ELSEVIER

Biochimica et Biophysica Acta 1372 (1998) 45–54

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Lipid interactions with human antiphospholipid antibody, β2-glycoprotein 1, and normal human IgG using the fluorescent probes NBD-PE and DPH

Daxin Tang [a], Douglas Borchman [a,*], Nigel Harris [b], Silvia Pierangeli [b]

[a] *Department of Ophthalmology and Visual Sciences, University of Louisville, 301 E. Muhammad Ali Blvd., Louisville, KY 40292, USA*
[b] *Morehouse School of Medicine, Atlanta, GA 30310, USA*

Received 21 August 1997; accepted 5 February 1998

Abstract

Recurrent venous thrombosis, arterial thrombosis and pregnancy losses are clinical manifestation associated with antiphospholipid antibody (aPL) that recognizes negatively charged phospholipid antigens. Enzyme-linked immunosorbent assays (ELISA) are generally used to determine the presence and specificity of aPL. In this paper, a fluorescence spectroscopy method has been applied, through monitoring the alteration of fluorescence intensity and anisotropy of a fluorophore that was incorporated in liposomes to explore the changes of molecular structure or configuration elicited by the binding aPL with phospholipid antigens. The bilayer surface was markedly ordered by aPL binding as indicated by the surface-sensitive probe NBD-PE. The binding of aPL on the bilayer surface is saturable. The saturation concentration of aPL is 40% (w/w, aPL/lipid) for cardiolipin membranes. The binding of aPL on cardiolipin took place in the absence of β2-GP1. The addition of β2-GP1 further increased the anisotropy and decreased the intensity of fluorescence. The binding of aPL is predominantly attributed to electrostatic interaction, but the configuration of the acyl chains of phospholipid also plays a role. It is found that the thermal history is important for aPL binding. The incubation at 37°C is more favorable for aPL binding than ambient temperature. Normal human serine (IgG-NHS) did not elicit any distinct change of NBD-PE fluorescence, which indicates it does not interact with the lipid. Published by Elsevier Science B.V.

Keywords: Antiphospholipid antibody; β2-GP1; Cardiolipin; Protein binding; Fluorescence; NBD-PE; DPH

1. Introduction

The association of anti-phospholipid antibodies (aPL) with recurrent thrombosis and pregnancy loss is well recognized [1–3]. Patients with this disorder are described as having the 'Antiphospholipid Syndrome' [4–6]. The mechanism by which these autoantibodies are induced, and the nature of the associ-

Abbreviations: IgG-NHS, normal human serum; aPL, antiphospholipid antibody; PC, phospholipid; ELISA, enzyme-linked immunosorbent assays; β2-GP1, β2-glycoprotein 1; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt; DPH, diphenylhexatriene; CL, cardiolipin; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; POPC, 1-palmitoy-2-oleoyl-3-*sn*-phosphatidylcholine; PC, bovine heart PC

* Corresponding author.

Published by Elsevier Science B.V.
PII S0005-2736(98)00028-5

ation of anti-phospholipid antibodies with thrombosis is unknown. There are several clues that these antibodies may play an integral role in the thrombotic process [7–15]. A central feature of antiphospholipid antibodies is that they influence coagulation reactions catalyzed by phospholipid. The so-called lupus anticoagulant reaction is due to the fact that these antibodies inhibit prothrombin to thrombin conversion [6,16]. This is a reaction catalyzed by phospholipid, and it has been demonstrated by several investigators that an increase of phospholipid in this system will inhibit the reaction [16–19].

Several investigators present evidence that antibodies with lupus anticoagulant activity may be specific for β2-glycoprotein 1 (β2-GP1), a β2-GP1–phospholipid complex [20,21], prothrombin [22], or a human prothrombin–phospholipid complex [23]. The conformation rather than the type of phospholipid may determine its antigenicity [24–26].

It is reasonable to suggest that the conformation adopted by coagulation proteins in the presence of phospholipid, and the conformation of phospholipid in the presence of coagulation proteins may be important in determining antibody binding and their resultant functional effects. It is also conceivable that by binding phospholipid with antibody, these antibodies and/or antigens may impose structural changes, which may enable circulating phospholipid to serve as templates for the coagulation reaction. In an effort to elucidate the structural interactions involved with the coagulation reactions, as a first step, we have studied the structure of β2-GP1, and cardiolipin interactions using infrared spectroscopy [27], and β2-GP1 interaction with calcium and cardiolipin using a head group-labeled fluorescent probe [28]. This methodology was developed in our laboratory to quantify lens protein–lipid binding [29]. In this study, β2-GP1, antiphospholipid antibody, lipid bilayer interactions were assessed using the fluorescent probe NBD-PE.

2. Materials and methods

2.1. Materials

β2-GP1 was purchased from Celsius Laboratories, (Cincinnati, OH). IgG was isolated from serum of a patient with the antiphospholipid syndrome (aPL) using affinity purification with cardiolipin liposomes and subsequent isolation of IgG with protein G Sepharose as described elsewhere [24,30,31]. Purity was determined by SDS-PAGE (single band at 150 kDa) and absence of contamination with β2GP1 was determined by immunoblot using a rabbit anti-human β2GP1 antiserum. Protein concentration was determined by the Lowry method [32]. Normal human IgG was obtained from a pool of normal health control sera ($n = 10$) by protein G Sepharose chromatography.

