Antibody Formation in Response to Liposomal Model Membranes Sensitized with N-Substituted Phosphatidylethanolamine Derivatives†

Kei-ichi Uemura, Robert A. Nicolotti, Howard R. Six, and Stephen C. Kinsky*

ABSTRACT: This investigation demonstrates that the immunogenicity of lipids, which is a known property of many naturally occurring glycosphingolipids and acidic phospholipids, can now be extended to include certain synthetic N-substituted phosphatidylethanolamine derivatives. Comparative experiments were performed with the dinitrophenylaminocaproyl derivatives of phosphatidylethanolamine (I), lysophosphatidylethanolamine (II), and glycerophosphorylethanolamine (III), and the fluorescein isothiocyanyl derivative of phosphatidylethanolamine (IV). Sera from immunized guinea pigs were assayed for their hemagglutination titer against sheep erythrocytes, which had been sensitized with trinitrophenyl or fluorescein residues, and the specificity of the reaction was confirmed with appropriate hapten inhibitors. In addition, we examined the ability of various immune sera to initiate complement-dependent release of trapped glucose marker from liposomal model membranes. Using these criteria, maximum antibody formation was observed when the immunogen preparation consisted of liposomes that had been actively sensitized with the amphipathic derivatives (I, II, or IV) prior to combination with complete Freund's adjuvant. The deacylated derivative (III) was not effective under these conditions, and passive sensitization with the fully acylated derivative (I) did not enhance its immunogenicity. These results are in accord with previous investigations of the structural features of dinitrophenylated phosphatidylethanolamine derivatives that enable them to sensitize liposomes to immune damage by antibody–complement; they suggest a correlation between the effectiveness of an immunogen preparation and the existence of lipid antigens in bilayer configuration. Liposomal model membranes, actively sensitized with N-substituted phosphatidylethanolamine derivatives, may constitute a useful alternative to conventional methods of obtaining specific antibodies to a hapten in which the latter is covalently linked to a high molecular weight water soluble carrier.

Immunization with natural membranes oftentimes elicits antibodies against lipid antigens. In this paper, we describe experiments showing that liposomal model membranes can provoke a similar immune response. Particularly significant is the fact that these liposomes contain as antigens certain N-substituted derivatives of phosphatidylethanolamine; to our knowledge, the immunogenicity of synthetic hapten–phospholipid conjugates has not been previously examined.

As background to this investigation, it is essential to briefly review some relevant immunological properties of these model membranes. Liposomes, which release trapped glucose marker in the presence of an appropriate antibody and complement source, have been employed extensively by this laboratory to examine the mechanism of complement dependent membrane damage (reviewed in Kinsky, 1972). Their applicability for this purpose was initially demonstrated using liposomes prepared from a total lipid extract of sheep erythrocytes which contained Forssman antigen as well as the phospholipids and neutral lipids localized in the cell membrane. In subsequent studies, such immunologically sensitive liposomes were generated by the incorporation of several natural occurring amphipathic substances into a lipid mixture of defined composition containing a phospholipid (lecithin or sphingomyelin), a sterol (cholesterol), and a charged amphiphile (dicetyl phosphate or stearylamine). This list of purified antigens included mammalian ceramides (such as Forssman, globoside I, and galactocerebroside), bacterial lipopolysaccharides (isolated from a variety of S and R form gram-negative organisms), and lipid A (obtained by degradation of the lipopolysaccharides).

More recently, we have synthesized a homologous series of dinitrophenylated derivatives of phosphatidylethanolamine and shown that a number of these compounds can be used in place of the natural lipid antigens to passively and/or actively sensitize liposomes to immune damage by antibody–complement (Uemura and Kinsky, 1972; Six *et al.*, 1973). Passive sensitization means that the derivative was added after the model membranes had been formed; this procedure proved effective only with Dnp-lysoPE.[1] Active sensitization means that the derivative was present at the time the liposomes were generated; this procedure worked not only with Dnp-lysoPE but also with Dnp-PE and Dnp-Cap-PE. In contrast, Dnp-GPE (which lacks both fatty acids of the parent phospholipid, PE) was ineffective either actively or passively, although liposomes prepared under conditions of active sensitization were visibly yellow due to trapping of this compound in the aqueous liposomal compartments. This finding indicated that an important prerequisite for liposomal sensitization was the retention of sufficient amphipathic character permitting proper insertion of the derivative into the liposomal bilayers in such

† From the Departments of Pharmacology and Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110. *Received November 13, 1973.* Supported by U. S. Public Health Service Research Grant AI-09319 and Training Grant NS-05221.

[1] Abbreviations used are: PE, phosphatidylethanolamine; lysoPE, lysophosphatidylethanolamine; GPE, glycerophosphorylethanolamine. Dnp-PE, Dnp-lysoPE, Dnp-GPE, Dnp-Cap-PE, Dnp-Cap-lysoPE, and Dnp-Cap-GPE represent the corresponding N-substituted derivatives with either a 2,4-dinitrophenyl (Dnp) or a 2,4-dinitrophenylaminocaproyl (Dnp-Cap) group; the complete structures of these compounds are illustrated in Uemura and Kinsky (1972) and Six *et al.* (1973). Fl-PE denotes the fluorescein isothiocyanyl derivative of phosphatidylethanolamine.

a way that its dinitrophenylated polar region is accessible to antibody (see discussion in Kinsky, 1972).

