Antibodies to phospholipids may have important physiological and biological functions. Lupus anticoagulants represent a subclass of anti-phospholipid antibodies which are characterized by their ability to prolong the clotting time in *in vitro* coagulation assays measuring partial thromboplastin time (PTT) (Thiagarajan, P., Shapiro, S. S., and DeMarco, L. (1980) *J. Clin. Invest.* 66, 397–405). In the present study, we produced hybridomas by fusing lymphocytes from 13 systemic lupus erythematosus patients with the GM 4672 lymphoblastoid line. Of the resulting 67 hybridoma autoantibodies, 14 (21%) were found to prolong a modified PTT assay, and 11 of these antibodies were analyzed further. Competition experiments, using a modified PTT assay, demonstrated that hexagonal phase phospholipids, including natural and synthetic forms of phosphatidylethanolamine, were able to neutralize the lupus anticoagulant activity of all 11 hybridoma antibodies. In contrast, lamellar phospholipids, such as phosphatidylcholine and synthetic lamellar forms of phosphatidylethanolamine, had no effect on the anticoagulant activity. Thus, these antibodies are capable of recognizing phospholipids on purely structural criteria. The demonstration that anti-phospholipid antibodies are able to distinguish between different structural arrangements of phospholipid may have important implications regarding the immunoregulation of autoimmune diseases.

**Antibodies to phospholipids represent a group of poorly characterized antibodies which may have important physiological and biological functions (1). These antibodies occur frequently in the sera of patients with a variety of autoimmune diseases, including systemic lupus erythematosus (SLE) and related connective tissue disorders (2). The mechanism(s) responsible for the production of these autoantibodies remains unknown. Recently, it has been found that antibodies against commonly occurring membrane lipids such as phosphatidylcholine, sphingomyelin, and possibly cholesterol are ubiquitous in normal (nonimmunized) human sera (3). Apparently, normal tissue is protected against attack by anti-phospholipid antibodies, but again, the mechanism remains obscure. One possible mechanism for the induction and/or pathogenesis of these antibodies involves alterations in the phospholipid architecture of the cell membrane. Such an explanation would seem plausible in light of the ability of membrane lipids to assume a variety of structures in addition to the bilayer phase (4). In fact, in certain pathological conditions, non-bilayer lipid structures have been demonstrated *in vivo* (5). Although such structures could conceivably play a role in antibody induction or pathogenesis, this possibility requires a clear demonstration that anti-phospholipid antibodies exist which are able to distinguish different polymorphic forms of the same lipid.**

**Our approach to this problem has involved the use of human hybridoma lupus anticoagulants derived from patients with SLE. Lupus anticoagulants are anti-phospholipid antibodies (6, 7) which are defined by their ability to prolong the normal clotting time in *in vitro* coagulation assays measuring the partial thromboplastin time (PTT). In these assays, platelet membranes are replaced by brain cephalin, a lipid extract rich in phosphatidylethanolamine. In this study, we show that the ability of lupus anticoagulants to prolong PTT values can be inhibited by the presence of hexagonal phase, but not bilayer phase, phosphatidylethanolamine. Hexagonal phase phosphatidylethanolamine, in contrast to the familiar bilayer phase, consists of hexagonally packed cylinders of lipid where the cylinders are composed of a central aqueous channel toward which the polar head groups are oriented (4). Thus, we describe here the ability of lupus anticoagulant antibodies to distinguish between different structural configurations of the same chemical species of phospholipid.**
and the chloroform removed by evaporation under dry nitrogen. The dried lipids were resuspended in 1.0 ml of 20 mM Hepes buffer (150 mM NaCl, pH 7.5), hydrated at a temperature above their transition temperature for 1-2 h and stored at 4 °C. Phosphate concentrations were determined by the method of Bartlett (10).

31P NMR spectra were obtained using a Bruker WP-200 FT-NMR spectrometer operating at 81.0 MHz for 31P. Lipids were dispersed in 10 mM Tris/HC1, 100 mM NaCl, 10% v/v HzO, pH 7, buffer by extensive vortexing at room temperature except for DPPE, which was heated to 70 °C before dispersal. Spectra were accumulated at 37 °C; 1-ss scans were employed using a 7-ns, 50-ms radiofrequency pulse, 20-KHz sweep width, and a 0.5-s interpulse delay in the presence of broadband proton decoupling. An exponential multiplicity corresponding to 50-Hz line broadening was applied to the free induction decay prior to Fourier transformation.

