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Influence of Phosphatidylethanolamine Concentration and Composition on the Detection of Antiphosphatidylethanolamine Antibodies by ELISA

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Background: Accumulating evidence supports a positive correlation between the presence of antiphosphatidylethanolamine (aPE) autoantibodies and clinical symptoms of antiphospholipid syndrome (APS). However, there is a lack of standardized ELISA-based method for detecting aPE. The current study was conducted to investigate the dependence of aPE ELISA on lipid concentration and composition of PE antigens. Methods: A range of ELISA conditions were examined by varying the concentrations of egg PE and by substituting egg PE with combinations of synthetic DOPE and DSPE. The physical properties of the synthetic PE species were also characterized. Results: Our data indicated that there are different optimal PE concentrations for conducting ELISA assays for cofactor-dependent and cofactor-independent aPEs. In addition, using a two-component synthetic lipid system, we demonstrated aPE ELISA readouts can be modulated to approach the performance level of egg PE, which is currently the most commonly used PE antigen. Conclusion: These data raised the possibility of ultimately replacing natural PE antigens with a blend of defined synthetic lipid species, thus overcoming a known variable factor in aPE detection. The outcome of this study will help pave the way to developing a standardized aPE test. J. Clin. Lab. Anal. 30: 689–696, 2016. © 2016 Wiley Periodicals, Inc.

Key words: antiphosphatidylethanolamine antibodies; antiphospholipid syndrome; phosphatidylethanolamine

INTRODUCTION

The clinical diagnosis of antiphospholipid syndrome (APS) requires the detection of persistent presence of circulating antiphospholipid antibodies (aPL) in association with clinical symptoms such as vascular thrombosis and recurrent pregnancy losses. The laboratory criteria of aPL for the classification of APS include lupus anticoagulant, anticardiolipin antibodies, and anti-β2-glycoprotein I antibodies (1–3). However, in some instances, patients with clinical manifestations highly suggestive of APS lack any of the previously mentioned aPL (4–8). These are referred to as seronegative APS.

Accumulating evidence has shown that antibodies directed against phosphatidylethanolamine (PE) lipids, a class of lipids with zwitterionic PE head group, are strongly associated with similar or identical clinical symptoms of APS, in the absence of the laboratory criteria of this syndrome (9–11). The investigation of anti-PE antibodies (aPEs) would impact the clinic diagnosis of unexplained thrombosis and recurrent pregnancy losses, therefore benefiting the treatment outcome of APS.

ELISA is the most common assay for the detection of aPE in patient serum samples. However, there is currently no standardized aPE ELISA protocol, and a number of variations in aPE ELISA conditions have been reported.
in aPE literature. It has been shown that different sources of PE impact ELISA signals to some extent. Other variations in ELISA assay are attributed to the material of microplates, buffer systems, and cofactor supplement used for aPE detection (12–15). Altogether, prior studies reveal an inconsistent comparison among aPE detection data from different laboratories and raised a need for standardization of the assay.

In the current study, in an effort to investigate an optimal assay condition for aPE detection, we examined the influence of antigen concentration and composition on the outcome of aPE ELISA. These results will contribute to a better understanding, and ultimately lead to the standardization of aPE ELISA assay conditions.

MATERIALS AND METHODS

Patients

The study was approved by the Northwestern University Institutional Review Board and was conducted under the NIH guidelines. All patient serum samples were referred for antiphospholipid antibody (Ab) testing and the selected samples were positive for aPE antibodies when tested by the HLA-Vascular Biology Laboratory at Franciscan St. Francis Health, Indianapolis. Twenty-four aPE-positive serum samples were included for this study. Ten were cofactor-dependent IgG, three were cofactor-independent IgG, three were cofactor-dependent IgM, and eight were cofactor-independent IgM. Blood was collected into serum separator vacutainer tubes without anticoagulant and serum was separated by centrifugation. Pooled normal serum control was included in each assay. Aliquots were stored frozen at –70°C until use.

Phospholipids

All phospholipids were purchased from Avanti Polar Lipids and used without further purification. The concentrations of phospholipid stocks were determined by the Bartlett method (16).

ELISA

Cofactor-Independent Patients

aPE ELISA assays were performed as described, with the following modifications (14). Briefly, microtiter plates (PolySorp, Thermo Fisher, Pittsburgh, PA) were coated with 50 µl of a solution containing various concentrations of egg yolk PE or equal molar amounts of dioleoyl PE (DOPE) in ethanol:chloroform (4:1) and dried by evaporation at room temperature (RT) in an extractor shielded from light. Each well was blocked for 2 h at RT with 10% BSA in PBS followed by 50 µl of patient serum diluted 1/100 in PBS containing 1% BSA for 1 h at RT. aPEs were assessed by using alkaline phosphatase conjugated polyclonal Ab against either human IgG or IgM. The plates were washed three times with PBS after blocking, serum and conjugate incubations. After color development with paranitrophenyl phosphate as substrate, optical density (OD) at 405 nm was measured. OD values of pooled normal serum control were subtracted from those of patient serum samples measured with the same PE concentration. For each patient sample measured with various PE concentrations, OD variations were expressed as the percentage of the highest OD value obtained among various PE concentrations.