It is important to note that, in the preceding investigations, glucose release from the model membranes was initiated by purified anti-dinitrophenyl immunoglobulins obtained from rabbits immunized with dinitrophenylated hemocyanin. Indeed, the synthesis of Dnp-Cap-PE was originally undertaken because the polar region of this derivative has a closer structural resemblance to ϵ-Dnp-lysine residues (the predominant antigenic determinants in the immunogen) than does Dnp-PE, and thus enabled us to compare the efficacy of low affinity IgG and IgM antibodies in producing complement-dependent damage of liposomes (Six *et al.*, 1973). However, for future studies, it seemed desirable to employ antibodies that were specifically directed toward the same compound used to sensitize the liposomes. These considerations prompted the current experiments to determine: (a) if dinitrophenylated phosphatidylethanolamine derivatives were immunogenic *per se;* (b) if the derivatives were immunogenic after incorporation into liposomes; (c) if the same structural features were necessary for immunogenicity as for liposomal sensitization to antibody–complement; (d) if other N-substituted derivatives could also function as antigens.

Experimental Section

Dinitrophenylated Derivatives. Dnp-PE and Dnp-Cap-PE were synthesized and purified as described previously (Uemura and Kinsky, 1972; Six *et al.*, 1973). Dnp-Cap-GPE was derived from Dnp-Cap-PE by the same alkaline hydrolysis procedure that has been used to convert Dnp-PE into Dnp-GPE (Uemura and Kinsky, 1972).

Dnp-Cap-PE was also employed as the substrate for the synthesis of Dnp-Cap-lysoPE in view of its succeptibility to enzymatic hydrolysis by snake venom (*Crotalus*) phospholipase A. Immediately before use, a commercial preparation of the enzyme (1 mg/ml, *ca.* 200 U/mg; Boehringer-Mannheim Corp., New York, N. Y.) was carefully adjusted to pH 7.5 with a small amount of 2 N KOH; 200 μl of the enzyme solution (*ca.* 175 μg of protein) was added to a biphasic reaction mixture that contained 1.5 ml of a diethyl ether solution of Dnp-Cap-PE (10 mM) and 0.55 ml of an aqueous solution of NaCl (300 mM), $CaCl_2$ (30 mM), EDTA (1.5 mM), and Tris (15 mM), pH 7.5) (*cf.* Wells and Hanahan, 1969). The tube was gently shaken at 30° with monitoring of the pH of the aqueous phase to make sure that it remained above 7; the progress of the reaction was followed by periodic chromatographic analysis of small aliquots (*ca.* 5 μl) of the organic phase on silica gel plates (0.25 mm thickness; Brinkmann Instruments, Des Plaines, Ill.) in a solvent system of chloroform–methanol–water (70:30:5 by volume). After 2 hr, more enzyme solution (80 μl) and buffered salt solution (170 μl) were added, and incubation was continued for 10 hr. At this time, visual estimation indicated an approximate 80% decrease in the amount of Dnp-Cap-PE with the concomitant appearance of a new yellow compound of anticipated slower mobility and the accumulation of free fatty acids at the solvent front.

The entire reaction mixture was then extracted twice with 2-ml portions of chloroform and twice with 2-ml portions of diethyl ether to remove virtually all of the yellow color from the aqueous phase. The organic extracts were combined, and the solvents were removed under a stream of nitrogen and subsequently dried *in vacuo*. The residue was redissolved in 0.8 ml of a 1:1 mixture of chloroform–methanol and this solution was applied to the origin of preparative thin-layer plates (20 × 20 cm, 2 mm thickness; Brinkmann). After development in the chromatographic solvent system described above, two distinct yellow bands were visible with R_F values of 0.59 and 0.45, respectively. Both bands contained significant amounts of phosphate but the faster one had the same mobility as previously reported for Dnp-Cap-PE (R_F of 0.60; Six *et al.*, 1973) and therefore corresponded to undegraded initial substrate. The desired product was isolated from the slower band by transfer of the latter to a chromatography column containing a small bed of washed Unisil (Clarkson Chemical Co., Williamsport, Pa.) to remove silica gel fines upon elution of the yellow compound with a 1:1 mixture of chloroform–methanol (Six *et al.*, 1973); the eluate was taken to dryness under reduced pressure at 40° and redissolved in 25 ml of chloroform.

Spectrophotometric analysis for dinitrophenyl residues (using a molar extinction coefficient of 17,000 at 345 nm) and phosphate determination (by a minor modification of the procedure of Gerlach and Deuticke (1963)) indicated a 66% yield of Dnp-Cap-lysoPE with a Dnp:phosphate ratio of 1.09. Purity of the compound was also confirmed by thin-layer chromatography in several solvent systems in which only a single yellow spot was observed and no contaminants were detectable by ninhydrin spray or iodine vapor; in this regard, it should be noted that Dnp-Cap-lysoPE had essentially the same mobility as Dnp-lysoPE which was employed as a reference compound and whose synthesis from dinitrofluorobenzene and lysophosphatidylethanolamine has been described elsewhere (Uemura and Kinsky, 1972). Moreover, identification of the new phospholipid derivative as Dnp-Cap-lysoPE was justified by the fact that low concentrations produced rapid and complete hemolysis of erythrocytes in contrast to the behavior of either Dnp-Cap-PE or Dnp-Cap-GPE (unpublished experiments).

