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Opposite β_2-glycoprotein I requirement for the binding of infectious and autoimmune antiphospholipid antibodies to cardiolipin liposomes is associated with antibody avidity

Claudia Maria Celli [a,*], Azzudin E. Gharavi [b], Hernan Chaimovich [a]

[a] *Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil*
[b] *Louisiana State University Medical Center, Department of Medicine, Section of Rheumatology, New Orleans, LA 70112-2822, USA*

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Abstract

The aim of this study was to investigate the interaction of antiphospholipid antibodies (aPL) from two different populations (patients with autoimmune or infectious disorders) with cardiolipin (CL) arranged in a defined bilayer. β_2-Glycoprotein I (β_2GPI), an apolipoprotein that plays a critical role in the aPL binding to phospholipids, was quantified by dot blot in purified IgG-aPL samples, further classified according to apparent avidity to CL. In solid-phase assays, β_2GPI increased, preferentially, the binding of low-avidity autoimmune aPL to CL but inhibited the binding of low-avidity syphilitic aPL. In the absence of β_2GPI, both autoimmune and infectious aPL induced the leakage of the entrapped fluorescent probe, carboxyfluorescein (CF), from small unilamellar vesicles containing CL. aPL-induced probe leakage was protein concentration-dependent and characterized by a lag-phase onset of 100–120 min. β_2GPI increased the leakage rate induced by low-avidity autoimmune aPL only and inhibited the leakage induced by all syphilitic aPL. The following conclusions were provided: (1) in the absence of β_2GPI, autoimmune and infectious aPL bind to CL in a bilayer, inducing liposome leakage; (2) the leakage mechanism induced by aPL is suggested to be intravesicular; (3) β_2GPI requirement for phospholipid binding in both solid and fluid phase is associated to aPL avidity; (4) CL alone or the CL–β_2GPI complex are the most likely epitopes for autoimmune aPL; (5) aPL from syphilis patients can only form the CL–aPL complex, supporting that β_2GPI is not (part of) the target epitope.

Keywords: Antiphospholipid antibody; β_2-Glycoprotein I; Autoimmune disease; Syphilis; Cardiolipin; Liposome leakage

1. Introduction

Antiphospholipid antibodies (aPL) occur with high frequency in autoimmune disorders such as systemic lupus erythematosus (SLE) [1] and antiphospholipid syndrome (APS) [2], in infectious diseases (syphilis) [3], and following some drug treatments [4,5]. Severe clinical complications such as venous and arterial thrombosis [6], thrombocytopenia [7] and intrauterine fetal death [8,9] are currently associated with autoimmune aPL, but not with aPL detected in syphilis patients. However, a high prevalence of aPL in patients with hepatitis C virus (HCV), in asymptomatic infection, has also been associated with throm-

* Corresponding author. Present address: Department of Cell Biology, The University of Texas – MD Anderson Cancer Center, 1515 Holcombe Boulevard, Box 173, Houston, TX 77030-4095, USA. Fax: +1-713-792-8747;
E-mail: clmcelli@hotmail.com

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bocytopenia, a history of thrombotic episodes and portal hypertension [10,11]. The pathogenesis and clinical significance of these autoantibodies remain unclear regardless that aPL are considered a family of related antibodies but with distinct properties [12].

A solid-phase immunoassay (ELISA), using cardiolipin (CL) as antigen, has been widely adopted for detection of aPL in patient sera [13]. The use of bovine serum as a sample diluent enhances the binding of aPL to target phospholipid [14,15]. This observation was rationalized by independent reports showing that a plasma protein, β_2-glycoprotein I (β_2GPI), plays a critical role in the binding of aPL to CL. A requirement of β_2GPI for autoimmune aPL binding to CL was observed, suggesting that aPL may bind to antigens shared by CL and β_2GPI [16]. It was also suggested that β_2GPI, rather than CL, was the target antigen for aPL from autoimmune disorders [17–19]. β_2GPI requisite for aPL binding was variable depending on the disease [20,21]. However, negatively charged phospholipid alone is still proposed as a potent target epitope for aPL [22–24]. Although active animal immunization studies have shown that CL alone does not induce aPL production, liposomes containing various lipids (cholesterol, dicetylphosphate, dipalmitoylphosphatidylcholine and lipid A) induced aPL production [25,26]. Immunization with human delipidated (or not) β_2GPI induces both aPL and anti-β_2GPI antibodies [27–29], generating an animal model for antiphospholipid syndrome [30]. In contrast to autoimmune aPL, aPL from syphilis bind to CL in the VDRL (Veneral Disease Research Laboratories) antigen containing CL, phosphatidylcholine (PC) and cholesterol, and exhibit little cross-reactivity with other negatively charged phospholipids [31,32]. The binding of infectious aPL to CL-coated ELISA plates is inhibited by β_2GPI [33,34]. These observations summarize some of the present controversies about the nature of the antigen responsible for aPL reactivity.

Although the precise physiological role remains obscure, β_2GPI is known to: (a) be associated with lipoprotein structures, especially chylomicrons [35], (b) bind to platelets and heparin [36], and (c) bind to negatively charged phospholipids [37]. β_2GPI has been reported to inhibit both platelet prothrombinase activity [38] and ADP-induced platelet aggregation [39]. Some of the known properties of β_2GPI and aPL relate both proteins to the pathogenesis of thrombosis in autoimmune disorders. Thrombi induced in the femoral veins of CD-1 mice were significantly larger and persisted longer in mice with aPL, independently of the production (or not) of anti-β_2GPI antibodies [23]. Because β_2GPI inhibits, in vitro, the contact activation system in blood coagulation, blocking of β_2GPI hemostatic response by aPL might predispose to thrombosis [40]. Affinity-purified IgG anticardiolipin antibodies from APS patients also inhibit the prothrombin–thrombin conversion reaction [41]. Protein C activation is inhibited by aPL predisposing to thrombosis [42]. It has also been shown that the binding of β_2GPI to PS on apoptotic cells is essential in mediating the immunophysiologic clearance of senescent non-self particles [43]. A dysfunction in the apoptotic process could create neoepitopes that then would stimulate the production of natural aPL. Therefore, the binding of β_2GPI and aPL to phospholipids interferes with coagulation and apoptotic pathways, though the mechanism(s) that induce the clinical features remain to be ascertained.