N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (NBD-PE) and diphenylhexatriene (DPH) were purchased from Molecular Probes (Eugene, OR). The concentration was determined using an extinction coefficient of 20,000 M^{-1} cm^{-1} at 460 nm and 91,000 M^{-1} cm^{-1} at 350 nm in chloroform, respectively.

Phosphate-buffered saline (PBS), alkaline-phosphatase-labeled antihuman immunoglobulin (IgG), *p*-nitrophenylphosphate, adult bovine serum (ABS), cardiolipin (CL), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC) and 1-palmitoy-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) were purchased from Sigma (St. Louis, MO). The concentration was determined as inorganic phosphate by the method used by Bartlett [33] or as specified by the manufacture.

2.2. Liposome preparation

Model membranes composed of phospholipid were used. Phospholipid and either of the fluorescence probes NBD-PE or DPH dissolved in chloroform were mixed together at a weight ratio of fluorescence probes to phospholipid of 1:200. The solution was dried under nitrogen, then lyophilized for 4 h to remove all chloroform. The mixture was suspended in buffer containing 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.5 at 25°C. After suspending the sample using a vortex mixer (Fisher Scientific), the lipid probe mixture was sonicated for 5 min at 40°C to form vesicles. After incubation at room temperature for 1 h, the suspension was divided equally into plastic cuvettes with antiphospholipid antibody (or antiphospholipid

antibody and β2-GP1) added. After mixing with a vortex mixer, the samples were incubated for 17 h at room temperature or 37°C. Binding was assessed using an ELISA assay and by fluorescence intensity and anisotropy measurements described below.

2.3. ELISA

The binding of anticardiolipin and antiphospholipid antibodies was determined by ELISA using cardiolipin or other phospholipids to coat the plates, as described elsewhere [34,35]. The levels of anticardiolipin antibodies were determined in GPL (Louisville APL Diagnostics, Louisville, KY).

2.4. Fluorescence intensity and anisotropy measurement

Fluorescence intensity and anisotropy measurements were made with an ISS PC1 photon counting spectrofluorometer (Champaign, IL) with polarization accessory unit. Steady-state fluorescence anisotropy, r, was measured as

$$r = (I_{\parallel} - gI_{\perp})/(I_{\parallel} + 2gI_{\perp})$$

in which $g = I_{\perp}/I_{\parallel}$; $I_{\perp}$ and $I_{\parallel}$ are the perpendicular and parallel components of fluorescence observed corresponding to the plane of polarization of the excitation beam, respectively. Total fluorescence intensity was calculated as

$$I_{\text{tot}} = I_{\parallel} + 2I_{\perp}$$

All measurements were made at the equilibrium temperature of 23°C or as indicated in the tables and figure legends. Samples with NBE-PE were excited at 460 nm, the emission was observed at 540 nm by a multiphotometer. Samples with DPH were excited at 350 nm, the emission was observed at 452 nm. The integration time was 5 s. The measuring error was less than 0.002 for anisotropy. The concentration of lipid used for fluorescence measurements is 0.26 mM in the phase transition study and 200 μg/ml in the protein binding study. The total volume of the mixture of lipid and proteins was 1.5 ml. Samples were stirred by a magnetic bar in the spectrometer cuvette through the fluorescence measurement.

3. Results

The fluorescent probe, NBD-PE was used to monitor changes in the bilayer surface environment caused by aPL binding. Addition of aPL to the cardiolipin membranes induced a dramatic decrease in the fluorescence intensity (Fig. 1a) and a dramatic increase in

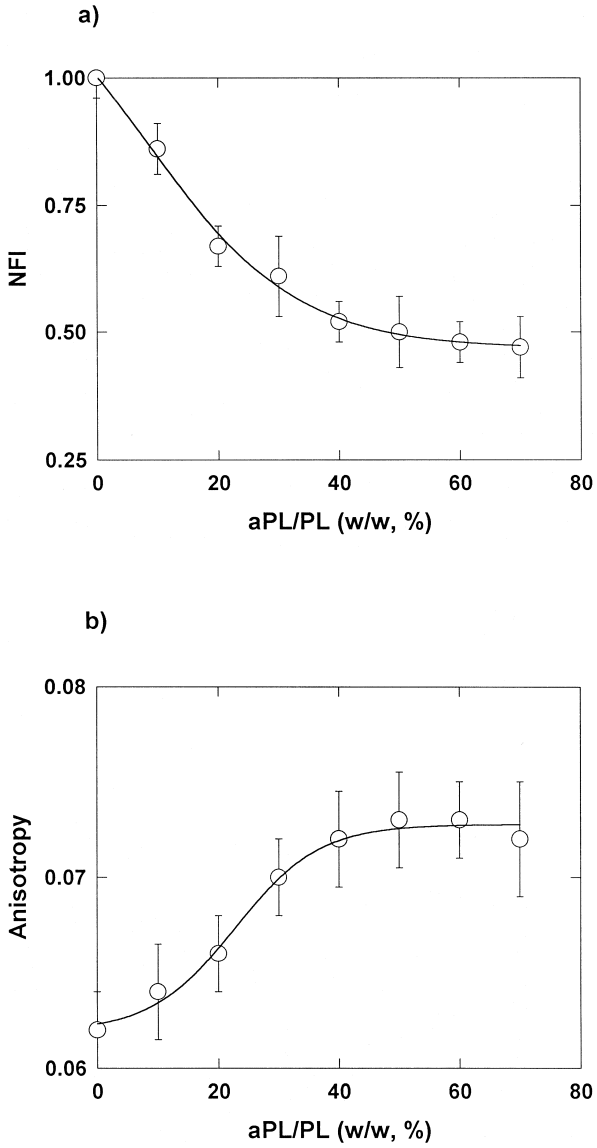

Fig. 1. (a) Decrease in normalized fluorescence intensity (NFI) and (b) increase in fluorescence anisotropy of NBD-PE upon increasing aPL. The liposomes were prepared from bovine heart cardiolipin. The measurements were completed at 23°C. The liposomes were bound by aPL at 23°C.