Fluorescein Isothiocyanyl Derivative of Phosphatidylethanolamine. Egg phosphatidylethanolamine (Cyclo Chemical, Los Angles, Calif.) (100 μmol) was dissolved in 10 ml of methanol containing 100 mM triethylamine; another solution was prepared containing 300 μmol of fluorescein isothiocyanate (isomer I; Sigma Chemical Co., St. Louis, Mo.) in 40 ml of methanol. The two solutions were mixed and incubated at room temperature under a nitrogen atmosphere with stirring; after 15 hr, the reaction had gone to completion as indicated by the disappearance of all ninhydrin positive material. The mixture was taken to dryness under reduced pressure and the residue subjected to preparative thin layer separation in chloroform–methanol–water (70:30:5 by volume) after being first redissolved in *ca.* 2 ml of a 1:1 chloroform–methanol mixture. When viewed with an ultraviolet light (maximum emission at 360 nm), the plates revealed several faintly and two intensely fluorescent bands. One of the latter (R_F of 0.68) corresponded to unreacted fluorescein isothiocyanate; the other (R_F of 0.44) was tentatively identified as Fl-PE because it was the only band which contained phosphate. The fluorescent material in this slower band was eluted with chloroform–methanol (1:1) as described above; this solvent mixture was also used to prepare stock solutions because Fl-PE, unlike the dinitrophenylated phosphatidylethanolamine derivatives, was not completely soluble in chloroform.

Complete details of the procedures employed to characterize Fl-PE will be described elsewhere (R. A. Nicolotti and S. C. Kinsky, manuscript in preparation) but the following should be noted. Phosphate determination indicated 80% yield (based on the initial amount of phosphatidylethanolamine) of a fluorescent compound that appeared pure when small quanti-

ties (0.4 nmol on the basis of phosphate content) were analyzed by thin-layer chromatography. However, when larger amounts (50 nmol) were examined chromatographically, the extreme sensitivity of the fluorescent detection procedure still revealed the presence of several minor contaminants presumably resulting from oxidation. Nevertheless, from measurements of the relative absorbancies of the major compound (Fl-PE) and the total contaminants after elution of the appropriate areas from the silica gel plates, it was determined that the Fl-PE was at least 90% pure.

Liposomes. Except where noted, all liposome preparations were actively sensitized by incorporation of appropriate amounts of the N-substituted phosphatidylethanolamine derivative into a basic lipid mixture containing sphingomyelin, cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.2, respectively. When the liposomes were to be used in the spectrophotometric assay for glucose release, they were generated by the method employed in earlier investigations (see, *e.g.*, Uemura and Kinsky, 1972; Six *et al.*, 1973). When the liposomes were to be used for immunization, this procedure was slightly modified in that the dried lipid film was dispersed in a sufficient volume of 150 mM NaCl (instead of 300 mM glucose) to yield a 25 mM (instead of a 10 mM) phospholipid suspension; also, the dialysis step to remove untrapped marker was omitted. As in previous publications, liposomes prepared under conditions of active sensitization are characterized on the basis of the per cent antigen (relative to sphingomyelin) that was present at the time the model membrane was formed; for example, liposomes actively sensitized with 10% Dnp-Cap-PE indicate that 100 nmol of this derivative were incorporated into the basic lipid mixture for every μmol of sphingomyelin.

Immunization. The liposomes were injected into the footpads of guinea pigs directly or after mixture with an equal volume of complete or incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). The guinea pigs (Eldridge Rabbitry, Antonia, Mo.) were females of random breed weighing approximately 300 g. Each animal received 50 μl/footpad of liposomes alone or 100 μl/footpad of liposome–adjuvant combination; under these conditions, immunization with 10% actively sensitized liposomes corresponded to a total dose of 0.5 μmol of N-substituted phosphatidylethanolamine derivative. The same procedure was employed when the guinea pigs were immunized with free derivative (*i.e.*, not incorporated into liposomes); in this case, the immunogen was prepared from a 2.5 mM suspension of the compound in isotonic saline. Blood was withdrawn by cardiac puncture 2–3 weeks later; the resulting sera were decomplemented by heating for 30 min at 56° and absorbed twice with one-fifth their volume of washed packed sheep erythrocytes (Mogul Diagnostics, East St. Louis, Ill.) prior to antibody titration.

Hemagglutination Assay. Anti-dinitrophenyl antibodies were detected by their ability to agglutinate sheep erythrocytes which had been sensitized with 2,4,6-trinitrophenyl residues by the procedure of Rittenberg and Pratt (1969) as modified by Hannestad *et al.* (1972). Fluorescein-sensitized erythrocytes were used to detect anti-fluorescein antibodies; they were prepared by a minor variation of the method of Matuhasi *et al.* (1971) in which 1 volume of washed packed cells was incubated for 4 hr at 4° with 2 volumes of fluorescein isothiocyanate solution (0.50 mg/ml of 150 mM NaCl–50 mM sodium carbonate (pH 9.0)). Before use, the cells were extensively washed with phosphate-buffered saline (77 mM NaCl–75 mM sodium phosphate (pH 7.2)) and suspended in the same buffer (supplemented with 1% fetal calf serum) at a 1% concentration.

Antibody titer was determined by hemagglutination assay in plates with V-shaped wells (Cooke Engineering; Alexandria, Va.). Each well contained initially 25 μl of the immune serum to be tested (twofold serial dilutions prepared in phosphate-buffered saline) and, when required by a particular experiment, 25 μl of a solution of the appropriate hapten inhibitor (also dissolved in this buffer); 25 μl of either sensitized or control (unsensitized) erythrocytes was then added. Plates were read after standing for 30 min at 37° and subsequent overnight incubation at room temperature. Each point in the figures denotes the antibody titer of serum from a single animal expressed as the logarithm (base 2) of the minimum dilution required to deposit the erythrocytes as a mat.

