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Anticardiolipin Antibodies Recognize β_2-Glycoprotein I Structure Altered by Interacting with an Oxygen Modified Solid Phase Surface

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Summary

Anticardiolipin antibodies (aCL) derived from the sera of individuals exhibiting the antiphospholipid syndrome (APS) directly bind to β_2-glycoprotein I (β_2-GPI), which is adsorbed to an oxidized polystyrene surface. Oxygen atoms were introduced on a polystyrene surface by irradiation with electron or γ-ray radiation. X-ray photoelectron spectroscopy revealed the irradiated surfaces were oxidized to generate C–O and C=O moieties. aCL derived from either APS patients or (NZW × BXSB)F_1 mice bound to β_2-GPI coated on the irradiated plates, depending on the radiation dose. Antibody binding to β_2-GPI on the irradiated plates was competitively inhibited by simultaneous addition of cardiolipin (CL)-coated latex beads mixed together with β_2-GPI but were unaffected by addition of excess β_2-GPI, CL micelles, or CL-coated latex beads alone. There was a high correlation between binding values of aCL in sera from 40 APS patients obtained by the anti-β_2-GPI enzyme-linked immunosorbent assay (ELISA) using the irradiated plates and those by the β_2-GPI-dependent aCL ELISA. Therefore, aCL have specificity for an epitope on β_2-GPI. This epitope is expressed by a conformational change occurring when β_2-GPI interacts with an oxygen-substituted solid phase surface.

Antiphospholipid antibodies (APA)[1] are a family of closely related immunoglobulins that react with anionic phospholipids. Recent studies would suggest these antibodies recognize complexes of anionic phospholipids and a variety of plasma proteins, including β_2-glycoprotein I (β_2-GPI), prothrombin, and proteins C and S (1–7). Anticardiolipin antibodies (aCL) and lupus anticoagulants are members of the APA family. Both aCL and lupus anticoagulants are found in sera of patients with SLE or related connective tissue diseases (8–14). The term, antiphospholipid syndrome (APS), has been proposed to explain the association of APA and a variety of clinical features, including arterial and venous thromboembolic events, recurrent spontaneous abortion or intrauterine fetal death, thrombocytopenia, and various neurologic defects (10, 11).

aCL are usually detected by either RIA or ELISA, using cardiolipin (CL) as a solid phase antigen (8, 9). In recent years, several groups (1–5) have shown that a 50-kD serum cofactor is required for aCL found in SLE patients to bind to plastic plates coated with CL. The cofactor has been identified as β_2-GPI by detecting an NH_2-terminal amino acid sequence of the purified protein (1, 5) and by cDNA cloning and sequencing (15). Further, the cofactor activity of β_2-GPI that provides the aCL binding in aCL ELISA was shown to be retained by recombinant β_2-GPI produced in an expression system utilizing baculovirus/*Spodoptera frugiperda* cells (16). We and others have also shown that such types of aCL are distinguished from aCL found in patients with infectious disorders, such as syphilis, malaria, hepatitis A, tuberculosis, or infectious mononucleosis, which react with solid phase and fluid phase CL in the absence of β_2-GPI (5, 17).

Thus, aCL found in SLE patients are not simply directed to the CL structure but the aCL binding requires the presence of β_2-GPI. However, the epitopic site(s) that aCL recognizes has not been immunologically defined. Three different hypotheses have been proposed to explain the aCL, CL, and β_2-GPI interactions: (*a*) the β_2-GPI–CL complex

[1] *Abbreviations used in this paper:* aCL, anticardiolipin antibodies; APA, antiphospholipid antibodies; APS, antiphospholipid syndrome; β_2-GPI, β_2-glycoprotein I; CL, cardiolipin; HRP, horseradish peroxide; PL, phospholipid; XPS, x-ray photoelectron spectroscopy.

is the structure recognized by aCL (1, 3–5); (*b*) the β_2-GPI is the actual target antigen for aCL but the epitope is cryptic in the absence of CL (5); and (*c*) the actual epitope for aCL appears on the native structure of β_2-GPI (2, 18, 19).

To distinguish among these possibilities, we used oxygen introduced onto polystyrene surfaces by irradiation with electron or γ-ray radiation. The introduction of polar groups to a polystyrene surface is accomplished by covalent coupling of oxygen atoms to polystyrene, using high energy irradiation with electron, γ-ray, or ultraviolet radiation or plasma treatment (20–22). aCL derived from either APS patients or (NZW × BXSB)F_1 mice, an animal model of human APS (23), bound to β_2-GPI coated on the irradiated plates, depending on the radiation dose. aCL binding to β_2-GPI adsorbed to oxygen-substituted solid phase was correlated well with that of β_2-GPI complexed to solid phase CL. Our results strongly suggest that aCL from APS recognize a modified form of β_2-GPI created by adsorption of the protein to a polyoxygenated surface, but not CL or native form of β_2-GPI, or an epitope structurally defined by both CL and β_2-GPI.