Despite progressing comprehension about the interaction of the complex CL–β_2GPI–aPL, the molecular details of antigen–antibody association in fluid phase remains obscure. Under various pathological circumstances, phospholipids in bilayers are probably present in microparticles or cell fragments circulating in vivo. Under this approach, phospholipid liposomes have been used as models for examining the interactions of lipids with various limbs of the immune system [44]. Small unilamellar vesicles are ultimate model systems to study lipid–protein interaction in fluid phase. First, lipid composition of SUV are effortlessly manipulated and defined. Thus, factors like surface charge, lipid head group specificity and membrane fluidity can be considered. Second, the sonication procedure generates stable liposomes that are essentially unilamellar, allowing the determination of several effects induced by protein–vesicle interaction. In order to study the interaction of aPL with negatively charged phospholipids in an appropriate and emblematic bilayer in fluid phase, we selected CL-containing liposomes as a model for the phospholipid antigen presentation. Both aPL from autoimmune diseases (such as SLE and APS) and

from infectious diseases (such as syphilis) induced the leakage of entrapped carboxyfluorescein from liposomes [45]. However, a great number of studies used either the sera or polyclonal aPL which possibly contain β_2GPI contamination. Here we investigated the interaction of purified aPL, assuredly β_2GPI-free, to CL-containing vesicles and associated the β_2GPI requirement for the aPL binding to CL with aPL apparent avidity. In addition, we examined the effects of added purified human β_2GPI on the aPL–vesicle interaction. We demonstrated that both aPL from autoimmune and infectious diseases induce the leakage of internal contents of CL-containing liposomes in the absence of β_2GPI. For aPL from autoimmune diseases, antibody avidity to CL is directly associated with β_2GPI requirement for efficient interfacial recognition or binding to CL in fluid phase. In contrast, β_2GPI inhibit the leakage rate induced by infectious aPL, independently of aPL avidity. The leakage mechanism induced by aPL is discussed.

2. Materials and methods

2.1. Chemicals

Cardiolipin from bovine heart, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidic acid (PA), bovine serum albumin (BSA), adult bovine sera (ABS), Tris buffer, phosphate-buffered saline (PBS), Triton X-100, alkaline phosphatase-conjugated goat anti-human immunoglobulin, and *p*-nitrophenylphosphate were all purchased from Sigma Chemical Co. (St. Louis, MO). Egg PC was purified as described [46]. 6-Carboxyfluorescein (CF) from Eastman Kodak Co. (New York, NY) was purified chromatographically [47]. All other chemicals were of analytical grade or better from regular commercial sources. Flat-bottom 96-well microtitration plates were obtained from ICN Biomedicals (Aurora, OH).

2.2. Patient sera

Ten sera from APS and SLE patients (following ACR criteria) were selected for study for being positive in anticardiolipin ELISA tests [13]. Sera from five patients with syphilis diagnosed on the basis of reactivity by VDRL and *Treponema* antibody-absorption (FTA-ABS) tests were evaluated. Sera from 10 healthy donors were used as negative controls.

2.3. Purification and characterization of IgG fractions of aPL

Antiphospholipid antibodies of the IgG isotype were isolated from human serum by protein G-Sepharose 4B affinity chromatography (Pharmacia) [48]. IgG fractions were eluted with 2% acetic acid, immediately neutralized with 1% (v/v) 2.5 M Tris (pH 8) and dialyzed against 0.1 M NaCl/10 mM Tris (pH 7.2). As a second purification step, IgG fractions were applied to Sepharose–heparin CL-6B fast-flow (Pharmacia) which retains contaminant β_2GPI. IgG fractions were recuperated in the column flow through and dialyzed against PBS, fractionated and frozen. All procedures were developed at 4°C. IgG isotype and purity was established by SDS–polyacrylamide gel electrophoresis. Protein concentration was controlled by absorbance measurements at 280 nm.

2.4. β_2GPI purification

β_2GPI was purified by a modification of the technique described in [49]. Briefly, human healthy serum was treated with perchloric acid (2.9% v/v) at 4°C. The mixture was stirred for 30 min and centrifuged at 10 000 rpm for 30 min at 4°C. The supernatant was neutralized to pH 6–7 with saturated sodium carbonate and dialyzed against 0.03 M NaCl/20 mM Tris (pH 7.1–7.2). β_2GPI was obtained by Sepharose–heparin CL-6B fast-flow (Pharmacia) affinity chromatography at 4°C, using 0.35 M NaCl/20 mM Tris (pH 8.5) to elute the protein. Purified β_2GPI was dialyzed against PBS (pH 7.4) and protein concentration was measured according to Morton [50].

2.5. Dot-blot immunoassay

Two μl of 0.5 mg/ml purified IgG-aPL were spotted onto 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) and dried at room tem-

perature (24 ± 1°C). Membranes were shaken in the blocking buffer (5% non-fat dry milk, 0.1% Tween-20, PBS, pH 7.4) for 45 min, washed with PBS for 10 min and then incubated with a 1:5 dilution in PBS of the yeast culture supernatant containing monoclonal anti-β_2GPI (A.E. Gharavi, in preparation) for 90 min under continuous agitation. Membranes were washed five times with PBS for 5 min, and incubated for 90 min with alkaline-phosphatase conjugated goat anti-mouse IgG (whole molecule) (Sigma) diluted 1:3000 in PBS. Membranes were washed five times with PBS for 5 min. Color development was visualized after incubation of membranes with the substrate solution (NTB and BCIP from Promega, Madison, WI). After positive spot development for the β_2GPI standard curve, membranes were dipped into distilled water to stop the reaction. The whole procedure was performed at room temperature (24 ± 1°C).