Miscellaneous Materials and Methods. Lipopolysaccharide from *Salmonella minnesota* (S form) was kindly provided by Dr. O. Lüderitz, Max-Planck Institute for Immunobiology, Freiburg, W. Germany. Dinitrophenylation of ovalbumin was performed following the procedures of Little and Eisen (1967); the fluorescein and tetramethylrhodamine derivatives of bovine serum albumin were prepared by minor modification of the methods described by Kawamura (1969) for the synthesis of fluorescent antibodies. The preparations used in this investigation contained approximately 9.7 dinitrophenyl, 6.3 fluorescein, and 7.8 tetramethylrhodamine residues per mole of protein as determined by spectrophotometric analysis. Release of trapped glucose marker from liposomes in the presence of immune sera and guinea pig complement was determined spectrophotometrically by the same assay employed in all of our previous studies (see, *e.g.*, Uemura and Kinsky (1972), Six *et al.* (1973), and references cited therein). Antibody absorption by liposomes was determined by the procedure described in the legend to Figure 5.

Results

Immune Response to Liposomes Sensitized with Dinitrophenyl Derivatives. Figure 1 shows that no anti-dinitrophenyl antibodies were produced when guinea pigs were immunized with a combination of complete Freund's adjuvant and unsensitized sphingomyelin–cholesterol–dicetyl phosphate liposomes. However, antibodies were elicited under these conditions when the liposomes were actively sensitized with Dnp-Cap-PE. This response was dose dependent: immunization with 10% sensitized liposomes (containing 0.5 μmol of Dnp-Cap-PE) yielded sera with a titer approximately 2^5 higher than did immunization with 1% sensitized liposomes. As indicated in Figure 1, sera of comparable titer were obtained when the guinea pigs received 50 μg of dinitrophenylated ovalbumin (containing 0.011 μmol of Dnp residues).

Figure 1 also shows that immunization with liposomes, which had been actively sensitized with 10% Dnp-PE, does not produce sera with a high titer. This result was not entirely unanticipated on the basis of other investigations. Davie and Paul (1972) have recently reported that, during development of the immune response in guinea pigs to dinitrophenylated guinea pig albumin, parallel increases occur in the avidity of lymphocytes for ε-Dnp-lysine and serum antibody affinity. We have previously demonstrated (Uemura and Kinsky, 1972; Six *et al.*, 1973) that Dnp-PE sensitized liposomes do not interact with low affinity rabbit anti-dinitrophenyl antibodies (K_0 for ε-Dnp-lysine of 10^5 l. mol^{-1}) which are formed early after immunization with dinitrophenylated hemocyanin (Eisen and Siskind, 1964). In contrast, the low affinity antibodies produce complement dependent damage of, and are bound by, liposomes sensitized with Dnp-Cap-PE. Accord-

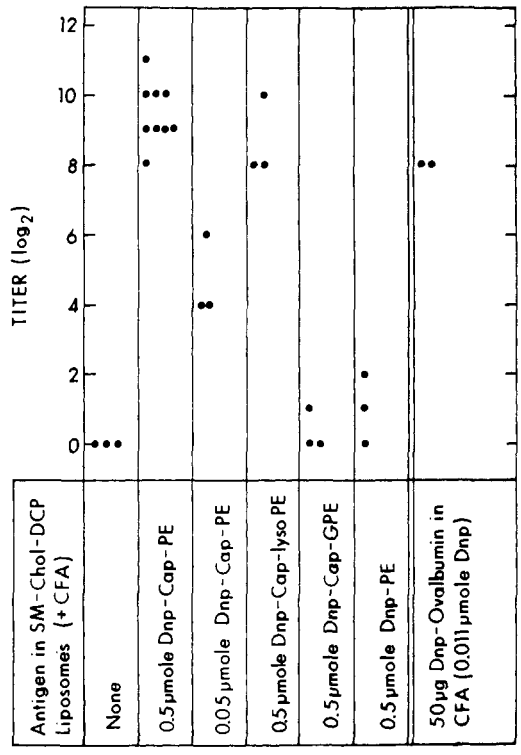

FIGURE 1: Immunogenicity of sphingomyelin–cholesterol–dicetyl phosphate (SM–Chol–DCP) liposomes, which were actively sensitized with various dinitrophenylated phosphatidylethanolamine derivatives, and mixed with complete Freund's adjuvant (CFA). The amounts of antigen specified in the figure correspond to the total immunizing dose of dinitrophenylated derivative received by each guinea pig; unsensitized liposomes (no antigen) contained 10% PE instead of a dinitrophenylated compound. For comparative purposes, animals were also immunized with dinitrophenylated ovalbumin; see text for additional details.

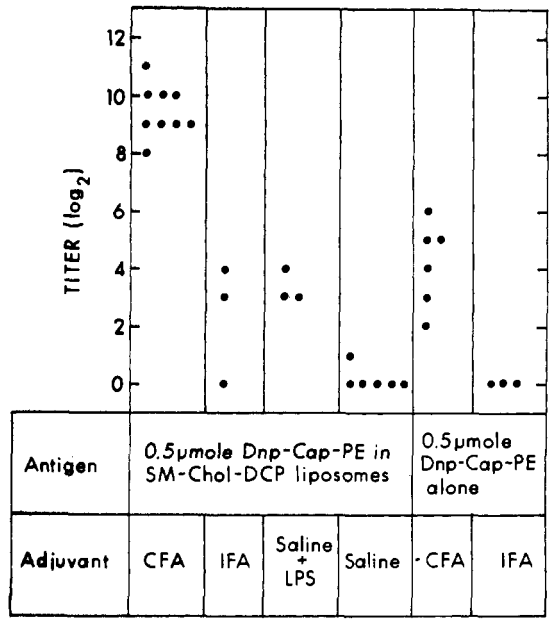

FIGURE 2: Adjuvant requirement for immunogenicity of free Dnp-Cap-PE or sphingomyelin–cholesterol–dicetyl phosphate (SM–Chol–DCP) liposomes which were actively sensitized with 10% Dnp-Cap-PE. Prior to immunization, the antigen preparations (sensitized liposomes or unincorporated derivative) were mixed with an equal volume of either complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), or the saline suspension of liposomes was supplemented with lipopolysaccharide (LPS) at a final concentration of 400 μg/ml.

ingly, the failure of Dnp-PE sensitized liposomes to induce as strong an immune response as Dnp-Cap-PE sensitized liposomes may reflect a correponding difference in binding of these derivatives to lymphocyte membrane immunoglobulin receptors.

