Antibodies to liposomal phosphatidylcholine and phosphatidylsulfocholine

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Antibodies against dimyristoyl phosphatidylsulfocholine or dimyristoyl phosphatidylcholine were raised in rabbits after injection of liposomes containing phosphatidylsulfocholine or phosphatidylcholine, cholesterol, and lipid A. The antibody activities were assayed by complement-dependent immune damage to liposomes and by a solid-phase, enzyme-linked immunosorbent assay using purified dimyristoyl phosphatidylcholine or dimyristoyl phosphatidylsulfocholine as antigen. Each antiserum raised against phosphatidylsulfolcho line reacted with liposomes containing phosphatidylcholine, and each antiserum raised against phosphatidylcholine reacted with liposomes containing phosphatidylsulfocholine. However, adsorption of dimyristoyl phosphatidylsulfocholine antiserum with liposomes containing dimyristoyl phosphatidylcholine removed all activity against dimyristoyl phosphatidylcholine, but did not eliminate antibody activity against dimyristoyl phosphatidylsulfocholine. These results indicate that the antisera against phosphatidylsulfocholine contained mixed populations of antibodies. Polyclonal antisera that have been appropriately adsorbed can therefore be obtained with a high degree of specificity for phosphatidylsulfocholine and such antisera can distinguish between phosphatidylsulfocholine and phosphatidylcholine.

Key words: liposomes, antibodies, phosphatidylsulfocholine, phosphatidylcholine.

L’injection à des lapins de liposomes composés de la phosphatidylsulfocholine ou de la phosphatidylcholine, de cholestérol et de lipid A, a produit des anticorps dirigés respectivement contre la dimyristoyl phosphatidylsulfocholine ou la dimyristoyl phosphatidylcholine. Nous avons évalué le titre de ces anticorps par la mesure des lésions causées aux liposomes par les anticorps et le complément et par un test immunoenzymatique ELISA utilisant les phospholipides purifiés comme antigènes. Chaque anticorps développé contre la phosphatidylsulfocholine a réagi avec les liposomes renfermant la phosphatidylcholine et chaque anticorps développé contre la phosphatidylcholine a réagi avec les liposomes renfermant la phosphatidylsulfocholine. Toutefois, l’adsorption d’un des antisérum dirigé contre la dimyristoyl phosphatidylsulfocholine avec des liposomes renfermant la dimyristoyl phosphatidylcholine a permis d’éliminer l’activité de cet antisérum avec la dimyristoyl phosphatidylcholine mais non son activité avec la dimyristoyl phosphatidylsulfocholine. Ces résultats montrent que l’antisérum contre la phosphatidylsulfocholine renferme non pas une seule population, mais un mélange de plusieurs populations d’anticorps. Il est donc possible qu’un antisérum polyclonal convenablement adsorbé puisse résulter en un antisérum doué d’un fort degré de spécificité envers la phosphatidylsulfocholine et capable de distinguer la phosphatidylsulfocholine de la phosphatidylcholine.

Mots clés : liposomes, anticorps, phosphatidylsulfocholine, phosphatidylcholine.

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Introduction

Most phospholipids are immunogenic and allow formation of antibodies directed against their polar head groups (Inoue and Nojima 1967; Kataoka and Nojima 1970; Guarnieri and Eisner 1974). In the studies cited above the antibodies were induced by immunizing with emulsions containing protein–phospholipid conjugates in the presence of an adjuvant such as complete Freund’s adjuvant (reviewed by Alving 1977). In recent years we have demonstrated that polyclonal or monoclonal antibodies against liposomal phospholipids can be raised by injecting liposomes containing LA as adjuvant into rabbits or mice (Schuster et al. 1979; Alving et al. 1980; Banerji and Alving 1981; Banerji et al. 1982; Wassef et al. 1984; Alving 1986).

In the present paper, we report on the production of polyclonal "antiliposome" antisera having reactivities with DMPC and DMPS. PSC is a sulfoxonium analog of PC, which is found in certain marine diatoms and algae (Bisseret et al. 1984). This sulfolipid was identified and synthesized by Kates and his colleagues and its physical properties were extensively investigated (Tremblay and Kates 1979, 1981; Bittman et al. 1984). Figure 1 shows the chemical structures of DMPC and DMPS that were the phospholipid antigens used in the immunizing liposomes.

