

Phospholipid in the hexagonal II phase is immunogenic: Evidence for immunorecognition of nonbilayer lipid phases *in vivo*

(nonbilayer lipid/autoantibodies/autoimmunity/systemic lupus erythematosus)

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ABSTRACT Immunization of mice with phosphatidylethanolamine in the hexagonal II phase but not the bilayer phase resulted in the induction of anti-phospholipid antibodies. These antibodies, which were strongly reactive with phosphatidylethanolamine and crossreactive with cardiolipin, had functional lupus anticoagulant activity and were characteristic of autoantibodies common in patients with autoimmune disease. Recognition of the hexagonal II phase by the afferent limb of the immune system suggests that nonbilayer phospholipids can arise in the course of membrane remodeling and induce the autoantibodies of disease.

Antibodies to phospholipids are associated with a variety of autoimmune diseases, including systemic lupus erythematosus and related rheumatic disorders (1-3). The mechanisms responsible for the production of these autoantibodies have not been elucidated. Although phospholipids have long been considered to be nonimmunogenic, only bilayer phase phospholipids have been studied (reviewed in refs. 4 and 5). Our findings that lupus anticoagulants (a subset of anti-phospholipid antibodies associated with thrombosis, thrombocytopenia, and multiple spontaneous abortions) recognize nonbilayer phase (hexagonal II) but not bilayer phase phosphatidylethanolamine (6-11) led us to ask whether the lipid polymorphic phase could modulate immunogenicity. Here we show that hexagonal II phase, but not bilayer phase, phosphatidylethanolamine is capable of inducing anti-phospholipid antibodies with lupus anticoagulant activity in mice. Hexagonal II phase lipid, as opposed to the familiar bilayer phase lipid, consists of hexagonally packed cylinders of lipid where the cylinders are composed of a central aqueous channel toward which the polar groups are oriented. The studies reported here thus demonstrate that phospholipid can be immunogenic and suggest that alterations in lipid phase *in vivo* may lead to the production of anti-phospholipid antibodies with associated pathological sequelae.

MATERIALS AND METHODS

Phospholipids. All phospholipids were purchased from Avanti Polar Lipids and used without further purification. Phospholipid suspensions were prepared in Hepes buffer (20 mM Hepes/150 mM NaCl, pH 7.5) as described (6).

Immunizations. Three-month-old female BALB/c mice (The Jackson Laboratory) were bled for preimmune sera and immunized intravenously with equimolar (0.02 μ mol per 100 μ l of injection) amounts of phospholipid diluted in saline, as determined by the method of Bartlett (12). Mice were given four injections at 2-week intervals and bled 12 days after the fourth injection.

ELISAs. ELISAs were performed as described (13), with the following modifications. Phospholipid-coated plates were blocked with 0.01 M phosphate-buffered saline at pH 7.3 (PBS) containing 0.3% gelatin and 10% (vol/vol) fetal bovine serum for 2 hr at 25°C. Sera were diluted 1:100 in PBS containing 0.3% gelatin and incubated for 3 hr at 25°C. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (polyvalent, Sigma) was incubated for 16 hr at 4°C. Autoimmune MRL *lpr/lpr* mouse serum served as the positive control and produced OD₄₀₅ values of >2.00 for all coated phospholipids.

Lupus Anticoagulant Antibody Activity. Blood collected from the immunized mice was anticoagulated with 3.8% buffered sodium citrate (9 volumes of blood: 1 volume of sodium citrate), and platelet-poor plasma was isolated by centrifugation in a Micro Centaur centrifuge (Accurate Chemical and Scientific, Westbury, NY) at 12,000 $\times g$ for 2 min. Plasmas were diluted 1:1 with citrated normal human plasma and were tested for lupus anticoagulant activity in a dilute activated partial thromboplastin time (APTT) assay, as previously described (6).

RESULTS AND DISCUSSION

After immunization of BALB/c mice with phospholipids, serum antibodies were detected by ELISAs. These assays are highly sensitive and are routinely used to measure naturally occurring anti-phospholipid antibodies (1, 14), but they are not necessarily phase-specific because the phospholipid antigen is immobilized on a solid support. Fig. 1 shows the levels of serum antibodies in mice after immunization with dioleoyl phosphatidylethanolamine (DOPE), 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE), or monooleoyl-phosphatidylethanolamine (MOPE; 1-oleoyl 2-lysophosphatidylethanolamine). When prepared for injection as a phospholipid suspension in saline, DOPE was nonbilayer, but POPE and MOPE were bilayer as confirmed by freeze-fracture microscopy, small-angle x-ray diffraction, and ³¹P NMR spectroscopy (6-11, 15). Only DOPE was immunogenic and induced antibodies reactive with itself and cross-reactive with POPE. These antibodies also reacted to a very limited extent with MOPE, a monoacyl lipid, which was expected to pack differently than the diacyl lipid POPE on the solid-support system (15).

