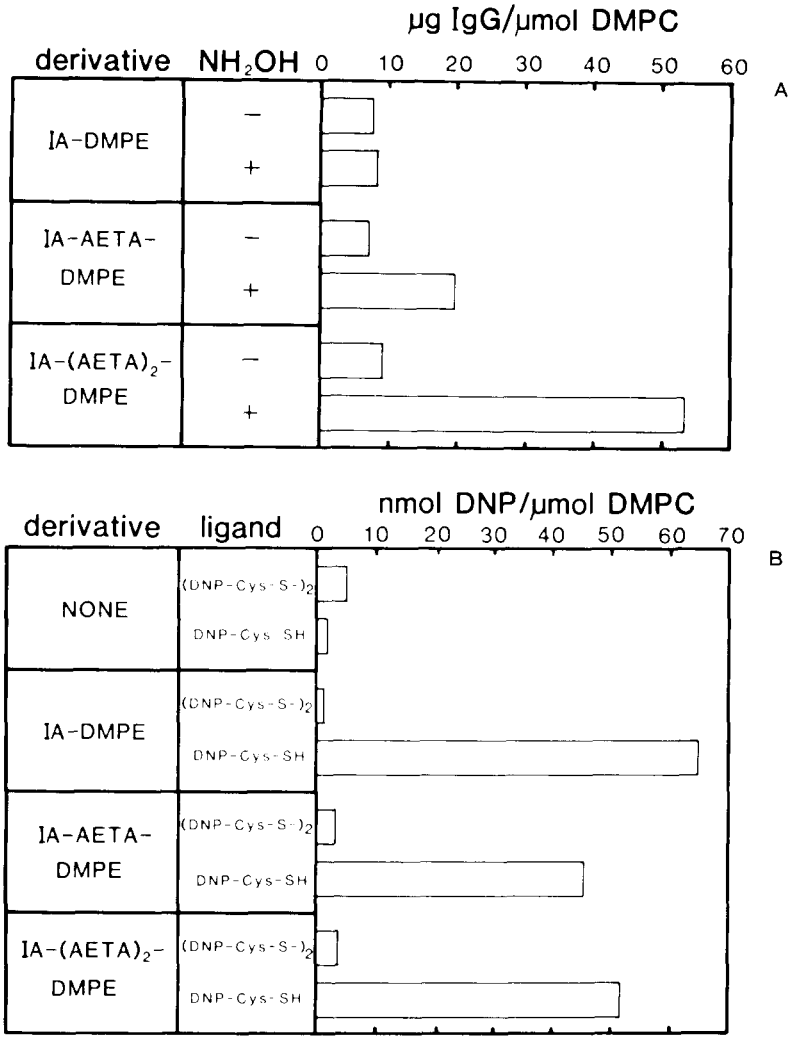

Fig. 5. Effect of spacer length on binding of IgG (A) and DNP-haptens (B) to liposomes. Liposomes were prepared with 5 mol% of the iodoacetylated DMPE derivatives indicated in the figure. (n.b., all liposomes contained 1 mol% biotinyl-$(AETA)_2$-DMPE.) *N,N'*-di-DNP-cystine is represented as $(DNP\text{-}Cys\text{-}S\text{-})_2$ and *N*-DNP-cysteine as DNP-Cys-SH.

underway. In this regard, it is noteworthy that we have not yet encountered evidence indicating that mice can mount a humoral response to AETA residues. This bears on one of the principal objectives of the current investigation: to devise a method by which sulfhydryl containing haptens can be stably attached to preformed liposomes via a nonimmunogenic bridge (see Introduction).