2.6. Enzyme-linked immunosorbent assays (ELISA) for aPL

The method for detecting aPL in ELISA was performed as described [48]. Briefly, ELISA plates were coated overnight at 4°C with 30 μl/well of 0.05 mg/ml CL in ethanol (or in methanol/chloroform 3:1 v/v for other phospholipids). The plates were blocked with 2% BSA/PBS for 60 min at 4°C. Appropriated amount of IgG was diluted in 10% ABS/PBS for the experiments in the presence of β_2GPI or in 2% BSA/PBS for those in the absence of β_2GPI, and incubated for 150 min at 4°C. Goat anti-human IgG (γ chain) alkaline phosphatase-conjugated antibody was applied for 1 h at 4°C and plates were developed with *p*-nitrophenylphosphate. Optical density (OD) was read at 405 nm in a Titertek ELISA reader.

2.7. Avidity

Avidity of IgG-aPL to CL on solid phase was estimated by ELISA by inhibition of aPL binding to CL by ionic strength increase [51,52]. The ELISA procedure was similar to that described above, modified by increasing NaCl concentrations added to the blocking step (2% BSA/PBS) and to the sample dilution (10% ABS/PBS), and in the washing buffer (PBS) within these steps.

2.8. Preparation and purification of the vesicles

Vesicle preparation method was slightly modified from a previously published technique [45,47]. Briefly, CL and PC dissolved in ethanol were evaporated under nitrogen and dried for 60 min under vacuum. The lipid film was resuspended in 10 mM Tris (pH 7.4) containing 0.05 M CF. The dispersion was sonicated using a Sonicator Virsonic 300 (Virtis Co., Gardiner, NY) equipped with titanium tip (iced bath, 1 min at 40% full power, 1 min off for eight or more cycles until transparency). The sonicated preparation was then centrifuged for 60 min at 15 000 rpm. Non-entrapped CF was removed from CF-containing vesicles by Sephadex G-25 fine filtration (Pharmacia) [45,47]. The final applied lipid concentration was 4.0 ± 0.5 μM, measured by phosphorus analysis [53].

2.9. Leakage from the intravesicle water compartment

CF, encapsulated within the vesicles at the highly self-quenching concentration, allows continuous monitoring of marker release from the vesicles [47]. The release of water-soluble vesicle contents after mixing aPL and/or β_2GPI with vesicles (always latest added) was evaluated by the increased CF fluorescence released into the milieu (0.47 M sucrose/10 mM Tris, pH 7.4). Fluorescence intensity was monitored with time (excitation at 490 nm, emission at 520 nm) in a 650-15 spectrofluorimeter (Perkin Elmer). To calculate the percent of CF leakage, the fluorescence of residual CF was taken as F_0 and the total fluorescence intensity, F_t was obtained from total vesicle lysis with Triton X-100 (0.5% v/v):

$$\%\mathrm{CF} = \{(F-F_0)/(F_t-F_0)\}\times 100 \quad (1)$$

where F is the fluorescence intensity at any time [45]. Vesicles were stable and spontaneous leakage of CF did not exceed 10% over the course of the experiments.

2.10. Static light scattering (SLS)

The radius of gyration (R_g) was obtained by static light scattering using a Dawn-F multiangle scattering photometer (Wyatt Instruments, Santa Barbara, CA)

[54]. The average R_g of the vesicles used here was 40 ± 20 nm.

3. Results

3.1. Purification of IgG isotype APL from patient sera

From patient sera with autoimmune or infectious diseases, we purified the aPL by standard procedures to obtain IgG isotype antibodies [48]. It was essential to determine β_2GPI contents in the purified IgG-aPL samples. To measure the β_2GPI contamination in the aPL samples we used a dot-blot assay adapted from the Western-blot technique which was sensitive to 1 ng/ml of β_2GPI. A monoclonal anti-β_2GPI antibody was applied for protein quantification (for details see Section 2). IgG-aPL samples purified only by protein G-Sepharose column contained ca. 100 ng β_2GPI per μg of IgG-aPL (Fig. 1, lane 2), emphasizing the need of a second purification step.

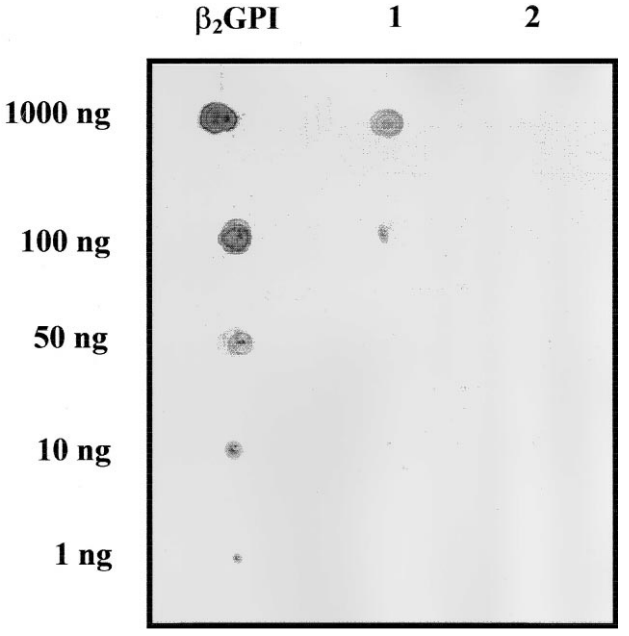

Fig. 1. β_2GPI detection by dot blot. Protein concentrations established on the left side represent human purified β_2GPI concentration applied as standard (column named β_2GPI) or the applied IgG-aPL concentration (columns 1 and 2). β_2GPI contamination was analyzed in aPL purified by Sepharose–protein G chromatography (lane 1) or by Sepharose–protein G followed by heparin–Sepharose chromatography (lane 2).