Because of the close resemblance of DMPS to DMPC, the question arose whether all of the antibodies in polyclonal
myristic acid. Fatty acid analysis was obtained by GLC on a column of silicic acid eluted with concentrated ammonium hydroxide (65:35:5, by volume). Fatty acid analysis by GLC showed the presence only of myristic acid (99.8%).

**Materials and methods**

**Source of lipids**

Lipids were purchased from the following sources: DMPC, Sigma Chemical Co. St. Louis, MO; CHOL, Calbiochem, La Jolla, CA; DCP, K and K Laboratories, Plainview, NY; LA (containing 1.1 μg/nmol LA phosphate) from *Salmonella minnesota* R595, List Biological Labs., Campbell, CA. DMPC was synthesized as described elsewhere (Tremblay and Kates 1979) and finally purified by chromatography on a column of silicic acid eluted with chloroform-methanol (1:1, v/v), followed by acetone precipitation. DMPC showed a single spot on TLC in chloroform - methanol - concentrated ammonium hydroxide (65:35:5, by volume). Fatty acid analysis by GLC showed the presence only of myristic acid (99.8%).

**Preparation of liposomes**

Complete details of liposome preparation are reviewed elsewhere (Alving et al. 1984). Briefly, multilamellar liposomes were prepared from a mixture of DMPC or DMPC, CHOL, and DCP in molar ratios of 2:1:5:0.22. Liposomes used for immunization lacked DCP, but contained 20 nmol of LA phosphate (22 μg LA)/μmol phospholipid (Schuster et al. 1979). Liposomes used for the complement-dependent immune damage assay contained DCP and also 12.5 nmol LA phosphate (14 μg LA)/μmol phospholipid where indicated. When GLUCER was incorporated in the liposomes, it was at a concentration of 150 μg/μmol phospholipid.

**Immunization schemes**

Male New Zealand white rabbits (2.5-3.0 kg) were immunized with 1.0 mL of liposomes containing 10 μmol of phospholipids. Each rabbit received a 0.5-mL dose, injected s.c. or i.m. at the caudal muscle of each thigh. The rabbits were immunized, bled, and boosted at the time intervals indicated in the respective figures. The following liposome compositions were used for immunization: group 1, DMPC-CHOL-LA; group 2, DMPC-CHOL-DCP. Each group was comprised of two rabbits.

**Assays of antibody activities**

Antibody activities against DMPC or DMPC were assayed both by complement-dependent immune damage to liposomes and by ELISA.

Complement-dependent immune damage to liposomes measured the release of trapped glucose from liposomes containing DMPC or DMPC owing to antibody-mediated complement-dependent damage to test liposomes. The assay has been described in detail elsewhere (Alving et al. 1984). Briefly, release of trapped liposomal glucose was enzymatically measured in a total volume of 1 mL, using a Tris-buffered assay solution containing hexokinase, glucose-6-phosphate dehydrogenase, ATP, NADP, and Ca^{2+} and...
Mg$^{2+}$. Each 5-μL aliquot of liposomes was incubated at room temperature with either 30 μL of rabbit antisera from different bleedings (Fig. 2) or with increasing volumes of rabbit antisera from the same bleeding (Fig. 4), and with 100 μL of GPS as a source of complement. Glucose release was detected after 30 min at room temperature by increased $A_{340}$ owing to reduction of the NADP. Data were expressed as percentage of trapped glucose released and a value of 5% glucose released represented a threshold for positive activity. The total amount of glucose originally trapped in the liposomes was determined by disrupting the liposomes with chloroform.

The general method for performing an ELISA with a lipid antigen has been described by Swartz et al. (1988). Appropriate lipid antigens were used to coat the wells of polystyrene plates (Immulon II, “U” bottom, Dynatech Laboratories, Alexandria, VA). Lipids were used at a concentration of 1 nmol per well with the exception of CHOL, which was used at a concentration of 1 μg per well. The ethanolic solution of lipid was evaporated, and the plates were further dried under high vacuum for 10 min. Plates were blocked with 110 μL per well of PBS containing 10% heat-inactivated FBS (Gibco Laboratories, Grand Island, NY). Plates were blocked for 1 h at room temperature or overnight at 4°C. Fifty microlitres of rabbit antisera diluted in PBS containing 10% FBS was added to the wells and incubated 3 h at room temperature or overnight at 4°C. Plates were then washed three times, for 5 min each, with PBS alone. Goat anti-rabbit IgG (H + L) - alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted in PBS containing 10% FBS was added at a concentration of 25 ng per well. Following 1 h of incubation at room temperature, the plates were again washed three times, for 5 min each, with PBS. Fifty microlitres of p-nitrophenyl phosphate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in diethanolamine buffer (2 mg/mL) was added to the wells and incubated for 1 h at room temperature in the dark. Absorbance at 405 nm was measured using a UVmax microplate reader (Molecular Devices, Palo Alto, CA). Values reported were adjusted by subtracting values in wells that contained antiserum but lacked antigen.