Targeted delivery of liposomes

The original synthesis of phosphatidylethanolamine derivatives for covalently linking antibodies to lipid bilayers (Refs. 1–4; see Introduction) subsequently enabled the laboratories of Papahadjopoulos and Leserman to demonstrate the antibody-mediated delivery of a drug (methotrexate), which was trapped in the aqueous compartments of liposomes, to selected target cells (see, for example Refs. 17–20). To circumvent the problem of drug leakage from such liposomes, we have recently synthesized methotrexate-γ-DMPE so that this potent cytotoxic agent could be anchored to liposomal bilayers; non-directed liposomes, which were sensitized with this derivative, can effectively inhibit the in vitro proliferation of both mouse and human cells [21,22]. These findings, in turn, have prompted an examination (in progress) of the antibody-directed targeting of liposomes, prepared with methotrexate-γ-DMPE, and, particularly, whether antibody direction may influence the pathway by which the phospholipid drug derivative enters cells and its metabolism.

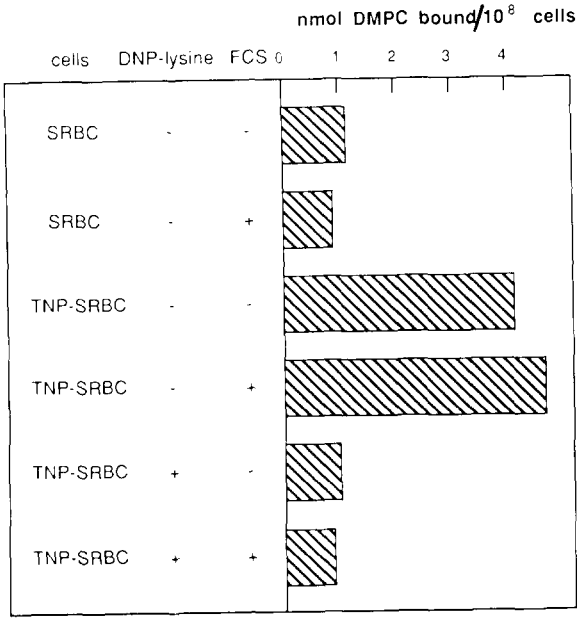

Fig. 6. Targeted delivery of liposomes mediated by rabbit IgG anti-DNP antibodies. Liposomes were prepared with 2.5 mol% IA-$(AETA)_2$-DMPE and 0.5 mol% biotinyl-S-S-DMPE (i.e., half the density of iodoacetylated and biotinylated derivatives usually incorporated), plus sufficient tritiated cholesterol to give $2 \cdot 10^6$ cpm/μmol DMPC. After incubation (standard protocol) with 200 μg of SAMSA-modified, non-radioactive, anti-DNP antibodies (5 mol sulfhydryl/mol IgG), the reaction mixture was left standing overnight at room temperature prior to precipitation of the liposomes with avidin (14 μg/ml). The thrice-washed liposomes were then resuspended in 950 μl of Hepes-NaCl-EDTA buffer (supplemented with 50 mM glycylglycine and adjusted to pH 8.2), plus 50 μl of 1 M dithiothreitol. This mixture was subsequently centrifuged (1000 $\times$ *g* for 15 min) to remove the small amount of liposomes that could not be dissociated from the avidin-biotin complex by reductive cleavage. Aliquots of the supernatant liposomes (containing 10 nmol DMPC) were added to 1 ml Hepes-NaCl-EDTA buffer containing 10^8 sheep erythrocytes (native or substituted with trinitrophenyl (TNP) groups) in the absence or presence of 0.5 mM *N*-ϵ-DNP-lysine and 5% fetal calf serum (FCS). After incubation for 2 h at room temperature, the erythrocytes were centrifuged as above, washed thrice with buffer, and then counted in a beta scintillation spectrometer to determine the quantity of liposomes (expressed as nmol DMPC) bound to the cells.

The relevance of the current experiments to this goal is illustrated by the use of a cleavable biotinylated-phosphatidylethanolamine derivative to isolated liposomes that bear covalently linked anti-DNP antibodies. Fig. 6 shows that these liposomes preferentially associate with sheep erythrocytes, which have been substituted with cross-reactive trinitrophenyl (TNP) residues; such association is inhibited completely by *N*-ϵ-DNP-lysine, further indicating that the antibodies have retained their immunological activity after being bound to liposomes via IA-$(AETA)_2$-DMPE.

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