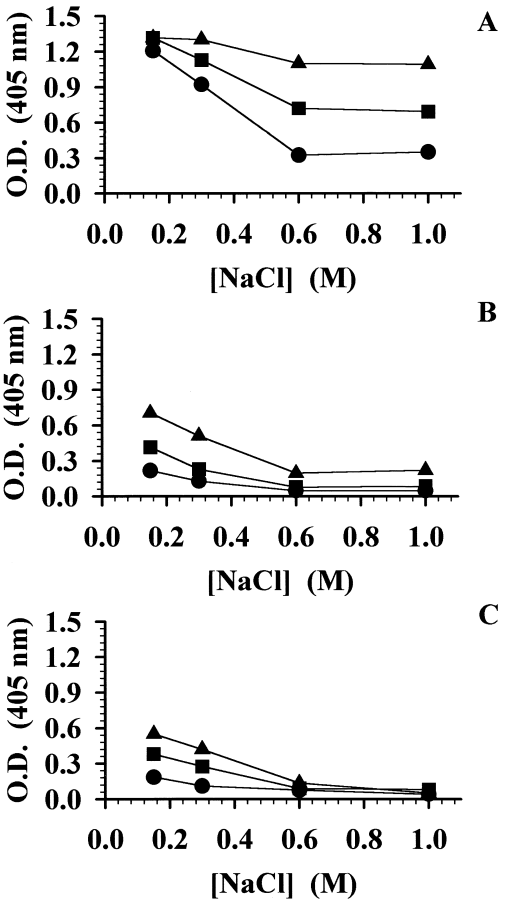

Fig. 2. Apparent antiphospholipid antibody avidity estimated by ELISA. Serial dilutions of purified IgG-aPL were applied to ELISA plates in the presence of increased salt concentrations (NaCl) in the diluent buffer (10% ABS/PBS) as described in Section 2. Representative patient with autoimmune disease showing high (A) or low (B) avidity to CL and a representative patient with syphilis shows low avidity to CL (C). ●, 15 μg/ml IgG-aPL; ■, 30 μg/ml IgG-aPL; ▲, 60 μg/ml IgG-aPL.

In order to eliminate β_2GPI contamination, the pretreated IgG-aPL samples were applied to a Sepharose–heparin column. By dot blot, we demonstrated that IgG-aPL purified here contained less than 1 ng of β_2GPI per μg of IgG-aPL (Fig. 1, lane 3). The same BSA used for the ELISA blocking steps was applied as negative control in the dot blot, revealing no β_2GPI contamination (data not shown). According to previous studies, the minimum β_2GPI concentration required to act as a cofactor for the autoimmune aPL binding to CL is 0.5–1.0 μg/ml β_2GPI per 70–100 μg/ml IgG-aPL, which was certainly not reached here due to β_2GPI contamination [48].

3.2. Apparent antibody avidity

The summation of attractive and repulsive forces involved in the interaction between an antigenic determinant and the homologous antibody combining site represents the antibody affinity. Antibody avidity, on the other hand, depends in part on affinity, but also involves other factors associated to binding such as antibody valency and electrostatic forces [51,52]. Because electrostatic forces govern the aPL binding to negatively charged phospholipid, we evaluated the apparent avidity of IgG-aPL by ELISA using increasing ionic strength as inhibitor agent for the IgG-aPL binding to CL. Fig. 2 shows the profile of significant IgG-aPL from an autoimmune patient of considered high- (Fig. 2A) and low-avidity (Fig. 2B) antibody and one representative profile of IgG-aPL from syphilitic patients (Fig. 2C). Three of 10 purified autoimmune aPL-IgG maintained similar OD values in the presence of 0.3 M salt and the IgG-aPL binding capacity to CL was inhibited only 15–20% by the addition of 1 M salt (Fig. 2A: 60 μg/ml IgG). The observed high association of the antigen–antibody complex distinguished these antibodies to be classified as high-avidity aPL in this study. For the other seven autoimmune IgG-aPL samples, 0.3 M salt, only double the salt concentration of the physiological milieu, inhibited 40% to 60% of aPL-binding capacity to CL (Fig. 2B). This percentage of inhibition, associated with no detectable aPL-binding at 0.6 M NaCl, defined the characteristic profile of low avidity aPL. All five aPL samples from patients with syphilis showed a low-avidity profile (Fig. 2C). Table 1 shows the general classification of the aPL samples according to CL avidity in the presence of β_2GPI.

The ELISA binding curves consist of a phase plateau at low antibody dilutions followed by a steep fall at high dilution that differentiates high- and low-affinity antibodies [52]. In order to confirm the previous avidity classification, we measured the CL-binding of all purified aPL within 10 to 100 μg/ml

Table 1
Apparent avidity associated to β_2GPI requirement for aPL binding to CL in solid phase[a]

Sample	Without β_2GPI[a]	With β_2GPI[a]	Slope[d]	Intercept[d]	Apparent avidity[e]
Blank[b]	0.08	0.090	–	–	–
Negative[c]	0.21	0.10	–	–	–
1. MH	1.24	1.31	0.0047	1.144	High
2. GB	1.18	1.30	0.0042	1.078	High
3. AR	0.95	1.09	0.0039	1.005	High
4. GP	0.45	0.75	0.0087	0.349	Low
5. WY	0.31	0.65	0.0114	0.170	Low
6. PT	0.52	0.72	0.0161	0.236	Low
7. AD	0.28	0.63	0.0102	0.185	Low
8. DZ	0.50	1.25	0.0071	0.486	Low
9. EA	0.26	0.46	0.0062	0.066	Low
10. NK	0.23	0.60	0.0101	0.232	Low
11. B5	0.50	0.35	0.0057	0.0124	Low
12. B16	0.59	0.25	0.0058	−0.0410	Low
13. B19	1.31	0.20	0.0018	−0.0021	Low
14. B20	0.24	0.06	0.0038	−0.0367	Low
15. B22	0.75	0.35	0.0065	0.0193	Low