In this regard, it is important to note (Figure 1) that liposomes actively sensitized with Dnp-Cap-lysoPE were as effective as those sensitized with Dnp-Cap-PE in provoking an immune response. In contrast, immunization with Dnp-Cap-GPE sensitized liposomes did not lead to antibody production. These results are particularly significant because they demonstrate that the immunogenicity of liposomes requires incorporation of an amphipathic derivative, *i.e.*, the same structural features that are necessary for active sensitization of liposomes to immune damage by antibody–complement.

Response to Other Immunogen Preparations. In the preceding experiments, the guina pigs were immunized with Dnp-Cap-PE sensitized liposomes in complete Freund's adjuvant (immunogen abbreviated Dnp-Cap-PE (L)/CFA; see footnote 2) because this procedure consistently yielded sera with the highest hemagglutination titer. However, as illustrated in Figure 2, we have occasionally observed that lower titer sera could be obtained with sensitized liposomes in incomplete Freund's adjuvant (Dnp-Cap-PE (L)/IFA). Immunization with sensitized liposomes in isotonic saline (Dnp-Cap-PE (L)/saline) was not effective but the failure of these preparations to elicit antibodies could be partially abrogated by the inclusion of lipopolysaccharide.

Figure 2 also shows that anti-dinitrophenyl antibodies were not formed upon immunization with unincorporated antigen combined with incomplete Freund's adjuvant (Dnp-Cap-PE/IFA) but were elaborated when the free antigen was administered with complete adjuvant (Dnp-Cap-PE/CFA). Thus, it was considered essential to establish whether the higher titer obtained with Dnp-Cap-PE (L)/CFA as immunogen was not simply due to the presence of sphingomyelin, cholesterol, and dicetyl phosphate (*i.e.*, the basic liposomal components). This possibility was rendered unlikely by the finding (Figure 3) that sensitization of liposomes under passive conditions does not increase the titer over that produced by Dnp-Cap-PE alone (no lipids). This observation is entirely consistent with our earlier contention that such compounds only function under active conditions in sensitizing liposomes to immune damage due to the fact that they possess a very low critical micelle concentration which does not favor insertion into preformed lipid bilayers. Moreover, the preceding results suggest that liposomal structure may largely survive emulsification with the adjuvant; if the model membranes had been extensively disrupted, we would have anticipated little difference in the immunogenicity of actively and passively sensitized liposomes.

In experiments designed to determine the influence of liposomal phospholipid composition, we observed one important difference between the response of these model membranes to antibody–complement and their effectiveness as immunogens. Figure 3 shows that immunization with actively sensitized lecithin–cholesterol–dicetyl phosphate liposomes did not result in a titer greater than that obtained with free Dnp-Cap-PE. However, these liposomes are susceptible to

[2] The shorthand notation employed to specify the composition of various immunogen preparations indicates the antigen in the "numerator" and, if followed by (L), means that it was incorporated into liposomes under conditions of active sensitization; the adjuvant is indicated in the "denominator."

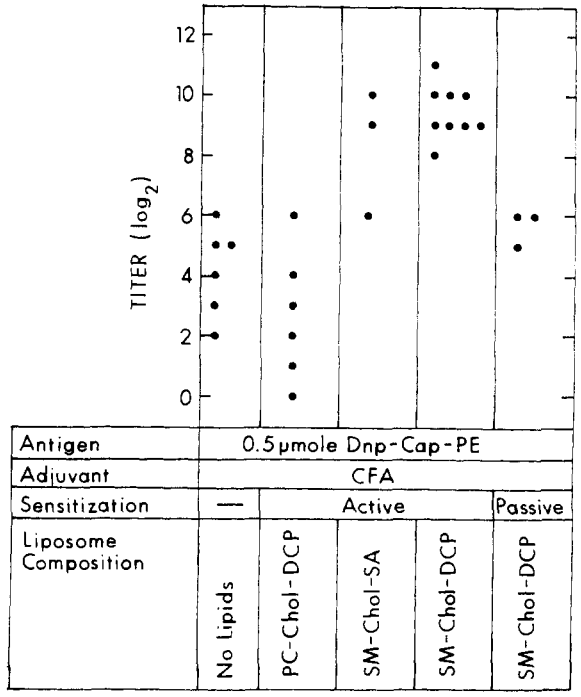

FIGURE 3: Effect of liposomal composition and sensitization conditions on immunogenicity. Sphingomyelin–cholesterol–dicetyl phosphate (SM–Chol–DCP) liposomes were actively sensitized with 10% Dnp-Cap-PE as described in the text. The identical procedure was employed for the active sensitization of liposomes in which lecithin (*i.e.*, phosphatidylcholine, PC) was substituted for sphingomyelin or stearylamine (SA) was substituted for dicetyl phosphate. Passive sensitization was performed by incubation for 30 min at room temperature of 100 μl of 50 mM liposomes (prepared from the basic lipid mixture of sphingomyelin, cholesterol, and dicetyl phosphate) with 100 μl of 5 mM Dnp-Cap-PE (suspended in 150 mM NaCl). Prior to immunization, both actively and passively sensitized liposomes were mixed with an equal volume of complete Freund's adjuvant (CFA) so that each guinea pig received 0.5 μmol of Dnp-Cap-PE. For comparative purposes (see text), an equivalent amount of free antigen in CFA (*i.e.*, not incorporated into liposomes) was also administered.