Production of Human-Human Hybridoma Lupus Anticoagulants—Fifty ml of venous blood was obtained from 13 patients who satisfied the revised American Rheumatism Association criteria for the classification of SLE (11). The plasma of two of these patients had elevated partial thromboplastin times, and another patient was positive in the Venerable Diseases Research Laboratories test for syphilis. The peripheral blood lymphocytes were isolated on a Ficoll-Hypaque density gradient and were fused with the GM 4672 human lymphoblastoid cell line (igG producing), obtained from the Cell Repository Institute of Medical Research, Camden, NJ, at a cell ratio of 1:1, using 44.4% polyethylene glycol. The fusion, plating, and cloning procedure have been described elsewhere in detail (12). All hybridoma antibodies were IgM immunoglobulins, as determined by a solid phase radioimmunoassay (13).

Detection of Hybridoma Lupus Anticoagulants—Hybridoma supernatants were tested for the production of lupus anticoagulants using a modified activated PTT assay (14). Fifty μl of hybridoma supernatant was diluted with an equal volume of freshly reconstituted Verify normal citrate (potted normal human plasma, General Diagnostics, Scarborough, Ontario) in a 13 X 100-mm borosilicate glass tube. One hundred μl of a 1/64 dilution of activated Thrombofax (Ortho Diagnostics Inc., Raritan, NJ) in Hepes buffer, which had been prewarmed to 37 °C, was added to 4672 human lymphoblastoid cell line (igG producing), obtained from the Cell Repository Institute of Medical Research, Camden, NJ, at a cell ratio of 1:1, using 44.4% polyethylene glycol. The fusion, plating, and cloning procedure have been described elsewhere in detail (12). All hybridoma antibodies were IgM immunoglobulins, as determined by a solid phase radioimmunoassay (13).

Inhibition = binding (without inhibitor) - binding (with inhibitor) X 100

Preincubation of Different Phospholipid Phase Systems with Lupus Anticoagulants at 37 °C. Effects on Partial Thromboplastin Times—

Preincubation of Egg PE(PC) and Egg PE with Hybridoma Lupus Anticoagulants at 37 °C. Effects on Partial Thromboplastin Times—

Preincubation of Egg PE(FC) and Egg PE with Hybridoma Lupus Anticoagulants at 43 °C: Effects on Partial Thromboplastin Times—

Results

Phospholipid Phase Assignments—The polymorphic phase preferences of the phospholipids used were determined employing the 31P NMR technique (4). Asymmetric 31P NMR spectra exhibiting a low field shoulder and high field peak are
Lupus Anticoagulants Recognize Hexagonal Phase Lipids

A

DOPE

EPE

PE(PC)

DOPE

B

BBPE

FIG. 1. 81.0-MHz $^{31}$P NMR spectra of aqueous dispersions of various species of phosphatidylethanolamines. A, DPPE, egg yolk phosphatidylethanolamine (EPE), dioleoylphosphatidylethanolamine (DOPE), and bovine brain phosphatidylethanolamine (BBPE) at 37 °C. B, phosphatidylethanolamine derived from PE(PC) at 37 and 43 °C.

FIG. 2. Titration of the inhibition of the anticoagulant activity of antibody 824 after preincubation with egg PE, egg PE(PC), or phosphorylethanolamine at 37 °C for 10 min. At this temperature, egg PE is hexagonal and egg PE(PC) is lamellar. Controls included anticoagulant antibody 824 + buffer, giving rise to PTT$_{max} = 71$ s, as well as controls without antibody 824, containing GM 4672 IgG or 1500 IgM incubated with buffer, phospholipid, or phosphorylethanolamine, which gave rise to mean PTT$_{min} = 61$ s ± 1. The ability of the phospholipids to inhibit the lupus anticoagulant activity was calculated using the formula,

\[ \% \text{ inhibition} = \frac{\text{PTT}_{max} - \text{PTT}(824 + \text{phospholipid})}{\text{PTT}_{max} - \text{PTT}_{min}} \times 100\%. \]

characteristic of bilayer phospholipids, whereas phospholipids in the hexagonal (H$_{II}$) organization give rise to spectra with reversed asymmetry which are approximately a factor of two narrower (4). As shown in Fig. 1A, DPPE assumes a bilayer organization at 37 °C, whereas dioleoyl-PE, bovine brain PE, and egg PE are in the hexagonal organization at these (and higher) temperatures. Egg PE(PC), which undergoes a bilayer to hexagonal (H$_{II}$) transition as the temperature is increased through 40 °C, adopts the bilayer phase at 37 °C and the hexagonal phase at 43 °C (Fig. 1B).