[a]aPL purified from 10 patients with autoimmune diseases (samples 1–10) and from five patients with syphilis (samples 11–15) were applied to CL-coated ELISA plates and the OD values in the absence and presence of β_2GPI are shown.
[b]'Blank' refers to OD values obtained in the absence of sera (substituted by buffer) that were already subtracted from the OD values presented for each sample above.
[c]'Negative' refers to OD values obtained by the average of 10 healthy donor-purified IgG antibodies.
[d]A serial dilution of purified IgG-aPL from each patient was tested for binding to CL in solid phase in the presence of β_2GPI. The slope and intercept values from the plot of purified IgG-aPL concentration versus the respective OD values for each patient are presented.
[e]Samples were classified as high- or low-avidity aPL samples according to the apparent avidity to CL in the ELISA assays.

of IgG concentration range by standard ELISA (0.15 M salt). OD values were proportional to the IgG-aPL dilution only in the case of low-avidity antibodies (compare different symbols at 0.15 M NaCl in Fig. 2). Every plot of IgG concentration by OD was linear, and the calculated slope and intercept for each curve is presented in Table 1. The intercept values are coincident with the OD value at the long phase plateau before the steep fall dilution point, which increases with low-avidity syphilitic aPL < autoimmune low-avidity aPL < autoimmune high-avidity aPL, respectively (Table 1). No notable changes are observed in the slope values among all aPL.

3.3. β_2GPI requirement for IgG-aPL binding to CL in ELISA

Although it is commonly accepted that β_2GPI affects aPL binding in CL-ELISA tests, there is contradictory data about aPL binding to CL in the absence of β_2GPI [12,16,22]. As indicated in Fig. 3, β_2GPI-free IgG-aPL bound to CL in the absence and presence of β_2GPI. Three autoimmune high-avidity aPL presented high OD values, demonstrating stronger binding to CL in the absence of β_2GPI (Fig. 3A). All seven autoimmune low-avidity aPL bound to CL in the absence of β_2GPI (Fig. 3B) but much higher IgG-aPL concentrations (> 80 μg/ml) were required to clearly distinguish a positive aPL binding from negative binding due to non-specific normal IgG antibodies. Taken together, these data confirm the binding of IgG-aPL to CL in the absence of β_2GPI, disassociated from aPL avidity. Table 1 shows the OD values obtained in the absence and presence of β_2GPI for aPL bound to CL. Our data showed a larger β_2GPI requirement for low-avidity aPL than for high-avidity aPL, which is in entire agreement with previous reports [48]. In addition, β_2GPI inhibited the CL-binding of aPL from syphilis patients (Table 1).

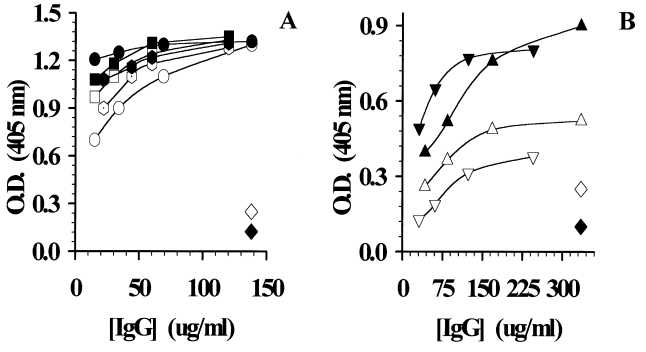

Fig. 3. Autoimmune aPL binding to cardiolipin in solid-phase assay (ELISA). Plot of the OD values obtained due to increasing concentrations of purified IgG-aPL in the absence (open symbols) or presence (full symbols) of β_2GPI. Three high-avidity aPL (MH, GB and AR) profiles (A) are compared with two representative low-avidity aPL (GP and WY) profiles (B). ●, IgG GB; ■, IgG MH; ⊙, IgG AR; ▲, IgG GP; ▼, IgG WY; ♦, IgG from healthy donors.

3.4. aPL binding to negatively charged phospholipids by ELISA

In order to investigate a possible association of aPL avidity with binding to several negatively charged phospholipids, all samples were tested for binding to PS, PA, PI, PE, and PC by ELISA in the presence of β_2GPI. In the absence of international standards for non-cardiolipin antibodies, we used as a cut-off value that of the mean OD from 10 negative controls (0.14 ± 0.06) plus 3 standard deviations. Thus, aPL binding to phospholipids was considered positive for those samples originating OD > 0.32. All 10 autoimmune IgG-aPL samples bound to CL and PA in the standard ELISA. Among the autoimmune IgG-aPL samples, 70% ($n = 7$) were positive for binding to PS, 10% ($n = 1$) for PG and 70% ($n = 7$) for PI. No autoimmune aPL presented significant binding to PE or PC. Among the syphilitic samples, 20% ($n = 1$) were positive for binding to PS, 60% ($n = 3$) for PA, 40% ($n = 2$) for PG, 60% ($n = 3$) for PI. Although no significant binding to PE was detected, 80% ($n = 4$) of the aPL from patients with syphilis recognized PC, a zwitterionic phospholipid (data not shown). These data confirm that the binding capacity of aPL to negatively charged phospholipids is higher for aPL from autoimmune than infectious disorders in solid-phase assays [3,14,32,34]. No pattern relating aPL avidity with lipid binding specificity was evident from these assays.

3.5. Membrane–protein interaction assay based on fluorescent probe leakage

To examine whether aPL from autoimmune dis-

eases can interact with CL in a well-defined bilayer in solution, we used CL-containing vesicles with entrapped carboxyfluorescein (CF). Affinity-purified IgG-aPL were incubated with CF-containing CL/PC vesicles in a salt-free buffer with 0.47 M sucrose to keep the osmotic pressure balance. Incubation of vesicles and aPL at 22 ± 1°C produced an extremely slow leakage with no significant difference between the leakage induced by IgG-aPL and by IgG from healthy donors (data not shown). Upon incubation at 29 ± 1°C, IgG-aPL induced remarkable CF leakage from vesicles containing either 20 mol% or 50 mol% CL. Fig. 4 shows the CF release kinetics for low- and high-avidity aPL provided from patient sera with SLE. A similar kinetic profile was induced by aPL from patient sera with syphilis (Fig. 6). A peculiar incubation lag phase extending up to 150 min was detected for all IgG-aPL. The lag phase was not discontinued by increasing autoimmune or infectious aPL concentrations (Fig. 4) or increasing CL contents in the membrane (data not shown). Incubation of aPL with vesicles containing 100% PC did not induce changes in the fluorescence intensity, confirming the specific aPL binding to CL (data not shown). These results demonstrate that β_2GPI-free IgG-aPL from autoimmune and infectious diseases interact with CL bilayers in solution, inducing the leakage of CL vesicles. This phenomenon is independent of the aPL avidity to CL.