Table 1
Protein–lipid binding parameters measured from fluorescence intensity of NBD-PE probe

Substrate bound to bovine heart cardiolipin and bovine heart PC [1:1 (w/w), 23°C, unless indicated]	Normalized fluorescence intensity parameters		
	ΔNFI	K_b	Cooperativity
aPL binding to cardiolipin (no PC)	−0.58 (0.03)	18.0 (1.3)	1.85 (0.22)
aPL	−0.285 (0.007)	14.7 (0.9)	2.4 (0.3)
aPL 37°C	−0.524 (0.044)	15.4 (2.7)	1.1 (0.252)
aPL, 4.0 μg β2-GP1	−0.626 (0.042)	< 12	N.S.
aPL, 6.3 μg β2-GP1	−0.689 (0.026)	1.87 (1.31)	1.1 (0.7)
IgG-NHS	N.S.*	N.S.	N.S.
IgG-NHS, 3 μg β2-GP1	−0.105 (0.003)	< 3	N.S.

*N.S. (not significant).

the fluorescence anisotropy (Fig. 1b). Note the plateau in the fluorescence anisotropy and intensity, which indicates saturable binding occurs above a aPL to cardiolipin ratio of 40% (w/w). Using Sigma Plot, version 2 (Jandel, San Rafael, CA), nonlinear regression analysis was used to fit the data in Fig. 1a,b to Eq. (1) below, that was derived from an equation used to quantify Michaelis–Menten kinetics for enzymes and lipid structural analysis [36].

$$\text{Equation: } Y = \min + \max/\left[1 + (K_b/X)^C\right] \qquad (1)$$

where Y = anisotropy or fluorescence intensity; X = ligand/phospholipid ratio as wt.%; min = minimum anisotropy or fluorescence intensity; max = maximum change in anisotropy or fluorescence; K_b = wt.% ratio for 50% binding, K_b; C = cooperativity of binding similar to 1/Hill coefficient, described by Michaelis–Menton kinetics. The broader the binding curve, the lower the cooperativity. Parameters for Fig. 1a,b are given in Tables 1 and 2, respectively. Using the fluorescent probe to quantify binding, a K_b of 18% (w/w) and a cooperativity of 1.8 was estimated.

To confirm the binding data determined by fluorescence, an ELISA assay was used to measure the binding of aPL with bovine heart cardiolipin antigen. Fig. 2 shows that O.D. at 410 nm increases upon the incorporation of aPL from 0 to 50% (w/w, aPL/cardiolipin), and a maximum saturation of aPL is found around 40% (w/w, aPL/cardiolipin) and a K_b of 11.7 and cooperativity of 1.2 was determined (Table 3). The binding data from the ELISA and fluorescence assays were identical (not statistically different, $p = 0.030$).

The effect of aPL binding on membranes composed of 50% (w/w) bovine heart cardiolipin and 50% (w/w) bovine heart PC also was studied by monitoring the change of NBD-PE fluorescence. All

Table 2
Protein–lipid binding parameters measured from anisotropy change of NBD-PE probe

Substrate bound to bovine heart cardiolipin and bovine heart PC [1:1 (w/w), 23°C, unless indicated]	Anisotropy parameters				
	ΔR	K_b	Cooperativity	R_{min}	R_{max}
aPL binding to cardiolipin (no PC)	0.0102 (0.0010)	24 (2.0)	4.0 (1.3)	0.063 (0.0006)	0.073
aPL	0.0106 (0.0012)	21 (2.8)	3.2 (1.2)	0.0709 (0.0009)	0.082
aPL, 37°C	0.032 (0.003)	23 (3.5)	1.50 (0.28)	0.086 (0.001)	0.118
aPL, 4.0 μg β2-GP1	0.039 (0.004)	19.2 (3.3)	1.6 (0.4)	0.086 (0.0002)	0.125
aPL, 6.3 μg β2-GP1	0.0432 (0.0060)	12 (2.7)	1.9 (0.7)	0.0726 (0.0043)	0.115
IgG-NHS	0.0094 (0.0075)	N.S.*	1.26 (1.27)	0.0972 (0.0016)	0.107
IgG-NHS, 3 μg β2-GP1	0.020 (0.002)	5.6 (1.2)	1.07 (0.38)	0.093 (0.001)	0.073

*N.S. (Not significant).