Materials and Methods

Reagents. CL (from bovine heart) was obtained from Sigma Chemical Co. (St. Louis, MO); BSA (ultrapure grade) from Life Technologies Inc. (Grand Island, NY); horseradish peroxidase (HRP) from Toyobo Co. (Osaka, Japan). All other chemicals were from commercial sources and of reagent grade.

Sera. aCL-positive sera were obtained from SLE patients who fulfilled the revised criteria of American Rheumatism Association in 1982. All patients exhibited significantly high levels of β_2-GPI–dependent aCL as compared with healthy subjects, and showed one or more of symptoms of APS, i.e., thromboembolic complications (venous and/or arterial), recurrent spontaneous abortion, and thrombocytopenia (10, 11).

Microtiter Plates. 96-well microtiter plates (plain polystyrene; MS-3496F) were obtained from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan). The plates were irradiated with electron beam (25 or 50 kGy) or with cobalt-60 γ-ray (6.3, 25, or 100 kGy) radiation under an ambient oxygen atmosphere.

Preparation of CL-coated Latex Beads. Polystyrene divinylbenzene beads of 6.4 ± 2.0 μm diameter in the form of a 10% uniparticulate dispersion in colloidal silica were obtained from Seradyn Inc. (Indianapolis, IN). Beads were first washed to remove colloidal silica and lyophilized as described (24). 200 mg of the decolloidal silica beads was dispersed in 2.0 ml of hexane/ethanol (1:1) and added to an ethanolic solution of CL (250 μg/100 μl). The beads were then briefly dispersed in a bath-sonicator and dried under a stream of nitrogen. The dry beads were redispersed in 3.0 ml of distilled water and washed three times by centrifugation at 300 *g* for 3 min. The amount of CL coated on the beads was measured according to the method of Bartlett (25).

Preparation of β_2-GPI. β_2-GPI was purified from normal human sera by sequential CL-affinity and ion exchange column and protein A–Sepharose column chromatography as described (12). Further, the preparation was delipidated by washing three times with butanol. The preparation resulted in a polymorphic 50-kD band detected by SDS-PAGE. Finally, an NH_2-terminal amino acid sequence of the preparations was determined by Edman degradation with a vapor phase protein sequencer (Model PSQ-1; Shimadzu Seisakusho, Kyoto, Japan). The NH_2-terminal sequence data demonstrated that the preparation contained homogeneous β_2-GPI. Analysis of the β_2-GPI preparation also revealed that the preparation did not contain any significant phospholipid contamination (25).

mAbs. A murine mAb, WB-CAL-1 (IgG2a, κ), was derived from (NZW × BXSB)F_1 (WB F1) male mice, an animal model of human APS (23). Another mAb directed to β_2-GPI, Cof-18 (IgG1, κ), was developed from BALB/c female mice immunized with Freund's adjuvant–emulsified human β_2-GPI. These antibodies were produced as ascites and were purified sequentially by ammonium sulfate precipitation and protein A–Sepharose affinity chromatography.

Surface Analyses. X-ray photoelectron spectroscopy (XPS) of polystyrene surface was analyzed with an ESCA spectrometer (JPS-9000MC; JEOL Ltd., Tokyo, Japan) utilizing Mgk$a_{1,2}$ radiation at 1,253.6 eV. Survey scan spectra (0–1,000 eV) were preliminarily performed for surface elemental analyses and then Cls spectra were taken at the analyzer's pass energy, 10 eV (resolution: 0.9 eV at Ag 3d 5/2 peak). The binding energy was calibrated to C-C binding energy (285.0 eV) of Cls spectra.

β_2-GPI–dependent aCL ELISA. β_2-GPI–dependent aCL ELISA was performed as previously described (5) with slight modification. CL in ethanol (2.5 μg/50 μl per well) was coated on the surface of wells of 96-well microtiter plates (plain polystyrene) by evaporation under nitrogen. The CL-coated wells were incubated with 50 μl of 10 mM Hepes, 150 mM NaCl (pH 7.4) containing 0.3% BSA (Hepes-BSA) for 1 h at 37°C, and were washed three times with 200 μl of PBS (pH 7.4) containing 0.05% Tween 20 (PBS-Tween). The wells were incubated with 50 μl of Hepes-BSA containing β_2-GPI (30 μg/ml) for 30 min at room temperature and were washed in the same manner. Then, the wells were incubated with 50 μl of diluted samples at room temperature for 30 min. After washing with PBS-Tween, the wells were incubated with 100 μl of HRP-labeled murine mAb against human IgG (G-02; Yamasa Corp., Choshi, Japan) or goat anti–mouse IgG for 30 min at room temperature. The wells were again washed and incubated with 100 μl of 0.3 mM tetramethylbendizine solution containing 0.003% of H_2O_2. After 10 min of incubation, the reaction was terminated by adding 100 μl of 2 N H_2SO_4 and the OD was measured at 450 nm.