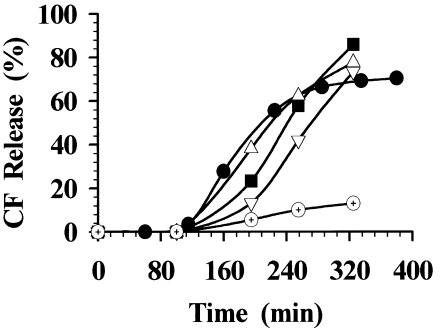

Fig. 4. Kinetics of carboxyfluorescein (CF) release from CL/PC (1:1 molar) vesicles induced by purified IgG-aPL. Autoimmune β_2GPI-free aPL were incubated with the vesicles in the absence of β_2GPI at 29 ± 1°C. Apparent high-avidity (full symbols) and low-avidity (open symbols) autoimmune aPL revealed similar kinetics profiles that completely diverge from the profile induced by IgG purified from healthy donors (⊕). Symbols represent purified aPL from different patients.

It is acclaimed that β_2GPI binds to negatively charged phospholipids in multilamellar vesicles [37]. In order to investigate whether β_2GPI alone induces leakage from unilamellar vesicles, human purified β_2GPI was incubated with CF-containing CL/PC vesicles. At 22 ± 1°C, no leakage was observed before 4 h incubation. At 29 ± 1°C, 1 to 10 μg/ml β_2GPI induced time-dependent CF release from vesicles containing CL/PC (1:4 mol%). The leakage rate was dependent on β_2GPI concentration (data not shown). Similar results were obtained with higher CL molar ratios (1:1 mol% CL/PC), although lower β_2GPI concentrations were required for a similar

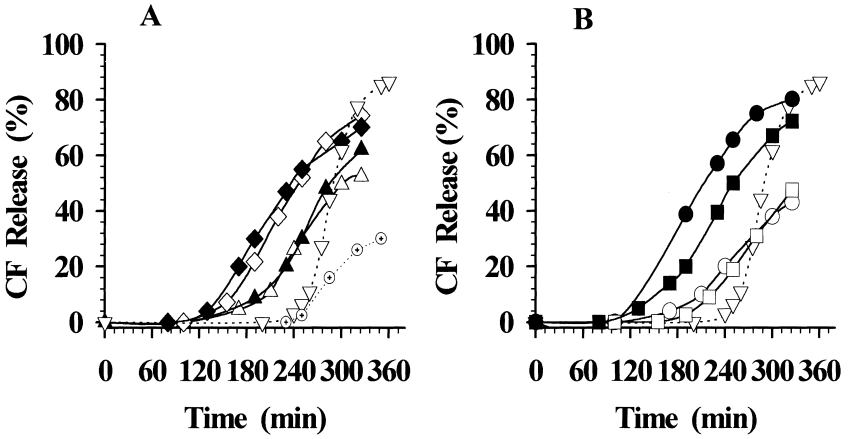

Fig. 5. β_2GPI effect in the kinetics of carboxyfluorescein (CF) release from CL/PC (1:1 molar) vesicles induced by autoimmune aPL. Vesicles were incubated with 10 μg/ml IgG-aPL in the absence (open symbols) or presence (full symbols) of 10 ng/ml of β_2GPI at 29 ± 1°C. Although the kinetics profile induced by apparent high-avidity autoimmune aPL was invariable (▲, IgG MH; ◆, IgG GB) (A), the presence of β_2GPI increased the kinetic rate induced by apparent low-avidity autoimmune aPL (●, IgG GP; ■, IgG WY) (B). ▽, 10 ng/ml β_2GPI; ⊕, 10 μg/ml IgG from healthy donors in the presence of β_2GPI.

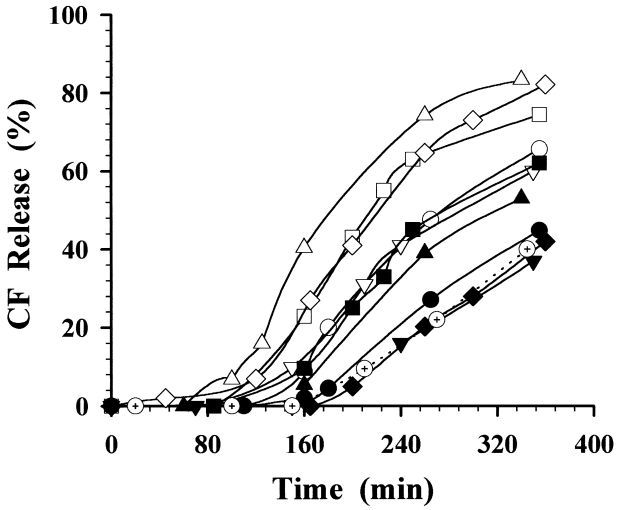

Fig. 6. β_2GPI effect on the kinetics of carboxyfluorescein (CF) release from CL/PC (1:1 molar) vesicles induced by aPL from syphilis. Vesicles were incubated with 10 μg/ml IgG-aPL in the absence (open symbols) or presence (full symbols) of 10 ng/ml of β_2GPI at 29 ± 1°C. β_2GPI inhibited the leakage rate induced by aPL from all syphilis patients (different symbols), reaching the basal rate induced by healthy donors in the presence of β_2GPI (⊕).

leakage profile. Fig. 5 shows a representative kinetic of CF release from CL/PC 1:1 vesicles induced by 10 ng/ml β_2GPI. No CF leakage was noticed using 100% PC vesicles. These results indicated that β_2GPI itself binds to CL vesicles in fluid phase, inducing the leakage of the vesicle contents.