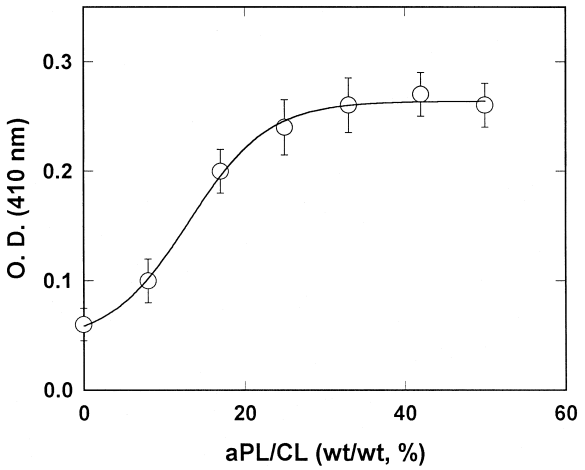

Fig. 2. The binding plot of cardiolipin liposome upon the amount of aPL detected by ELISA at 23°C.

of binding parameters from fluorescence intensity and fluorescence anisotropy are listed in Tables 1 and 2, respectively. From the fluorescence intensity, the K_b was calculated to be 18.0 (without PC) and 14.7 with 50 wt.% PC; from the fluorescence anisotropy, it was 24.0 without PC and 21.0 with 50 wt.% PC, respectively. This indicates that aPL binds with the same sensitivity with or without 50% (w/w) PC. The ΔNFI with 50% PC was statistically twofold lower ($p = 0.0001$) compared with the ΔNFI for cardiolipin alone. This indicates that the capacity to bind aPL is twofold lower in cardiolipin vesicle with 50% PC. Following the above experiment, an additional 6.3 μg β2-GP1 was added into each sample and incubated at room temperature overnight. β2-GP1 caused no significant change in the fluorescence intensity in the absence of aPL, but caused a significant ($p = 0.0001$) twofold increase in the binding capacity (ΔNFI) of aPL to cardiolipin/PC vesicles (Table 1). The K_b was also significantly lower ($p = 0.0001$) with 6 μg β2-GP1 present.

The aPL binding was also tested with the zwitterionic phospholipid DMPC and DPPC as the lipid matrix. DPPC has a higher phase transition temperature (43°C, gel/liquid crystalline phase) than DMPC (25°C, gel/liquid crystalline phase). Binding of aPL was quantified at 23°C and 48°C for comparison with cardiolipin (Table 4). In comparison with cardiolipin, the ΔNFI value (binding capacity) was 10 times lower for DPPC alone (Table 4), which indicates that very little, if any, aPL binds to DPPC. The ΔNFI value was two times larger when DPPC is in the liquid–crystalline phase, but the binding capacity was still five times lower than for cardiolipin alone (Table 4). Since aPL binds only slightly to DPPC and DMPC in any phase, the 50% decrease in the binding capacity of aPL to cardiolipin when PC is present is elucidated, and suggests that aPL binds only to cardiolipin when PC is present. After normalization, the data that were obtained at different temperatures, before and after the addition of β2-GP1, was identical (Table 4). From the data above, we conclude that neither DPPC nor DMPC bound significantly with aPL or β2-GP1.

Using our fluorescence probe technique, the binding activity of aPL that tested positive in the ELISA assay was compared to an IgG from normal human sera (IgG-NHS). IgG-NHS was incorporated into liposomes composed of cardiolipin and bovine heart PC (1:1, w:w) (Table 1). IgG-NHS caused an insignificant change in the anisotropy and fluorescence intensity of the NBD-PE probe; thus, no significant IgG-NHS binding was observed. The addition of 3.0 μg β2-GP1 in each sample induced a discernible increase in the binding capacity (ΔNFI) of aPL; however, the changes in the binding capacity, ΔNFI, for β2-GP1/IgG-NHS was 6 times smaller than for aPL. This suggests that even with β2-GP1, IgG-NHS binds only slightly to cardiolipin/PC vesicles.

The thermal history of samples influenced the binding of aPL to the membranes composed of

Table 3
aPL-cardiolipin binding parameters measured from ELISA assay

	ELISA assay parameters			
	$\Delta O.D._{max}$	K_b	Cooperativity	$O.D._{min}$
aPL binding to: bovine heart cardiolipin, 23°C	0.247 (0.049)	11.7 (1.2)	1.2 (0.5)	0.060 (0.011)

Table 4
aPL binding to DPPC measured from changes in NBD-PE fluorescence probe characteristics

Conditions	Normalized fluorescence intensity parameters			
	ΔNFI	K_b	Cooperativity	
23°C	−0.053 (0.035)	< 12	N.S.*	
48°C	−0.13 (0.22)	< 12	N.S.	
2.5 μg	< 12	*N*.S.		
β2-GP1, 48°C	*N*.S.			
	Anisotropy parameters			
	Δ*R*	K_b	Cooperativity	R_{min}
23°C	N.S.	N.S.	N.S.	0.118 (0.005)
48°C	N.S.	N.S.	N.S.	0.060 (0.002)
2.5 μg β2-GP1, 48°C	0.017 (0.037)	N.S.	N.S.	0.063 (0.002)

*N.S. (not significant).

cardiolipin and bovine heart PC (1:1. w/w). As seen in Table 1, when aPL cardiolipin/PC binding at 23°C is compared with the binding at 37°C, the binding capacity, ΔNFI, was significantly two times higher ($p < 0.0001$) at 37°C. There is no significant difference between the K_b.