Cofactor-Dependent Patients

aPE ELISA assays were performed as described previously for plasma protein dependent patients, with the following modifications (14). After being coated with phospholipids, ELISA wells were blocked for 2 h at RT with 10% BSA in TBS followed by 50 µl of patient serum diluted 1/100 in TBS with 1% BSA in the presence or absence of 10% adult bovine plasma (ABP) for 1 h at RT. aPEs were then assessed by using alkaline phosphatase conjugated polyclonal Ab against human IgG.

DSC Measurements

DSC (differential scanning calorimetry) measurements were made using a TA Instruments (New Castle, DE) Q20 differential scanning calorimeter. Mixed samples were...
made by adding appropriate amounts of DOPE and distearoyl PE (DSPE) to a sample pan for a total lipid sample of a few milligrams. High-purity cyclohexane (Sigma Aldrich, St. Louis, MO.) was added to dissolve and mix the lipids; the cyclohexane was evaporated off and the weight of the sample pan checked to insure all of the solvent had evaporated. An amount of deionized water matching or exceeding the lipid weight was added and the sample was sealed using a Tzero hermetic lid and Tzero press (TA Instruments). Samples were heated and cooled at 0.2°C/s to ensure mixing and were then heated and cooled at least twice at rates of 0.1, 0.05, 0.02, and 0.01°C/s. The phase transition temperature seen on heating was plotted versus the temperature seen on cooling and fit to a straight line; the equilibrium transition temperature was found by determining the intersection of this line and the line where the heating and cooling temperatures are equal.

RESULTS AND DISCUSSION
Influence of PE Concentration on aPE Detection
In order to determine the optimal concentration of antigen in aPE ELISA, wells were coated with a series of concentrations of egg yolk PE ranging from 1 to 400 μg/ml. aPEs or normal control serum were assessed with alkaline phosphatase conjugated polyclonal Ab to either IgG or IgM. For each sample, the OD values of pooled normal serum indicating the level of nonspecific binding were systematically subtracted from the OD values obtained with patient serum at the same PE concentration. OD values of each patient serum sample at different PE concentrations, ranging from 1 to 400 μg/ml, were normalized to the highest OD value obtained for the patient. Figure 1 shows an example of normalized independent aPE reactivities as a function of egg yolk PE or synthetic DOPE concentrations from a representative patient serum. With
antigen concentrations increasing from 1 to 400 μg/ml, aPE reactivities increased and peaked at around 5 μg/ml and then gradually decreased.

Among the 24 serum samples tested, 13 of them were cofactor dependent, which required ABP to achieve optimum ELISA readouts. Besides such a difference in ELISA procedure, these two types of serum samples also showed differences in the plot of normalized aPE reactivities versus PE concentrations (Fig. 2). Comparatively, cofactor-dependent samples required a greater antigen concentration to achieve appreciable signal levels. For instance, 1 μg/ml PE hardly resulted in a detectable reactivity with cofactor-dependent aPE samples (Fig. 2A and B), whereas independent samples already showed a decent level of reactivity (Fig. 2C and D). It became apparent that there is a difference between dependent and independent samples in the antigen concentration required to achieve maximal aPE reactivity: the concentration of PE for plasma protein dependent serum was within a board range from about 20 to 100 μg/ml (Fig. 2A and B), whereas the concentration of PE for independent samples to reach maximum readouts fell in a much narrower range around 5 μg/ml (Fig. 2C and D). For cofactor-dependent samples, adding ABP in ELISA not only enhanced the reactivity with PE but also right shifted the curve of normalized aPE reactivities versus PE concentrations. Figure 3 showed a representative curve of normalized aPE reactivities with increasing PE concentrations in the absence (○) or presence (●) of ABP. Data are mean ± SD, n = 3.
concentrations for ELISA in the absence or presence of ABP.

It is notable that different Ab isotypes did not result in significant variations in the optimal range of PE concentrations for ELISA readout. More specifically, cofactor-independent aPE serum samples consisted of three IgG and eight IgM isotypes. When ELISA reactivity as a function of PE concentration is plotted based on individual isotypes (i.e., IgG vs. IgM), the optimal range was consistent between the two isotypes (Fig. 4A vs. C and B vs. D). For cofactor-dependent samples, there were ten IgG and three IgM isotypes. Again, the optimal PE concentration for ELISA readout was comparable between the two isotypes (Fig. 5A vs. C and B vs. D).