**Results**

**Antibodies to DMPC and DMPSC**

The production of anti-DMPC or anti-DMPSC antiserum, as determined by complement damage to liposomes containing DMPC or DMPSC, is shown in Figs. 2A and 2B, respectively. Anti-DMPSC serum apparently cross-reacted with liposomes containing DMPC (Fig. 2A), and anti-DMPSC serum apparently cross-reacted with liposomes containing DMPSC (Fig. 2B). Anti-DMPC serum activity and cross-reactivity against DMPC in anti-DMPSC serum peaked at 2–3 weeks (Fig. 2A). When the sera were tested with liposomes containing DMPC, the activity of anti-DMPSC serum against DMPC was much higher and more prolonged after boosting than the cross-reacting activity in anti-DMPC serum (Fig. 2B). Despite the differences observed, the data indicated that the patterns of antibody activities were similar for antibodies elicited by DMPC and DMPSC, and there appeared to be extensive cross-reactivity between both groups. However, from the data presented it was impossible to determine if reactivity of an antiserum with both antigens was due to cross-reactivity or to mixtures of monospecific antibodies.

It should be pointed out that GLU CER was included in the test liposomes because of previous work with “anti-liposome” antibodies that showed that the presence of ceramide in the test liposomes nonspecifically increased the amount of glucose released from the liposomes and increased the sensitivity of the glucose release method for detecting antibodies (Banerji and Alving 1981). This is a nonspecific amplification technique that does not indicate the presence of antibodies to GLU CER.

**Immune reactivities with purified antigens**

A solid-phase ELISA employing either purified DMPC or DMPSC as the antigen demonstrated that antibodies that bound to either of these two phospholipids could be obtained (Fig. 3). Animals immunized with liposomes containing DMPC had similar reactivities to both DMPC and DMPSC (Fig. 3A). In contrast, animals immunized with liposomes containing DMPSC showed higher binding to DMPSC compared with DMPC (Fig. 3B). All animals had detectable binding to both DMPC and DMPSC when comparisons were made with preimmunization serum samples which were barely detectable.

**Adsorption studies**

The specificity of one of the antisera to DMPSC was further examined by adsorption of the antiserum with liposomes containing DMPC. The antiserum that was obtained at 6 weeks after immunization was adsorbed with liposomes containing DMPC–CHOL–DCP–LA. This antiserum originally had shown substantial reactivity with liposomes containing DMPC at 6 weeks (Figs. 2 and 3), but following adsorption the antibody activity against DMPC was completely removed while the antibody activity against DMPSC was not affected, when tested by complement-dependent immune damage to liposomes (Fig. 4). When the antiserum was tested by ELISA using individual liposomal
In this paper we have described the production of antibodies to the bulk liposomal phospholipid, either DMPC or DMPSC, obtained after injection of the respective liposomes containing LA into rabbits. The chemical structure of DMPC differed from DMPSC by the replacement of the quaternary ammonium group of DMPC by a sulfonium group (Fig. 1). The antisera obtained reacted with both phospholipids and it was initially impossible to determine whether multiple populations of monospecific antibodies were induced or whether cross-reacting antibodies were present in the antisera. However, the possibility of multiple specificities was suggested by the observation that allowing the boosting immunization the antisera induced by immunizing with DMPSC showed much higher titers against DMPSC compared with DMPC liposomes (Figs. 2B and 3B). It seemed likely that the apparent cross-reactivities exhibited by anti-DMPSC antiserum when tested with DMPC and DMPSC could have been due, at least partially, to different antibody populations. To test this hypothesis we adsorbed the antiserum with liposomes containing DMPC and tested for residual activity against DMPC or DMPSC liposomes (Fig. 4) or with the individual liposomal constituents as antigens in ELISAs (Fig. 5). It is clear that the antibody activity against DMPC, but not against DMPSC, was entirely removed by adsorption with DMPC (Figs. 4 and 5). The data indicate that we were dealing with populations of different antibodies, and a subpopulation could be identified that had the ability to distinguish DMPSC from DMPC.