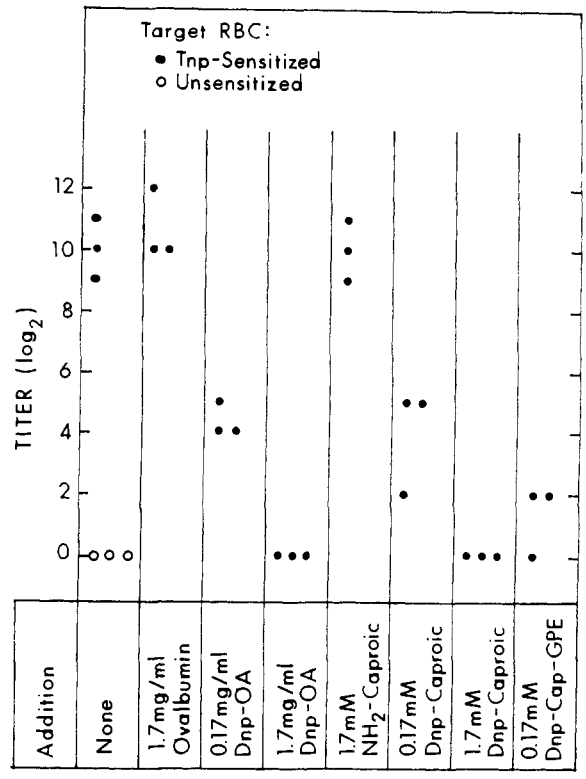

FIGURE 4: Effect of various hapten inhibitors on the hemagglutination titer of sera obtained from guinea pigs immunized with sphingomyelin–cholesterol–dicetyl phosphate liposomes, which were actively sensitized with 10% Dnp-Cap-PE, and mixed with complete Freund's adjuvant. The target cells were either unsensitized or sensitized with trinitrophenyl (Tnp) groups, and the materials tested for inhibition were incorporated into the wells at the final concentrations indicated in the figure. Abbreviations used are: Dnp-OA, dinitrophenylated ovalbumin; NH_2-caproic, ε-amino-*n*-caproic acid; Dnp-caproic, *N*-dinitrophenyl-ε-amino-*n*-caproic acid.

complement-dependent damage (indicating incorporation of Dnp-Cap-PE into the bilayers) and, in common with lecithin liposomes that have been sensitized with other amphipathic antigens, oftentimes release more trapped glucose marker than the corresponding sphingomyelin liposomes (Kinsky, 1972). This phenomenon has been attributed to a greater stability of sphingomyelin–cholesterol as opposed to lecithin–cholesterol bilayers; the greater stability of sphingomyelin liposomes may partially explain why they are better immunogens than lecithin liposomes. Figure 3 also demonstrates that the net charge of sphingomyelin liposomes has no appreciable effect on their immunogenicity; thus, high titer sera were produced when Dnp-Cap-PE was employed to actively sensitize postively charged liposomes prepared with stearylamine substituted for dicetyl phosphate (Figure 3).

Additional Properties of Immune Sera. Figure 4 shows that high titer sera from animals immunized with Dnp-Cap-PE (L)/CFA did not agglutinate unsensitized erythrocytes. Agglutination of trinitrophenyl sensitized cells could be inhibited completely by appropriate concentrations of dinitrophenylated ovalbumin and dinitrophenylaminocaproic acid; neither ovalbumin nor aminocaproic acid had any effect indicating that inhibition was specific for the dinitrophenyl group. As expected on the basis of its close structural similarity to the antigen, Dnp-Cap-GPE has proven to be the most potent hapten inhibitor so far tested (Figure 4).

Precipitin analysis indicated that the high titer immune sera employed in the preceding experiment contained anti-dinitrophenyl antibodies in the range of 180–300 μg/ml. A value of 265 μg/ml was obtained by absorbing pooled sera with Dnp-Cap-PE sensitized liposomes and measuring the amount of protein bound to the washed liposomal pellet (Figure 5); the validity of this procedure is indicated by the fact that essentially no protein was attached to unsensitized liposomes. Subsequent experiments have shown that the antibodies can be eluted from the liposomes with solutions of Dnp-Cap-GPE or other haptens, and this is currently being used as a preliminary step in their purification so that they may be eventually characterized in terms of immunoglobulin class, affinity constant, heterogeneity index, etc.

Effect of Immune Sera on Liposomes in the Presence of Complement. Striking confirmation of the results obtained by hemagglutination assay was provided by comparing the ability of various immune sera to initiate complement-dependent release of glucose from liposomes sensitized with Dnp-Cap-PE. Figure 6 shows that maximum marker loss was observed with very small quantities (*ca.* 6 μl) of sera derived from animals immunized with Dnp-Cap-PE (L)/CFA (curve 1). Significantly greater amounts (more than 50 μl) were necessary in the case of sera obtained from guinea pigs immunized with either Dnp-Cap-PE/CFA (curve 2) or Dnp-Cap-PE (L)/IFA (curve 3). Glucose release did not occur in the presence of sera from animals immunized with the following: Dnp-Cap-GPE (L)/CFA (curve 4); PE (L)/CFA (curve 5); Dnp-Cap-PE