### Influence of PE Composition on aPE Detection

Currently, aPE ELISA assays involve variable reagents and protocols among laboratories, which contribute to inconsistent comparisons of aPE ELISA data. The source of antigen PE has been considered as one of the parameters that affect the sensitivity and reproducibility of ELISA (14). Egg yolk is currently the most common source of antigen PE used in aPE ELISA. PE compositions from nature sources, such as the egg, brain or bacteria, can be highly variable, differing in fatty acid compositions, such as the length of the fatty acid chains, as well as the ratios and locations of saturated to unsaturated bonds in acyl chains. The differences between manufacturers or even different batches from one manufacturer could affect the packing and conformation of lipid molecules, contributing to uncertainties in aPE detection. To this end, synthetic lipids are chemically homogenous. A goal of the current study was to explore the possibility of identifying a blend of synthetic lipids with defined composition to mimic the antigenic properties of natural PE. When DOPE alone was compared with egg yolk PE in the current study, as shown in Figure 1, PE origin does not appear to influence the dependence of aPE readouts as a function of PE concentrations. However, the absolute OD value obtained at 5 μg/ml egg yolk PE was higher than the value at same molar concentration of DOPE (Fig. 6). According to existing literature on phospholipid analysis, egg yolk...
Fig. 6. Normalized aPE reactivities for binary DOPE/DSPE mixtures (●) and egg yolk PE (■). aPE reactivities were measured using 5 μg/ml egg yolk PE or equal molar amount of PE containing binary mixtures of DOPE and DSPE. aPE reactivities with different PE compositions were normalized to aPE reactivities with 100% DOPE. Data are mean ± SD, n = 4. * P < 0.5, ** P < 0.05, *** P < 0.005.

PE consists of different species of PE molecules, including about 60% saturated and 40% unsaturated PE. The length of fatty acid chain also varies, with around 20% C₁₆, 60% C₁₈, 10% C₂₀, and 10% C₂₂ (17). As shown in Figure 6, by adding different percentages of DSPE into DOPE, aPE reactivities can be modulated in a composition-dependent fashion. The lipid compositions of 75% DOPE and 25% DSPE, or 50% DOPE and 50% DSPE show an increased reactivity compared to 100% DOPE, raising the likeli-

Fig. 7. A diagram showing distinct physical phases of lipids. In the lamellar phases, the lipid sheets stack to form multilamellar vesicles. In the inverted hexagonal phase, multiple cylinders of lipids stack, and are surrounded by a bounding monolayer in order to keep the hydrophobic tails away from aqueous environment.

hood that the antigen PE from natural sources, such as the egg, can eventually be substituted with synthetic lipid compositions that can ensure consistency and optimized ELISA readouts.

Phase analysis was conducted to characterize the physical properties of the two-component lipid mixture. PE lipids, or lipids with PE head groups, typically form the phases illustrated and described in Figure 7. At lower temperatures, a frozen or gel lamellar (L_α) phase is formed. At intermediate temperatures, the fluid lamellar (L_α) phase is present and at elevated temperatures the lipids form the nonlamellar H_{II} phase. The temperatures at which these phases occur depend decisively on the tail structure (18). DOPE has two identical monounsaturated tails and an L_α-H_{II} phase transition of 3°C (19). DSPE has two identical saturated tails; DSPE has an L_β-H_{II} phase transition of 100°C. Egg PE generally exhibits a L_β'-L_α transition around 5°C and a L_α-H_{II} transition at around 30°C (20).

A single lipid system at equilibrium generally forms only one phase at a given temperature; systems composed of two lipids at equilibrium can form two phases at a given temperature. The phase diagram for DOPE/DSPE mixtures at 6.25, 12.5, 25, 50, 17, and 100% DOPE balanced with DSPE is shown in Figure 8. At higher temperatures, around or above the melting temperature of DSPE, the lipid mixtures form a fluid phase; at lower temperatures, around or below the melting temperature of DOPE, the lipid mixtures form solid phases. In between these extremes, they phase separate into a solid, DSPE-rich phase and a liquid, DOPE-rich phase.

These properties can guide our interpretation of the normalized reactivities of DOPE/DSPE mixtures shown in Figure 7. A general trend is that DSPE-rich mixtures have lower reactivities than DOPE-rich mixtures. We can hypothesize that the fluid nature of DOPE at the temperature at which the ELISA assays were conducted made DOPE more reactive with the aPE. However, as pure DOPE goes to the inverted hexagonal phase, the apparent drop in reactivity may be due to the fact that most of the DOPE forms cylinders that might be inaccessible to the aPE, leaving only the bounding monolayer available to interact with the aPE (Fig. 8). As such, the current data demonstrated that aPE ELISA reactivities can be modulated by adjusting the physical forms of lipid mixtures, and that the ELISA data were consistent with phase measurements using DSC. These data are indicative that, with future studies, the blend of synthetic lipids may ultimately replace PE antigens from natural sources, thus eliminating a major factor of uncertainty in aPE ELISA.

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