We next asked whether the induced anti-phospholipid antibodies had lupus anticoagulant activity. Lupus anticoagulant antibodies are identified by their ability to prolong the clotting time in *in vitro* phospholipid-dependent coagulation assays, in particular those measuring APTTs (16, 17). This phenomenon arises because these antibodies bind to the

Abbreviations: APTT, activated partial thromboplastin time; DOPE, dioleoyl phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; MOPE, monooleoyl phosphatidylethanolamine (1-oleoyl 2-lysophosphatidylethanolamine).

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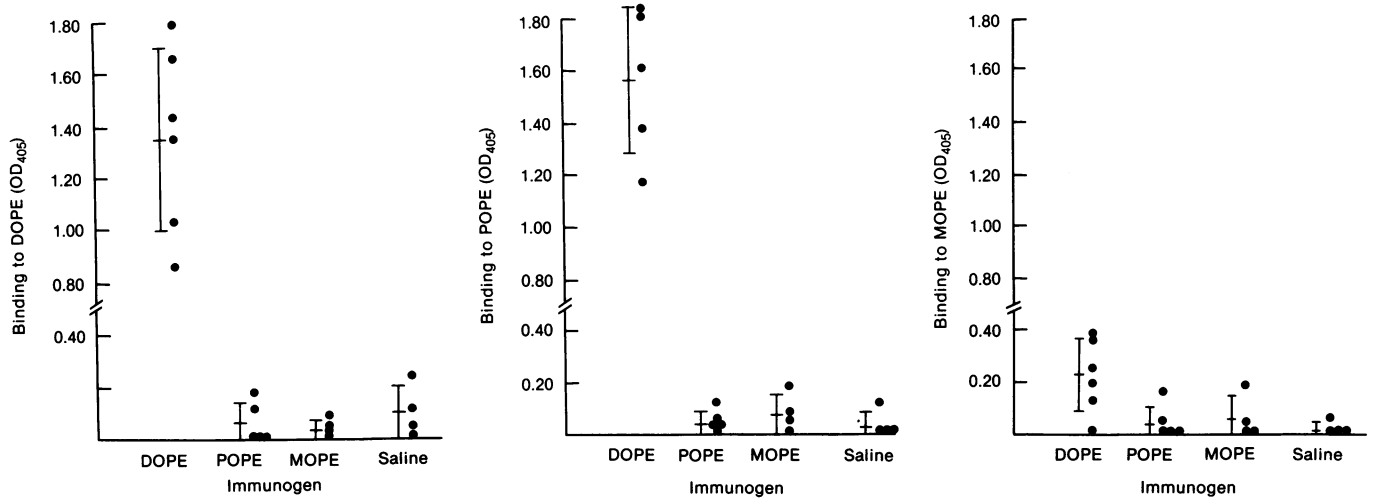


FIG. 1. Serum antibodies reactive with DOPE (Left), POPE (Center), and MOPE (Right) in mice immunized with nonbilayer (DOPE) or bilayer (POPE and MOPE) lipid. Binding of serum antibodies to DOPE, POPE, and MOPE was detected in solid-phase ELISAs. Each point represents the mean OD_{405} value of duplicate samples of serum from an individual mouse minus the OD_{405} value for the preimmune serum from that mouse on the same ELISA plate. The bars denote the mean OD_{405} value ± 2 SD for each group of mice. Only mice injected with nonbilayer lipid (DOPE) produced antibodies.

phospholipid portion of the prothrombin activator complex provided by the exogenously added brain cephalin in the assay system (17–22). We measured APTTs in platelet-poor plasma drawn from each immunized mouse. As shown in Fig. 2, significantly prolonged APTT values were observed only in mice injected with nonbilayer lipid (DOPE). These values exceeded the mean APTT value obtained from plasma drawn from control mice immunized with normal saline and were not corrected by a 1:1 dilution with normal citrated human plasma. This dilution method is widely used to confirm the presence of lupus anticoagulant antibodies (23–25).

Studies of lupus anticoagulant activity in patients have revealed a large ($\approx 70\%$) but incomplete overlap with antibodies reactive with cardiolipin (26). Some lupus anticoagulant and anti-cardiolipin antibodies have also been shown to crossreact with denatured DNA (9, 27–32). As shown in Fig. 3, the antibodies that we were able to induce in mice by immunization with DOPE exhibited a similar pattern of reactivity. Strong crossreactivity with cardiolipin was observed, but little or no binding to denatured DNA could be

detected. No correlation was found between antibody titers to DOPE or cardiolipin and lupus anticoagulant activity. A similar lack of correlation and variation in levels of lupus anticoagulant activity have been noted in other studies both in mice (33) and humans (9, 34, 35) and have been attributed to subsite specificities (5, 33).