DPH probe was utilized to explore the influence of aPL binding on the flexibility of acyl chains in membranes with cardiolipin. The DPH fluorescence probe has been extensively adopted to study the anisotropy or polarization of the interior of membranes. We measured samples that were incubated at ambient temperature and 37°C, respectively. aPL binding caused no obvious change in anisotropy of DPH suggesting that binding has no effect on the hydrocarbon chains of the membranes.

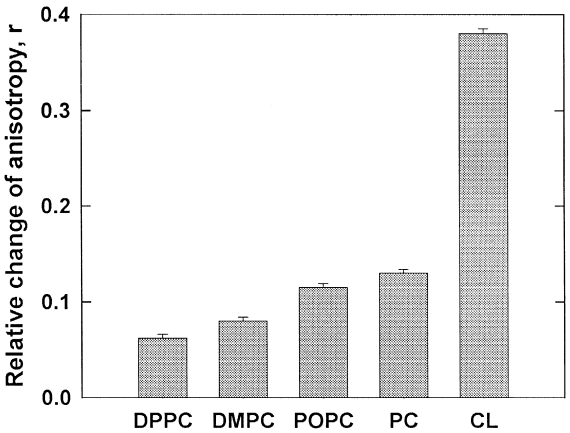

Fig. 3. The comparison of binding of aPL to membranes that are composed of homogeneous liposomes. The ratio of aPL to phospholipid is 65% (w/w). The unit of *Y* axis is the relative increase of anisotropy, *r*, defined as follows: $[r_{with} - r_{free}]/r_{free}$, in which r_{with} = anisotropy of NBD-PE where aPL present, r_{free} = anisotropy of NBD-PE where aPL is absent. Spectroscopy measurements and binding equilibration were done at 23°C.

To determine if the configuration (or the unsaturation of the acyl chains) of phospholipid influence the binding of aPL, aPL was bound to liposomes prepared from DOPC, bovine heart PC, DMPC and DPPC. Judging from the relative increase in the anisotropy of the NBD-PE probe upon aPL binding, aPL binds in following order: DPPC < POPC = bovine heart PC ≪ CL (Fig. 3).

To test if oxidating conditions influenced the binding of aPL to cardiolipin, argon purging was omitted from all protocols. The results obtained without argon were identical to these obtained with argon.

4. Discussion

The disorder of recurrent venous thrombosis, arterial thrombosis (strokes, myocardial infarction) and pregnancy loss was recognized in young women with systemic lupus erythematosus (SLE). The abnormality accompanying this disorder is a production of antiphospholipid antibodies. The disorder is called the 'Antiphospholipid Syndrome'; however, the mechanism of action of antiphospholipid antibody in this syndromes is unknown. In a first attempt to elucidate the interactions involved with coagulation, the binding of aPL to a few select lipid systems and

the effect of β2-GP1 on this binding was determined and compared to the binding of IgG from healthy humans. It is necessary to systematically study the condition of aPL binding appearance and the influence of aPL binding on the structure of antigens and membranes with antigens.

In the past, the interactions of phospholipid and antiphospholipid antibody has been determined by an ELISA assay. Although the ELISA assay is very sensitive for the binding, it does not reveal molecular configuration or structure change during the binding process. In this paper, fluorescence spectroscopy was used to confirm the binding of phospholipid with antiphospholipid antibody. The fluorescence method overcomes the disadvantage of the loss of lipid during washing in ELISA, directly giving information about interaction between liposome and aPL. Fluorescence probes can be located in different positions of lipid membrane and information, such as the change of molecular configuration during the binding, can be obtained by monitoring changes of fluorescence intensity and anisotropy upon the addition of aPL.

In our experiments, two different fluorescent probes were used. NBD-PE was used to monitor the influence of aPL binding on the head group of phospholipid antigens in membranes, because the fluorescent moieties of NBD-PE are situated in the head group region of the phospholipid bilayer and are thus sensitive to the change of the surface environment of membranes [29]. DPH was used to study the effect of aPL binding on the hydrocarbon chains of phospholipid, because DPH is distributed in the hydrophobic region of membranes and is sensitive to change in lipid order at various depths in the hydrophobic bilayer interior [37]. We found that binding did not influence the anisotropy or fluorescence intensity of the DPH probe, indicating that the hydrophobic interior of the bilayers is not affected by aPL binding, and that large packing changes between vesicles did not occur.

Cardiolipin was the first antigen found that binds with aPL [1]. In this paper, the binding of aPL with cardiolipin was confirmed by NBD-PE fluorescence spectroscopy.

The increase of probe fluorescence anisotropy and the decrease of probe fluorescence intensity is attributed to the binding of aPL to cardiolipin. The increase of NBD-PE fluorescence anisotropy reflects a decrease of flexibility in the head group region of the lipid bilayer in the vicinity of aPL. We have found that the binding of aPL to cardiolipin is saturable with a K_b of 18 μg aPL/100 μg cardiolipin. The incorporation of PC (50% by weight caused a 50% decrease in the binding capacity (ΔNFI) of aPL (Table 1). The K_b determined by a decrease in fluorescence intensity was lower than that determined by the increase in anisotropy data, because it is likely that a greater amount of aPL binding was necessary to elicit a change in the mobility wobble (anisotropy) of the probe.