3.6. β_2GPI requirement on aPL binding to CL-containing vesicles

Because both purified aPL and β_2GPI induce the leakage of vesicle contents, we investigated whether β_2GPI affected aPL-induced vesicle leakage (Fig. 5). Resulting vesicle leakage induced by aPL due to β_2GPI addition was dependent on the origin of aPL. In the case of autoimmune aPL, the leakage rate induced by low-avidity aPL increased upon addition of low concentrations of β_2GPI (Fig. 5A). The lag phase decreased from 240 min (considering 1 ng/ml β_2GPI only) or from 150 min (considering 10 μg/ml aPL only) to 100–110 min on vesicles exposed to both β_2GPI and aPL (Fig. 5B) considering CL/PC 1:1 vesicles at 29 ± 1°C. Similar effects were observed for all seven low-avidity aPL, using vesicles with 20% or 50% CL at 29 ± 1°C (data not shown). In contrast, vesicle leakage induced by high-avidity aPL was not affected by the presence of β_2GPI (Fig. 5A), not even for the high-avidity aPL (patient GB in Fig. 5A) that showed increase in binding to CL induced by β_2GPI addition in the ELISA assay (Fig. 3A). These data show that aPL avidity is related to β_2GPI requirement for CL-binding in solution. On the contrary, purified β_2GPI inhibited the CF release induced by all five purified aPL from patients with syphilis (Fig. 6), at 20% or 50% CL/PC vesicles. The kinetics profile for syphilitic aPL obtained in the presence of β_2GPI, for 60% of the samples (3/5), achieved the same rate as that induced by β_2GPI alone (Fig. 6).

3.7. Light-scattering measurements

Protein binding to phospholipid vesicles can result in a membrane defect, vesicle aggregation or vesicle fusion ([55,56] and references therein). The last two phenomena commonly result in increase of vesicle size or aggregate. We monitored vesicle size (radius of gyration) by SLS to verify whether the internal content leakage induced by aPL was due to vesicle fusion or aggregation. No changes in the hydrodynamic radius of CL/PC vesicles with bound aPL were detected through time, suggesting that the recorded leakage induced by aPL was due to the aPL binding to CL in a single vesicle.

4. Discussion

The role of β_2GPI in the binding of aPL to anionic phospholipids has certainly provoked intense research in the aPL field, regarding β_2GPI as either cofactor for the phospholipid binding or being (part of) the epitope itself. A vast majority of these studies used solid-phase assays, a technique that may not be providing the closest phospholipid arrangement to that existent in vivo. Here we report new aspects of the interaction of the target epitope and aPL from infectious and autoimmune disorders using a biomimetic model and strictly purified proteins. We demonstrated that aPL from autoimmune or infectious disorders interact with CL in solid or fluid phase in assured absence of β_2GPI. The evaluation of aPL apparent avidity demonstrated that β_2GPI requirement for binding to CL is: (1) associated to

aPL avidity to CL and (2) opposite to the aPL pathological origin (infectious or autoimmune).

The binding of β_2GPI-free aPL to CL was first evaluated when CL was presented in a solid-phase assay. We demonstrated that β_2GPI-free purified IgG-aPL from autoimmune and infectious disorders bind to CL in ELISA assays. The aPL–CL binding is dependent on aPL concentration and aPL avidity to CL. Approximately 10 μg/ml of IgG-aPL were enough to distinguish high-avidity aPL from normal IgG in a modified ELISA assay. However, higher concentrations of low-avidity aPL (>80 μg/ml) are required to distinguish positive aPL binding to CL from the non-specific binding of IgG from normal controls. These results may explain particular controversies when no detectable aPL binding to CL in the absence of β_2GPI was noticed by ELISA, likely because of the appliance of low IgG concentrations (4 μg/ml [16], 17 μg/ml [17], 8–23 μg/ml [57]).

We detected the binding of very low concentrations of aPL purified from three different autoimmune patients, suggesting the existence of high-avidity antibodies (Table 1) [52]. Indirect competition ELISA assay, a convenient method for estimating antibody affinity, was based on the electrostatic interactions among aPL–CL–β_2GPI to evaluate aPL avidity [58]. The characterization of the strength of antigen–antibody interaction distinguished aPL in two groups: high- or low-avidity aPL. Patients with syphilis presented exclusively low-avidity aPL. However, 30% of the patients with autoimmune disorders presented high-avidity aPL. Other infectious disorders, like malaria and AIDS, have also been associated to the presence of low-avidity aPL, when avidity was compared to autoimmune aPL features [59]. These data suggest a possible association between aPL pathological origin and its avidity, also likely to be associated with thrombosis in autoimmune aPL but not in infectious diseases. However, further studies are required to clearly correlate thrombosis origin in autoimmune disorders. Therefore, measurements of apparent aPL avidity may be of substantial value for subsequent aPL studies as well as on patient care.

In light of several reports showing the binding of antibody to β_2GPI in the absence of phospholipid, we tested the binding of all purified aPL to β_2GPI by ELISA [17–19,57]. Particularly two of three autoimmune high-avidity purified aPL bound to β_2GPI presented in solid phase in the absence of additional phospholipids (data not shown). This observation suggests that the so-called high-avidity aPL are anti-β_2GPI antibodies. Due to inhibition of β_2GPI binding to negatively charged phospholipids by increasing salt concentrations in the avidity assay, β_2GPI concentration should be decreasing at these experimental conditions. If high-avidity aPL bind specifically to β_2GPI, the detectable binding should also decrease with increasing salt concentrations, which was not verified. In addition, aPL from syphilis patients did not bind to β_2GPI in the absence of phospholipids and the aPL binding to CL was inhibited by increasing salt concentrations, in contrast to the hypothesis that the autoantibodies studied here could be anti-β_2GPI antibodies. From another point of view, these data suggest that aPL that recognized the complex CL–β_2GPI and β_2GPI itself present a higher affinity binding than those aPL that bind only to the complex.