It has been known for some time that anti-phospholipid antibodies can be induced experimentally following injection of bilayer-forming lipids in combination with immunogenic carriers or adjuvants (4, 5, 33). The most salient feature of the work we report here concerns the demonstration that pure phosphatidylethanolamine alone, in the absence of carriers or adjuvants, is immunogenic in a phase-related fashion. The biological relevance of this finding relates to the fact that a significant fraction of phospholipids comprising cell membranes, including but not limited to unsaturated species of phosphatidylethanolamine, will adopt nonbilayer structures in isolation (36). These lipids, which most likely play a normal regulatory role, are stabilized in the membrane-forming bilayer configuration *in vivo* by bilayer-forming lipids and

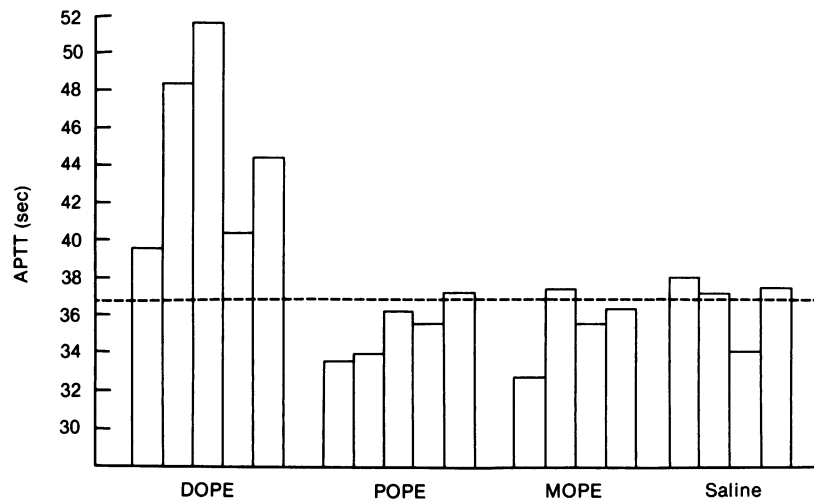


FIG. 2. Lupus anticoagulant antibody activity detected by measurement of APTT in plasma of mice immunized with nonbilayer (DOPE) or bilayer (POPE and MOPE) lipid. Plasmas were tested on two separate occasions and values, expressed in seconds, were reproduced with a mean variation of ± 2 sec. The dashed line across the bar graph indicates the mean APTT value of the group of mice injected with saline. The APTT values of all five mice immunized with DOPE exceeded this control group value, whereas none of the mice injected with either POPE or MOPE were positive for lupus anticoagulant antibody activity.

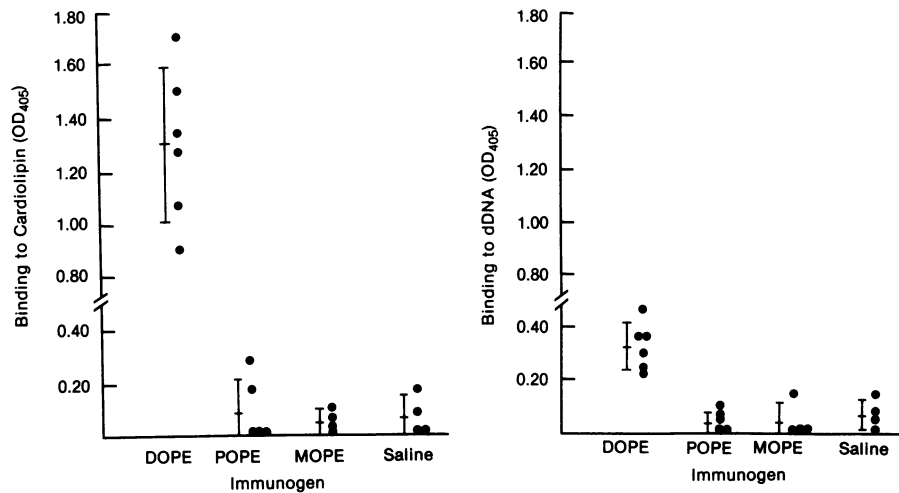


FIG. 3. Serum antibodies reactive with cardiolipin (*Left*) and denatured DNA (dDNA) (*Right*) in mice immunized with nonbilayer (DOPE) or bilayer (POPE or MOPE) lipid. Binding of serum antibodies to cardiolipin and denatured DNA was detected by using solid-phase ELISAs. Each point represents the mean OD₄₀₅ value of duplicate samples of serum from an individual mouse minus the OD₄₀₅ value for the preimmune serum from that mouse on the same ELISA plate. The bars indicate the mean \pm 2 SD for each group of mice. Only mice injected with nonbilayer lipid (DOPE) demonstrated high levels of antibodies reactive with cardiolipin. In contrast, sera from all mice showed little or no reactivity with denatured DNA.

proteins (36–38). Loss of stabilization would result in the formation of nonbilayer structures. The immunogenicity of these structures, a topic we have begun to address here, might likely form the etiological basis of a variety of autoimmune diseases.

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