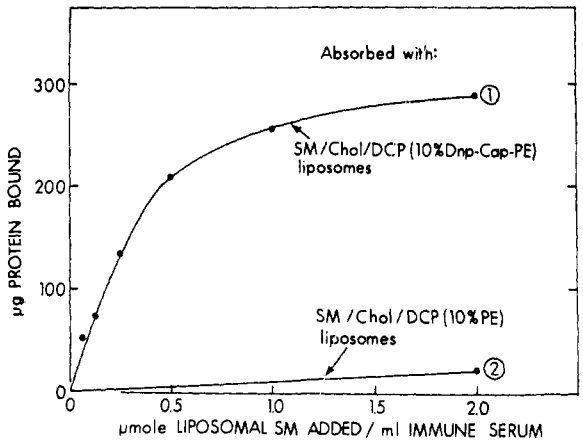

FIGURE 5: Absorption of immune serum by liposomes. Pooled serum was obtained from guinea pigs that had been immunized with sphingomyelin–cholesterol–dicetyl phosphate (SM–Chol–DCP) liposomes, actively sensitized with 10% Dnp-Cap-PE, in complete Freund's adjuvant. An identical liposome preparation (curve 1), as well as one sensitized with 10% PE (curve 2), was used for absorption by the following procedure. Immune serum (400 μl) was added to test tubes containing various amounts of liposomes to yield the ratios indicated on the abscissa and sufficient 150 mM NaCl to give a final volume of 2 ml; control tubes contained liposomes but no immune serum. After incubation for 2 hr at room temperature, the liposomes were recovered by centrifugation (27,000*g* for 30 min at 2°) and washed once with 1 ml of cold 150 mM NaCl. Protein was eluted from the washed liposomal pellet with 1 ml of 0.1 N NaOH and the eluate clarified by centrifugation as above. The differences in absorbancy at 278 and 360 nm between experimental and control eluates was measured and from these values the amount of protein (antibody) was calculated using the empirical formula of Eisen *et al.* (1967).

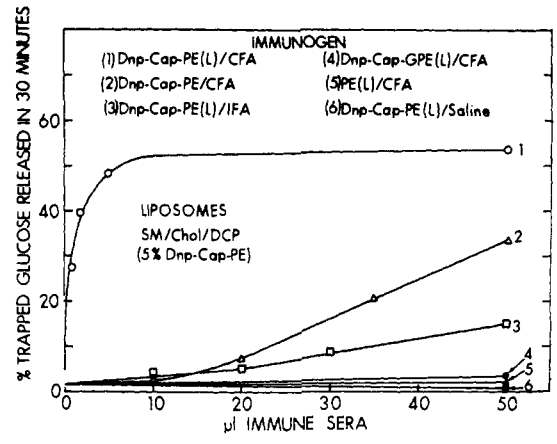

FIGURE 6: Complement-dependent release of glucose from Dnp-Cap-PE sensitized liposomes. Sphingomyelin–cholesterol–dicetyl phosphate (SM–Chol–DCP) liposomes, actively sensitized with 5% Dnp-Cap-PE and containing trapped glucose marker, were prepared as described in Uemura and Kinsky (1972) and Six *et al.* (1973). These papers should also be consulted for details of the spectrophotometric assay by which marker release was measured in the presence of 125 μl of guinea pig serum (complement source) and the various amounts of immune sera (antibody source) indicated on the abscissa. Pooled immune sera were obtained from guinea pigs immunized with the preparations, each containing 0.5 μmol of PE or dinitrophenylated phosphatidylethanolamine derivative, specified in the figure (see footnote 2).

(L)/saline (curve 6). Although not indicated in the figure, it is important to note that essentially no loss of trapped marker (less than 2.5%) was observed when any of the immune sera (50 μl) were assayed in cuvets that contained guinea pig serum which had been decomplemented by heating at 56° for 30 min. Furthermore, none of the immune sera promoted complement-dependent glucose release from unsensitized liposomes and loss of marker required higher amounts when the liposomes were sensitized with a dinitrophenylated phospholipid that was not the same as the derivative employed for immunization. For example, 50 μl of sera (obtained from animals immunized with Dnp-Cap-PE (L)/CFA) released only 19% of trapped glucose from liposomes sensitized with 5% Dnp-PE (*cf.* Figure 6, curve 1).

Immunization with Fl-PE Sensitized Liposomes. The efficacy of Dnp-Cap-PE (L)/CFA as an immunogen prompted analogous experiments with at least one other N-substituted phosphatidylethanolamine derivative to determine if it could also function as an antigen when incorporated into liposomes. For this purpose, we synthesized Fl-PE because it has been previously demonstrated with rabbits that fluorescein protein conjugates can elicit anti-fluorescein antibodies that can also be detected by hemagglutination assay using sensitized erythrocytes (Matuhasi *et al.*, 1971). The most significant findings to date are summarized in Figure 7 showing that anti-fluorescein antibodies are indeed produced in guinea pigs after immunization with Fl-PE (L)/CFA. Antibody formation is dependent upon the incorporation of Fl-PE into the liposomes as indicated by the fact that sera obtained from animals immunized with Dnp-Cap-PE (L)/CFA do not agglutinate fluorescein-sensitized erythrocytes. Conversely, the sera of guinea pigs immunized with Fl-PE (L)/CFA cannot agglutinate either unsensitized or Tnp sensitized cells. The most convincing evidence for the specificity of these antibodies is the observation that a conjugate of bovine serum albumin with tetramethylrhodamine, which is structurally quite similar to fluorescein, does not function as a hapten inhibitor whereas, under identical conditions, a conjugate with fluorescein blocks agglutination completely.

Discussion

Immunological activity has been well documented for two diverse groups of naturally occurring lipids: the glycosphingolipids (*e.g.*, ceramides and gangliosides) and acidic phospholipids (*e.g.*, cardiolipin and phosphatidylinositol). For example, mixed micelles of these compounds with auxiliary lipids (such as lecithin and cholesterol) fix complement in the presence of, or are agglutinated by, antibodies produced in response to immunization with appropriate cell membranes or subcellular particulate fractions (Rapport and Graff, 1969; Inoue and Nojima, 1967; Kataoka and Nojima, 1970). In addition, although the highly purified compounds are very poor immunogens, antibodies against them can be elicited by immunization with the mixed micelles and a heterologous protein (such as serum albumin or methylated serum albumin) plus an appropriate adjuvant (see references cited above and also Hakomori, 1972).