There are at least two possibilities that could explain the quenching of NBD fluorescence by aPL. One explanation is that the NBD-PE molecules may phase-separate from the major lipids during the binding of aPL. Such a phase separation would increase the local concentration of NBD-PE, and lead to fluorescence self-quenching. This phenomenon was observed when lipids containing 1 mol% NBD-PE are in the gel phase [38]. A second explanation is that the quenching of NBD-PE fluorescence after aPL binding may arise from the exposure of NBD fluorophore moiety to water (a quencher). The fluorophore moiety for NBD is hydrophobic and located at the bilayer surface, as demonstrated by quenching experiments [39] and spin-labeled lipids [40]. The binding of aPL in the environment of NBD moieties may make the head groups of phospholipids more rigid as indicated by anisotropy results, forcing the NBD towards the water that leads to a quenching of fluorescence.

The thermal history of the incubation of the samples is another important factor that influences aPL binding to phospholipid antigens. When the same samples (50 wt.% cardiolipin and 50 wt.% PC) were incubated at 23°C, less apparent binding was observed as evidenced by a small ΔNFI and ΔR value (Tables 1 and 2). When same samples were incubated at 37°C for the same period, the ΔNFI and ΔR values for NBD-PE increased twofold (Tables 1 and 2). This indicates that physiological temperature is more favorable to the binding of aPL to membranes containing cardiolipin.

There are several different opinions about the role of β2-GP1 in aPL binding [41]. The first is that β2-GP1 alone is the antigen recognized by anticardiolipin antibodies. Galli et al. [42], using antibodies affinity purified from two patients with

Antiphospholipid Syndrome reported direct antibody binding to β2-GP1. The second is that anticardiolipin antibodies bind a cardiolipin–β2-GP1 complex. McNeil et al. [43] found no direct binding of affinity-purified anticardiolipin antibodies to β2-GP1 by column chromatograph, but they reported that β2-GP1 was an absolute requirement for binding to cardiolipin. They proposed that these antibodies bind epitopes shared by cardiolipin and β2-GP1. The third opinion is that the β2-GP1 enhances binding to cardiolipin of some patients' sera [41,44,45]. In our experiments, an obvious increase of fluorescence anisotropy and a marked decrease of fluorescence intensity were found after addition of β2-GP1 into aPL/(cardiolipin + bovine heart PC) comparing with aPL/(cardiolipin + bovine heart PC) alone; however, we also found a similar increase of anisotropy and a similar decrease of NBD-PE fluorescence intensity when β2-GP1 was incorporated into cardiolipin/bovine heart PC mixture. The binding of β2-GP1 on acid phospholipid depends predominantly on the electrostatic interaction of the acidic head group of phospholipid, and the positively charged amino acid histidine [46,47]. Based on our results, β2-GP1 enhances the binding of aPL, which must occur via a conformational change. Although we show that β2-GP1 can bind to CL alone, the change in probe ΔNFI and xxanisotropy are much greater when aPL and β2-GP1 are together than when the system is saturated with aPL or β2-GP1 alone. Our results eliminate the possibility proposed by Galli et al. [42] that β2-GP1 alone is the antigen for aPL, since aPL alone can bind with phospholipid at appropriate incubation condition even in the absence β2-GP1.

Through the comparison of binding data using DMPC, DPPC, POPC, bovine heart PC and cardiolipin as antigen with aPL present and aPL absent, we have found that the anisotropy of NBD-PE was raised by the incorporation of aPL to a different extent (Fig. 3). The relative change in anisotropy follows the order: cardiolipin ≫ bovine heart PC > POPC > DMPC > DPPC. The negatively charged head group of cardiolipin greatly enhances binding compared with all forms of phosphatidylcholine (Fig. 3). DPPC is completely ordered at 23°C and binds aPL less than DMPC (Fig. 3). Thus, hydrocarbon chain order and aPL binding are correlated. It is possible that the ability of bound aPL to raise the anisotropy of NBD-PE in DPPC is less than DMPC, since DPPC is more ordered, and the anisotropy is already as high as possible. The influence of the acyl chains on aPL binding was explored by Levy et al. [48] using the ELISA method. They found the potential role of the fatty acid chains on a PL binding by monitoring the effect of degradation of phosphatidylethanolamine by loss of an acyl chain group to lyso phosphatidylethanolamine [49].

IgG-NHS does not change the fluorescence intensity and anisotropy of NBD-PE in CL/PC, PC and DMPC liposome. This is a hint that 'Antiphospholipid Syndrome' is attributed to the binding of aPL to lipid membranes. Weak binding of IgG-NHS is observed when β2-GP1 is added, but even with β2-GP1, the extent of IgG-NHS binding is much less than for aPL.

Acknowledgements

This work is supported by a grant from the Jewish Hospital, Louisville KY and USPHS grant EY07975 and an unrestricted grant from Research to Prevent Blindness.