Anti-β_2-GPI ELISA. Antibodies directed to β_2-GPI were also assayed by ELISA. Wells of 96-well microtiter plates (plain or irradiated polystyrene plates) were coated with 50 μl of β_2-GPI (10 μg/ml dissolved in 10 mM Hepes, 150 mM NaCl, pH 7.4, (Hepes buffer), for 16–20 h at 4°C. After the incubation, the β_2-GPI–coated wells were washed three times with 200 μl of PBS-Tween, and then were incubated with 200 μl of Hepes buffer containing 3% gelatin (0143-01; Difco Laboratories, Detroit, MI) for 1 h at room temperature. After washing in the same manner, the wells were incubated with 50 μl of diluted samples for 1 h at room temperature. The wells were again washed and incubated with 100 μl of HRP-labeled murine anti–human IgG or goat anti–mouse IgG for 1 h at room temperature. The color was developed and the OD was measured as described above.

Results

Specificity of Antibodies Defined by β_2-GPI–dependent aCL ELISA and anti-β_2-GPI ELISA. Table 1 presents the data for SLE-1, WB-CAL-1, and Cof-18 binding to surfaces coated with either CL or β_2-GPI. SLE-1 serum was obtained from a patient with APS. WB-CAL-1 is a monoclonal autoanti-

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Table 1. *Binding Profiles of aCL and Anti-β_2-GPI Antibodies*

Antibody	Binding to CL coated on plates		Binding to β_2-GPI on plates
	$-\beta_2$-GPI	$+\beta_2$-GPI (15 μg/ml)	
SLE-1	0.023	1.072	0.043
WB-CAL-1	0.021	1.159	0.033
Cof-18	0.030	1.103	1.213

50 μl of SLE-1 serum (200-fold dilution), WB-CAL-1 antibody (0.5 μg/ml), or Cof-18 antibody (0.5 μg/ml) were added to the wells. These values indicate OD at 450 nm detected by the β_2-GPI–dependent aCL ELISA and by the anti-β_2-GPI ELISA using plain polystyrene plates.

body derived from WB F_1 mice. Cof-18 is a murine mAb against β_2-GPI. To avoid the influence of endogenous β_2-GPI, these mAbs were purified and SLE-1 serum was finally diluted 200-fold. The antibodies SLE-1 and WB-CAL-1 bound to CL-coated plates (plain polystyrene) when the plates were previously or simultaneously incubated with β_2-GPI, but not to β_2-GPI–coated plates directly. The antibody Cof-18 bound to either β_2-GPI–coated plates directly or CL-coated plates when the plates were previously incubated with β_2-GPI. These results confirm that SLE-1 and WB-CAL-1 antibodies recognize the complex of β_2-GPI and CL, and that Cof-18 antibody is directed to β_2-GPI.

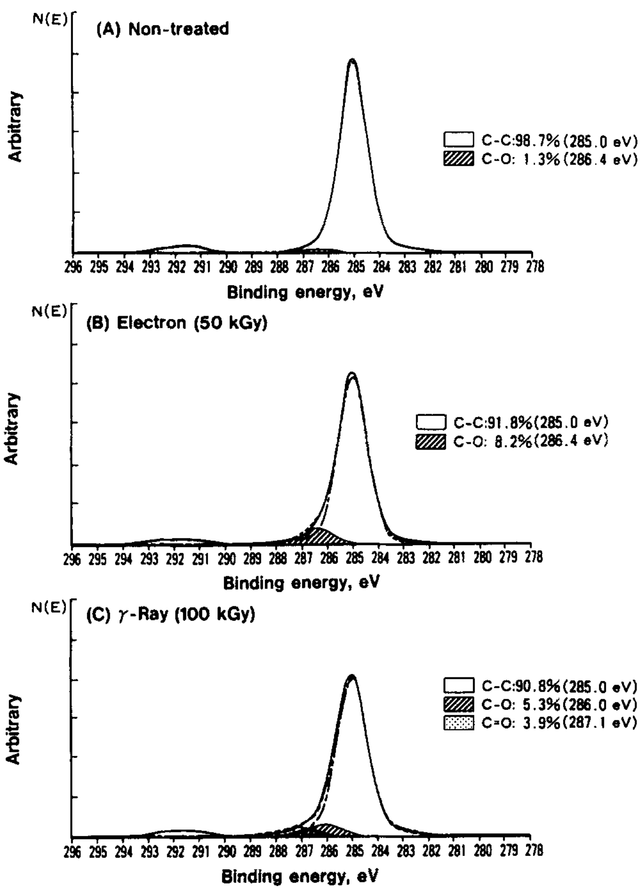

Figure 1. Analyses of molecular chains on the polystyrene surfaces. The values indicate atomic mol% of molecular chains on the polystyrene surfaces calculated from Cls spectra and binding energy (in parentheses). The XPS was analyzed as described in Materials and Methods.