Titration of the Inhibition of the Anticoagulant Activity of a Hybridoma Lupus Anticoagulant by Egg PE at 37 °C—A titration curve of the inhibition of the anticoagulant activity of hybridoma lupus anticoagulant 824 by egg PE is shown in Fig. 2. Inhibition of the anticoagulant activity by egg PE, which adopts the hexagonal phase at 37 °C, occurred when greater than 2.1 nmol of phosphorus was added and was complete at 68 nmol of phosphorus. On the other hand, egg PE(PC), which is lamellar and liquid crystalline at 37 °C, and phosphorylethanolamine, the polar head group of PE, failed to inhibit anticoagulant activity over the same range of concentrations. Monomyristoyl PE, monopalmitoyl PE, phosphatidylcholine, and phosphatidylinositol also did not inhibit the anticoagulant activity of this antibody (data not shown).

Titration of the Inhibition of the DNA Binding and Rheumatoid Factor Activities of Hybridoma Antibodies by DNA, Immunoglobulin, and Hexagonal and Lamellar PE—In order to ensure that hexagonal PE was not inhibiting the lupus anticoagulant activity by nonspecific interactions with hybridoma IgM antibodies, similar competition experiments using hexagonal and lamellar PE and other IgM hybridoma antibodies were performed in two other assay systems.

Fig. 3A shows the inhibition of the binding of hybridoma antibody 1206 to denatured DNA (dDNA) by dDNA, dioleoyl-PE (DOPE), and DPPE at 37 °C for 1 h. Controls included the hybridoma antibodies preincubated with buffer only (maximal binding in the absence of inhibitors). The percentage inhibition of maximal binding in each assay was calculated using the formula,

\[ \% \text{ inhibition} = \frac{\text{binding (without inhibitor)} - \text{binding (with inhibitor)}}{\text{binding (without inhibitor)}} \times 100\%. \]
binding activity of this antibody was achieved with the addition of 10 nmol of phosphorus of dDNA, while equivalent and 16-fold higher amounts of dioleoyl-PE and DPPE did not show any inhibition of the DNA-binding activity.

Similarly, the rheumatoid factor activity of hybridoma antibody 18141 (Fig. 3B) was inhibited by 70% with the addition of 10 μg of human IgG, but was unaffected by incubation with up to 100 nmol of phosphorus of dDNA, dioleoyl-PE, or DPPE.

**Effects of Phospholipid Phase Structure on Lupus Anticoagulant Activity**—Fig. 4 shows the effects of different PE phospholipid phase structures on the PTT of all 11 hybridoma lupus anticoagulant antibodies, as well as the 1500 IgM control. Hybridoma lupus anticoagulants incubated with buffer had PTT values ranging between 66 and 72 s. This represented a prolongation of 6–12 s over the IgM control, which had a mean PTT of 60 s. There was no correlation between activity and hybridoma IgM concentration, which ranged from 0.03–74.0 μg/ml.

Bovine and egg PE (which are hexagonal phase phospholipids at 37 °C) did not have any effect on the PTT assay itself, as their addition did not change the PTT of the control 1500 IgM supernatant. However, the addition of these phospholipids to the hybridoma lupus anticoagulants resulted in PTT values equivalent to the 1500 control for all 11 antibodies, demonstrating complete inhibition of lupus anticoagulant activity (p < 0.0005). Dioleoyl-PE (hexagonal phase at 37 °C) also completely inhibited the activity of all 11 hybridoma anticoagulants (p < 0.0005) but did not affect the control supernatant. Five of the 11 antibodies were inhibited by 2.7 nmol of dioleoyl-PE (one-half the amount of bovine PE added), while the other 6 required higher amounts of dioleoyl-PE (6.75–67.5 nmol of phosphorus) for complete inhibition. In contrast, DPPE and egg PE(PC) (both lamellar at 37 °C) caused no statistically significant inhibition of lupus anticoagulant activity at concentrations 1.5–48-fold that of dioleoyl-PE.

**Effects of Preincubation of Hybridoma Lupus Anticoagulants with Egg PE(PC) at 37 and 43 °C**—The previous results may be taken to suggest that the anticoagulant antibodies bind to hexagonal phase lipids as opposed to lipids in the lamellar organization. It is possible, however, that the observed effects may be due to the different molecular species of the phospholipids tested. In order to investigate this, we employed egg PE(PC), which adopts the lamellar phase at 37 °C and the hexagonal II phase at 43 °C (Fig. 1B). It was reasoned that if the antibodies are sensitive to structure, egg PE(PC) at 43 °C should be bound by the anticoagulant antibodies, whereas egg PE(PC) at 37 °C should not.