References

[1] E.N. Harris, Antiphospholipid antibodies and cardiovascular disease, in: R.B. Francis (Ed.), Atherosclerotic Cardiovascular Disease, Hemostasis, and Endothelial Function, Marcel Dekker, New York, 1992, pp. 237–276.
[2] D.A. Triplett, J.T. Brandt, Lupus anticoagulants: misnomer, paradox, riddle, epiphenomena, Hematol. Pathol. 2 (1988) 121–143.
[3] P.E. Love, S.A. Santoro, Antiphospholipid antibodies: anticardiolipin and lupus anticoagulant in systemic lupus erythematosus (SLE) and in non-SLE disorders: prevalence and clinical significance, Ann. Intern. Med. 12 (1990) 682–698.
[4] E.N. Harris, Syndrome of the Black Swan, Br. J. Rheumatol. 26 (1987) 324–326.
[5] R.A. Asherson, A 'primary' antiphospholipid syndrome?, J. Rheumatol. 15 (1988) 1742–1746.
[6] D. Alarcon-Segovia, J. Sanchez-Guerrero, J. Rheumatol. 16 (1989) 482–488.
[7] E.N. Harris, J.K.H. Chan, R.A. Asherson, V.R. Aber, A.E. Ghavari, G.R.V. Hughes, Thrombosis, recurrent fetal loss, thrombocytopenia; predictive value of IgG anticardiolipin antibodies, Arch. Intern. Med. 88 (1990) 112–116.
[8] M.D. Lockshin, M.L. Druzin, T. Qatar, Prednisone does not

prevent recurrent fetal death in women with antiphospholipid antibodies, Am. J. Obstet. Gynecol. 160 (1989) 439–443.
[9] B.M. Alving, C.F. Barr, D.D. Tang, Correlation between lupus anticoagulants and anti cardiolipin antibodies in patients with prolonged activated partial thromboplastin times, Am. J. Med. 88 (1990) 112–116.
[10] S. Loizou, M.A. Byron, H.R. Englert, M.J. Walport, G.R.V. Hughes, Association of quantitative anti-cardiolipin antibody levels with fetal loss, Q. J. Med. 68 (1998) 525–531.
[11] D.W. Branch, D.J. Dudley, M.D. Mitchell, K.A. Creightari, J.M. Abbott, E.H. Hammond, R.A. Daynes, Immunoglobulins G fractions from patients with antiphospholipid antibodies causes fetal death in Balb/c mice: a model for autoimmune fetal loss, Am. J. Obstet. Gynecol. 163 (1990) 210–216.
[12] M. Blank, J. Cohen, V. Toder, Y. Shoenfeld, Proc. Natl. Acad. Sci. U.S.A. 88 (1993) 3069–3073.
[13] R. Bakimer, P. Fishman, M. Blank, B. Srednc, M. Djaidetti, Y. Shoenfeld, Induction of primary antiphospholipid syndrome in mice by immunization with a human monoclonal anticardiolipin antibody (H-3), J. Clin. Invest. 89 (1992) 1558–1563.
[14] H.R. Smith, C.L. Hansen, R. Rose, R.T. Canoso, Autoimmune MRl-lpr/lpr mice are an animal model for the secondary antiphospholipid syndrome, J. Rheumatol. 17 (1990) 911–915.
[15] A.E. Gharavi, Anti-phospholipid syndrome in mice, in: E.N. Harris, T. Exner, G.V.R. Hughes, R.A. Asherson (Eds.), Phospholipid Binding Antibodies, CRC Press, Boca Raton, FL, 1991, pp. 403–416.
[16] M. Galli, S. Beguin, T. Lindout, C.H. Hemker, Inhibition of phospholipid and platelet-dependent prothrombinase activity in the plasms of patients with lupus anticoagulants, Br. J. Haematol. 72 (1989) 549–555.
[17] J.T. Brandt, Assay for phospholipid-dependent formation of thrombin and factor Xa: a potential method for quantifying lupus anticoagulants, Thromb. Haemost. 66 (1991) 453–458.
[18] A.J. Marcus, H.L. Ullman, L.B. Safier, Lipid composition of subcellular particles of human blood platelets, J. Lipid Res. 10 (1969) 108–115.
[19] D.A. Triplett, J.T. Brandt, Lupus anticoagulants: misnomer, paradox, riddle, epiphenomena, Hematol. Pathol. 2 (1988) 121–143.
[20] J.D. Oosting, R.H.W.M. Derksen, T.I. Entjes, B.N. Bouma, P.G. de Groot, Lupus anticoagulant activity is frequently dependent on the presence of β2 glycoprotein1, Thromb. Haemostasis 67 (1992) 499–502.
[21] M. Galli, P. Comfurius, T. Barbui, R.F.A. Zwaal, E.M. Bevers, Anticoagulant activity of beta 2-glycoprotein 1 is potentiated by a distinct subgroup of anticardiolipin antibodies, Thromb. Haemostasis 68 (1992) 297–300.
[22] R.A. Fleck, S.E. Rapaport, L.V.M. Rao, Anti-prothrombin antibodies and the lupus anticoagulant, Blood 72 (1989) 512–519.
[23] E.M. Bevers, M. Galli, T. Barbui, P. Camfurius, R.F.A. Zwaa, Lupus anticoagulant IgG's prothrombin, Thromb. Haemostasis 66 (1991) 629–632.
[24] E.N. Harris, A.E. Gharavi, A. Tincani, J.K.H. Chan, G. Balestrieri, G.R.V. Hughes, Affinity-purified anti-cardiolipin antibodies and anti-DNA antibodies, J. Clin. Lab. Immunol. 17 (1985) 155–162.
[25] E.N. Harris, A.E. Gharavi, G.D. Wasley, G.R.V. Hughes, Use of an enzyme-linked immunosorbent assay and of inhibition studies to distinguish between antibodies to cardiolipin from patients with syphilis of autoimmune disorders, J. Infect. Dis. 157 (1988) 23–31.
[26] J. Rauch, M. Tannenbaum, H. Tannenbaum, H. Ramelion, P.R. Cullis, C.P.S. Tilcock, M.J. Hope, A.S. Janoff, Human hybridoma lupus anticoagulants distinguish between bilayer and non-bilayer phase lipid systems, J. Biol. Chem. 261 (1986) 9672–9677.
[27] D. Borchamn, E.N. Harris, S.S. Pierangeli, O.P. Lamba, Interactions and molecular structure of cardiolipin and beta 2-glycoprotein 1 (β2-GP1), Clin. Exp. Immunol. 102 (1995) 373–378.
[28] D. Borchamn, D. Tang, N. Harris, S. Pierangeli, The structure of cardiolipin-phosphatidylcholine membranes and the effect of calcium and beta2-GP1 binding as explored by the fluorescence probe NBD-PE, submitted.
[29] D. Borchman, D. Tang, Binding capacity of α-crystalline to bovine lens lipids, Exp. Eye Res. 63 (1996) 407–410.
[30] S.S. Pierangeli, E.N. Harris, G.H. Goldsmith, A.E. Gharavi, D.W. Branch, W.L. Dean, Are immunoglobulins with lupus anticoagulant activity specific for phospholipids?, Br. J. Haematol. 85 (1993) 124–132.
[31] S.S. Pierangeli, G.H. Goldsmith, S. Krnic, E.N. Harris, Difference in functional activity of anticardiolipin antibodies from patients with syphilis and the antiphospholipid syndrome, Infect. Immunol. 62 (1994) 4081–4084.
[32] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[33] G.R. Bartlett, Phosphorus assay in column chromatography, J. Biol. Chem. 234 (1959) 466–468.
[34] E.N. Harris, Annotation: antiphospholipid antibodies, Br. J. Haematol. 74 (1990) 1–9.
[35] E.N. Harris, S.S. Pierangeli, D. Birch, Anticardiolipin wet workshop report: Vth International Symposium on Antiphospholipid Antibodies, Am. J. Clin. Pathol. 101 (5) (1994) 616–624.
[36] D. Borchman, Y. Oriki, O.P. Lamba, W.C. Byrdwell, M.C. Yappert, Age and regional structural characterization of lipid hydrocarbon chains from human lenses by infrared, and near-infrared Raman, spectroscopies, Biospectroscopy 2 (1996) 113–123.
[37] B.R. Lentz, D.A. Barro, M. Hoechli, Cholesterol–phosphatidycholine interactions in multilamellar vesicles, Biochemistry 19 (1980) 1943–1954.
[38] J. Wylie, R.E. Pagano, Kinetics of soluble lipid monomer diffusion between vesicles, Biochemistry 20 (1981) 2783–2789.