The data presented in this report are therefore consistent with our previous studies which show that although liposomes function as complete antigens, the ability of antibodies to differentiate between liposomes having different, but similar, phospholipid compositions can be quite considerable. Specificities of antibodies raised in this fashion against individual phospholipids were previously observed with polyclonal antisera (Schuster et al. 1979; Alving 1986) or monoclonal antibodies (Banerji et al. 1982; Wassef et al. 1984). For example, a monoclonal antibody to PIP reacted 1000-fold more strongly with DMPC-CHOL-DCP-PIP liposomes than with DMPC-CHOL-DCP liposomes (Wassef et al. 1984). The present study demonstrates the utility of adsorbing antiserum with liposomes to increase the specificity of antisera, by selectively removing populations of antibodies that cross-react with phospholipids that are closely similar to the immunizing antigen.

The ability to prepare specific antisera that recognize DMPSC, but not DMPC, complements previous research which has demonstrated that antibodies to phosphatidylcholine can be induced by utilizing liposomal LA as an

![Fig. 4](image-url)  
**Fig. 4.** Adsorption of antibody activity against liposomes containing DMPC. Antiserum (1 mL) from a rabbit immunized with liposomes containing DMPC (6 weeks bleeding, see Figs. 2 and 3) was incubated for 30 min at room temperature with 0.4 mL liposomes composed of DMPC-CHOL-DCP-LA. The liposomes were removed by centrifugation (27 000 × g, 10 min). Glucose release from liposomes containing DMPC (●) or DMPSC (○) using the adsorbed serum is shown in A. Activity of an equal amount of unadsorbed serum against the same liposomes is shown in B. The data shown have been corrected for background activity by subtracting the values obtained for preimmune sera.

![Fig. 5](image-url)  
**Fig. 5.** Activities of adsorbed and nonadsorbed serum against each individual liposomal component, as determined by ELISA. Aliquots of serum from a rabbit immunized against DMPC-CHOL-LA were adsorbed with liposomes lacking DMPSC, but containing DMPC, as described in Fig. 4. The activities of the resulting adsorbed antisera and of an equal amount of unadsorbed antiserum were determined by using a serum dilution of 1:25. Values reported have been corrected for background activity and nonspecific binding by subtracting absorbance values of preimmune sera.
adjuvant (Schuster et al. 1979). Autoantibodies to phosphatidylcholine have also been induced by immunizing mice with bromelain-treated mouse erythrocytes (Cox and Hardy 1985), and monoclonal antibodies that recognize the trimethylammonium head group of phosphatidylcholine have been induced by this technique (Poncet et al. 1985). Antibodies to DPPC were also induced in guinea pigs by emulsifying DPPC with bovine serum albumin and complete Freund's adjuvant (Niedieck and monoclonal myeloma protein and with antilecithin sera from mice immunized with bromelain-modified RBC are specifically inhibited by bromelain-modified RBC (Cox et al. 1985). The antibodies that had such specificity were undoubtedly part of a larger population of antibodies that had specificity for the trimethylammonium head group (Niedieck et al. 1987; Urbaneja et al. 1987). Differential reactivity of the TEPC 15 antibody occurred with monolayers of phosphatidylcholine and phosphatidylethanolamine (Urbaneja et al. 1987).

The present study supports the concept that considerable specificity can be exerted by antibodies directed against the head group of phosphatidylcholine. Replacement of the trimethylammonium group with dimethylsulfonium provided sufficient alteration of the head group to allow induction of antibodies that differentiated the two head groups. The antibodies that had such specificity were undoubtedly part of a larger population of antibodies that cross-reacted to varying degrees with both DMPC and DSPC. These data, therefore, are compatible with the concept that the antigen binding sites of antibodies to phospholipids have capacities to recognize patterns of membrane surfaces. Binding of subpopulations of polyclonal antibodies can be influenced by subtle chemical changes of liposomal phospholipid polar head groups, and a high degree of specificity can be achieved by adsorption by antiserum with appropriate liposomes.