The present study demonstrates that the immunogenicity of lipids can now be extended to include certain synthetic N-substituted phosphatidylethanolamine derivatives. In the case of the dinitrophenylated derivatives, the available evidence suggests a correlation between the effectiveness of an immunogen preparation and the existence of these compounds in bilayer configuration which presumably corresponds to the orientation of natural lipid antigens in cell membranes. Thus, to obtain maximum antibody formation, it was necessary to employ the amphipathic derivatives (*i.e.*, Dnp-Cap-PE or Dnp-Cap-lysoPE) for the active sensitization of liposomal model membranes. We have not yet performed extensive

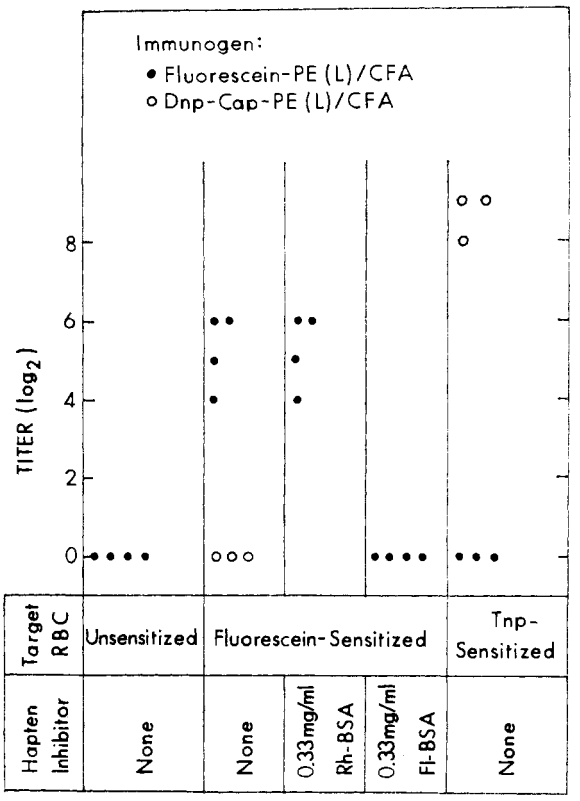

FIGURE 7: Immunogenicity of sphingomyelin–cholesterol–dicetyl phosphate liposomes (L), which were actively sensitized with 10% fluorescein-PE, and mixed with complete Freund's adjuvant (CFA). Hemagglutination assay was performed using either unsensitized, fluorescein sensitized, or trinitrophenyl (Tnp) sensitized erythrocytes (RBC) as target cells. For comparative purposes (see text), sera obtained from guinea pigs immunized with 10% Dnp-Cap-PE actively sensitized liposomes were also assayed against fluorescein and Tnp sensitized erythrocytes. Hapten inhibition was determined by incorporating either the fluorescein or the tetramethylrhodamine isothiocyanyl conjugate of bovine serum albumin (abbreviated Fl-BSA and Rh-BSA, respectively) into the wells at the final concentration indicated in the figure.

experiments to determine whether the levels of antibody can be increased by secondary immunization with these liposomes or other preparations; it is noteworthy, however, that skin testing with either actively sensitized Dnp-Cap-PE liposomes, Dnp-Cap-PE alone, dinitrophenylated proteins, or dinitrofluorobenzene has so far failed to provide convincing evidence for a delayed hypersensitivity reaction in guinea pigs which have produced antibodies (results not shown). Also, it should be emphasized that our attention has been confined to randomly bred guinea pigs and it must still be established whether other animals (particularly inbred strains) behave in the same way. Such experiments may indicate whether Dnp-Cap-PE can be exploited similarly to synthetic dinitrophenylated amino acid polymers in investigations concerned with the genetic control of the immune response and immunological tolerance.

Further extension of this approach to the synthesis of other phospholipid derivatives appears warranted by the fact that liposomes sensitized with Fl-PE also give rise to specific antibodies. Indeed, all of the chemical reactions that have been employed to modify aminoalkyl agarose for the preparation of affinity adsorbants (Cuatrecasas, 1972) would seem to be suitable for the synthesis of an unlimited number of N-substituted phosphatidylethanolamine derivatives. Because most of these reactions can be performed in organic solvents exclusively, it should be possible to prepare immunoreactive, as well as immunogenic, derivatives of water insoluble biologically active substances that may be potentially applicable to a number of problems. For example, many drugs belong in this category and the present experiments (*cf.* Figure 6) suggest that marker release from liposomes sensitized with "drug-substituted" phosphatidylethanolamine derivatives may serve as a convenient assay procedure for detecting serum antibodies against these pharmacological agents.

Conversely, immunization with such liposomes may constitute a useful alternative for obtaining antibodies (*e.g.*, for use in immunoassay) to more conventional methods of immunogen preparation which depend on prior covalent linkage of hapten to a high molecular weight water soluble carrier such as protein. Furthermore, in these classical immunogens, various hybrid antigenic determinants may be formed as the result of hydrophobic interaction between the hapten and appropriate domains (*i.e.*, amino acid residues) in the protein. In liposomes, however, the hapten is not only situated in a more homogeneous environment but the interposal of hydrophilic groups (*i.e.*, phosphate) between the hydrophobic hapten (*e.g.*, dinitrophenyl) and the bilayer backbone structure may markedly limit hybrid determinant formation. Accordingly, the possibility that restricted antibodies are produced as a consequence of liposomal immunization is now under investigation.

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