[39] K. Hong, P.A. Baldwin, T.M. Allen, D. Papahadjopoulos, Fluorometric detection of the bilayer-to-hexagonal phase transition in liposomes, Biochemistry 27 (1988) 3947–3955.
[40] A. Chattopadhyay, E. London, Spectropic and ionization properties of *N*-(1-nitronenz-2-oxa-1,3-diazol-4-yl)-labeled lipids in model membranes, Biochim. Biophys. Acta 938 (1988) 24–34.
[41] S. Pierangeli, G. Goldsmith, E.N. Harris, A.E. Gharavi, D.W. Branch, W.I. Dean, Arthritics Rheum. 35 (1992) S69, Abstract.
[42] M. Galli, P. Comfurius, C. Maasen, H.C. Hemker, M.H. Debiets, P.J.C. Van Bred-Vriesman, T. Barbui, R.F.A. Zwaal, E.M. Bevers, Anticardiolipin antibodies directed not to cardiolipin but to a plasma protein cofactor, Lancet 335 (1990) 1544–1547.
[43] H.P. McNeil, R.J. Simpson, C.N. Chesterman, S. Krilis, Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: β2 glycoprotein 1, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 4120–4124.
[44] E. Matsuura, Y. Igarashi, M. Fujimoto, K. Ichikawa, T. Koike, Anticardiolipin cofactors and differential diagnosis of autoimmune disease, Lancet 336 (1990) 177–178.
[45] L.R. Sammaritano, M.D. Lockshin, A.E. Gharavi, Antiphospholipid antibodies differ in aPL cofactor requirement, Lupus 1 (1992) 83–90.
[46] S.A. Lauer, U. Hempel, A. Gries, K.H. Frank, Amino acid sequence of the region of beta 2-glycoprotein 1 (gp1) which mediates binding of autoantibodies to the cardiolipin–gp1 complex in humans, Immunology 80 (1993) 22–28.
[47] J. Hunt, S. Krilis, The fifth domain of β2-glycoprotein 1 contains a phospholipid binding site (cys281-cys288) and a region recognized by anticardiolipin antibodies, J. Immunol. 152 (1994) 653–659.
[48] R.A. Levy, A.E. Gharavi, L.R. Sammaritano, L.V. Habina, M.D. Lockshin, Fatty acid chain is a critical epitope for antiphospholipid antibody, J. Clin. Immunol. 10 (1990) 141–145.
[49] T. Qamar, A.E. Gharavi, R.A. Levy, M.D. Lockshin, Lysophosphatidylethanolamine is the antigen to which apparent antibody to phosphatidylethanolamine binds, J. Clin. Immunol. 10 (1990) 260–263.