Egg PE(PC) and egg PE were incubated with the hybridoma anticoagulants at 43 °C and subsequently removed by Millipore filtration and the filtrate used in the PTT assay. Fig. 5 shows the effect of the preincubation of the 11 hybridoma lupus anticoagulants with egg PE(PC) at 37 and 43 °C and egg PE at 43 °C. Egg PE(PC) is lamellar at 37 °C and did not inhibit the lupus anticoagulant activity. However, egg PE(PC) assumes a hexagonal configuration at 43 °C (Fig. 1B) and, in this arrangement, dramatically inhibited the anticoagulant activity of the same lupus anticoagulants. In parallel experiments, egg PE was substituted for egg PE(PC) and it was found that at 43 °C, egg PE was less capable of inhibiting anticoagulant activity than at 37 °C, where it removed 100%...
of the lupus anticoagulant activity (Fig. 4). Thus, antibody binding to hexagonal lipid was found to be partially inhibited at elevated temperatures. Antibody heated to 43 °C in the presence of buffer retained all of its lupus anticoagulant activity.

**DISCUSSION**

The results of this study show that certain hybridoma antibodies derived from SLE patients can prolong normal PTT assay values and that this prolongation can be inhibited by the presence of hexagonal (H₃) but not lamellar phase phosphatidylethanolamine. Three points of interest concern the mechanism whereby these antibodies can increase clotting times, the reasons why they exhibit a preferential interaction with H₃ phase phosphatidylethanolamines, and the possible biological significance of antibodies which interact with “nonbilayer” lipid structure. We discuss these points in turn.

The ability of the anticoagulant antibodies to prolong PTT values is not understood in detail. It is, however, well known that initiation of clotting requires the exposure of “inner monolayer” lipids such as PE and the negatively charged lipid phosphatidyserine on the outer monolayer of platelets (18). A similar surface is presumably provided by the cephalin dispersion in the in vitro PTT assay, and it may be that the presence of the anticoagulant antibodies impedes the binding of blood-clotting factors, thus prolonging the assay time. The ability of hexagonal phase (but not bilayer phase) PE to inhibit this prolongation clearly suggests a specific affinity of these anticoagulants for H₃ phase lipid. Other hybridoma IgM autoantibodies with anti-DNA and rheumatoid factor activities cannot be inhibited by preincubation with hexagonal (H₃) phosphatidylethanolamine. Thus, these results support the “specific” recognition and interaction of the hybridoma lupus anticoagulants with hexagonal phase PE.

With the exception of DPPE, all of the phospholipids examined were in the fluid (liquid crystalline) state at the experimental temperatures employed. Thus, the inability of DPPE to reduce PTT values could be related to the crystalline (gel state) nature of this lipid species at 37 °C (19). However, the fact that egg PE(PC) was also incapable of inhibiting anticoagulant activity at 37 °C, where it exists in a liquid crystalline lamellar phase, clearly indicates that the overall polymorphic phase is the determining factor, rather than the gel or liquid crystalline nature of the acyl chains. The inability of phosphatidylethanolamine and monomysristoyl or monopalmitoyl phosphatidylethanolamine to affect prolongation of the PTT values would also suggest that the anticoagulant-PE interaction does not solely involve a chemical interaction at the headgroup level. A remaining possibility concerns the nature of the interface between the H₃ phase aggregates and the aqueous medium. In particular, it is logical to suggest that this interface consists of a monolayer of lipid which surrounds the aggregated hexagonally packed cylinders characteristic of the H₃ phase (20). This monolayer would likely exhibit different packing properties than those observed in a bilayer system, possibly leading to increased antibody recognition and binding to epitopes unmasked on the ethanolamine portion of the molecule.

The results of this study indicate that SLE patients can exhibit antibodies which interact preferentially with, and are possibly specific for, H₃ phase lipid structure. Current studies are aimed at demonstrating similar antibody specificity for non-bilayer lipidic particle structure (21). The possibility of antibodies against non-bilayer lipid aggregates may initially appear surprising. However, whereas the normal function of a cell membrane relies on the presence of a largely (if not exclusively) bilayer lipid organization, many membrane lipids preferentially adopt non-bilayer hexagonal phase or lipidic particle (21) structure. The long term presence of such structures in vivo could well represent lesions requiring recognition and clearance.

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