We demonstrated that, in the absence of β_2GPI, purified IgG-aPL, from 10 different patients with autoimmune disorders and five with syphilis, interact to CL in a bilayer model, inducing the leakage of the internal contents from the vesicles. In agreement with aPL binding in the absence of β_2GPI, purified aPL from two patients with autoimmune diseases were shown to bind in glass microspheres coated with CL, PC and cholesterol [60]. Similar results were reported using mouse aPL induced by liposomes containing Lipid A [61].

We demonstrated, for the first time, that aPL induce the leakage of the internal contents of CL-containing vesicles. Interestingly, and consistent with preceding reports [45], the lag phase for the probe release onset was independent of IgG-aPL concentration, but strictly dependent on aPL avidity and β_2GPI concentration. Although high β_2GPI concentrations (50 μg/ml) decreased the lag phase, it was not completely eliminated (data not shown). Increasing CL vesicle contents did not affect the lag phase, indicating that CL density is not a factor that completely limits the leakage onset induced by aPL. In addition, the aPL binding to CL-containing vesicles did not enhance vesicle size. These findings strongly suggest that the leakage induced by aPL is not a consequence of vesicle aggregation or fusion. Based

on similar experiments, we suggest that the vesicle content leakage induced by β_2GPI alone is not due to fusion or aggregation of the vesicles.

Antibody binding to phospholipids in solid-phase assays is temperature dependent [62]. IgG-aPL binding to CL bilayer was also notably sensitive to temperature; in particular the lag-phase span for vesicle leakage decreased from 400–500 min at 22 ± 1°C to 120–150 min at 29 ± 1°C. Increasing temperature alters membrane fluidity from gel to fluid phase, providing more phospholipid mobility [63]. The straight dependence of CL mobility for aPL binding, either in solid or fluid phase, suggests that aPL detain more than one molecule of CL in the lipid-binding site. Calculations of the relative size of one IgG binding site and of one CL molecule suggested that eight CL polar heads were required for complete interaction [64]. These recordings indicate that local changes in a single vesicle membrane structure occur upon antibody binding, implying that an intravesicular mechanism is responsible for vesicle content leakage induced by aPL.

We analyzed the β_2GPI cofactor activity in the binding of aPL to CL in the fluid-phase model. The mechanism analysis of vesicle leakage provided evidence supporting a different epitope for autoimmune and infectious aPL. The probe leakage kinetic, expressed by an apparent rate constant (k_{app}), is dependent on the interaction of an antibody molecule and a single probe-containing vesicle. In the case of aPL from syphilitic patients, the binary aPL–vesicle complex generates a higher k_{app} than the k_{app} representing the β_2GPI–vesicle complex, since a higher leakage rate is induced by syphilitic aPL than that induced by β_2GPI alone. However, from the analysis of the vesicle leakage in the presence of both β_2GPI and syphilitic aPL, it can be conclude that k_{app} induced by β_2GPI is higher than k_{app} induced by aPL due to the preponderance of the former over the effect induced by aPL alone. There is no formation of the ternary vesicle–β_2GPI–aPL complex in this case, but competition between β_2GPI and aPL for the negatively charged surface, clearly demonstrated that β_2GPI is not part of the epitope recognized by aPL from infectious diseases like syphilis. This phenomenon is not dependent on aPL avidity and is in good agreement with previous proposed epitopes for syphilitic aPL [12,31,32]. In contrast, k_{app} induced by high-avidity autoimmune aPL was apparently higher than (or similar to) that induced by β_2GPI alone, since the addition of β_2GPI did not substantially alter the k_{app} induced by these aPL. In this case, there was a preponderance of k_{app} induced by high-avidity autoimmune aPL. For low-avidity autoimmune aPL, the β_2GPI–aPL complex induced a higher k_{app} than aPL or β_2GPI by itself, suggesting the formation of a ternary vesicle–β_2GPI–aPL complex. Although different k_{app} were induced by high- and low-avidity autoimmune aPL, these findings confirmed that β_2GPI is part of the epitope for (low-avidity) autoimmune aPL.

Binding of aPL to its targets in vivo may be characterized by a greater degree of complexity than in vitro. In vivo targets of aPL identified to date include monocytes [65], cultured HUVEC [66], surface of activated platelets [67], and apoptotic cells [68]. The binding of β_2GPI to the newly exposed anionic phospholipid on apoptotic cells is suggested to generate a ligand by which apoptotic cells may be recognized by macrophages for phagocytic clearance [68]. Thus, the β_2GPI–aPL complex formed on apoptotic cell surface may contribute to the development of autoimmunity by interfering with the normal cell clearance and by the prominent autoantigens formed. This hypothesis is supported by our results that distinctly showed a stable complex aPL-lipid-β_2GPI resistant to high ionic strength. In addition, the lag phase disclosed that the binding of aPL and/or β_2GPI to the vesicle outer surface is a prolonged process, which is in accord with explanations of the origin of the aPL antigen. The fact that infectious aPL do not recognize β_2GPI on a negatively charged surface may also associate the thrombi formation in autoimmune disorders with the β_2GPI–aPL complex formation [23].

In conclusion, we demonstrated that, in the absence of β_2GPI, human purified IgG-aPL bind to CL in solid-phase assay and to CL in a bilayer structure in fluid phase. Our data confirm the existence of two different populations of autoimmune aPL. In autoimmune diseases, β_2GPI requirement for the phospholipid binding is dependent on the apparent avidity of the autoimmune aPL. Cardiolipin alone or the complex CL–β_2GPI are the most likely epitopes for autoimmune aPL, CL alone, and certainly not β_2GPI, is the only epitope identified for infectious

aPL. Further investigations are required to determine the exact epitope for the different aPL populations and the association with the clinical features of the diseases.

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