Introduction of Oxygen Atoms onto the Polystyrene Surface. XPS data revealed that the plain polystyrene surface was composed entirely of aliphatic carbons (Fig. 1). The Cls peak at 285.0 eV was exhibited a full width at half-maximum of 1.29 eV. No nitrogen and a trace of oxygen (1.3 atomic mol%) was detected on the surface. Once polystyrene plates were irradiated with electron (50 kGy) or γ-ray (100 kGy), an increase of intensity in Ols peak and an apparent shoulder on the high binding energy side of the Cls peak were observed. The increased full width at half-maximum for the Cls spectra was accompanied by an increase in the shoulder on the Cls peak (1.37 and 1.43 eV, respectively). The Gaussian/Lorentzian (80:20) curve fitting was used for analyses. A peak at 286.4 eV corresponding to single bond of carbon to oxygen (C–O, 8.2 atomic mol%) appeared in the high binding energy Cls shoulders of the electron (50 kGy)-irradiated plates, and the shoulder of γ-ray (100 kGy)-irradiated plates were further resolved into two peaks, representing a C–O bond (5.3 atomic mol%) at 286.0 eV and a double bond to oxygen (C=O, 3.9 atomic mol%) at 287.1 eV. These results indicate that the irradiation with electron or γ-ray radiation could covalently introduce oxygen atoms onto the surfaces of polystyrene plates caused by producing oxidized carbons (alcohol, ether, and/or carbonyl groups).

aCL Binding to β_2-GPI Coated on the Irradiated Polystyrene Surface. We initially examined antibody binding to β_2-GPI coated on polystyrene surfaces irradiated under the various dose conditions of electron or γ-ray. Increased binding of SLE-1 and WB-CAL-1 antibodies to the irradiated plates depending on the radiation dose are shown in Fig. 2, *A* and *B*. The maximal binding of either SLE-1 or WB-CAL-1 antibodies to β_2-GPI–coated plates irradiated with 100 kGy of γ-ray radiation showed an OD of 1.161 and 1.087, and their control binding to β_2-GPI coated on nontreated plates gave an OD of 0.070 and 0.043, respectively. In contrast, Cof-18 antibody appreciably bound to nontreated plates (an OD of 0.917) and a slightly increased binding was observed (Fig. 2 *C*).

To study the molecular basis of the specificity of aCL, a competitive anti-β_2-GPI ELISA using the irradiated polysty-

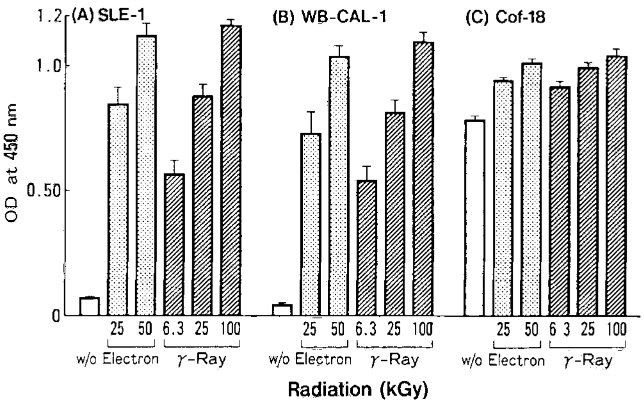

Figure 2. Antibody binding to β_2-GPI coated on the irradiated polystyrene plates. SLE-1 serum (200-fold dilution), WB-CAL-1 antibody (0.5 μg/ml), or Cof-18 antibody (0.5 μg/ml) was incubated in β_2-GPI–coated wells of which polystyrene plates were previously irradiated or not. Each bar represents the mean and SD (n = 5).

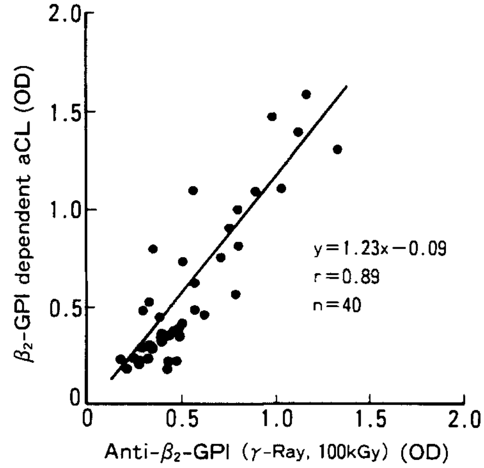

Figure 4. Correlation between antibody values in sera from APS patients obtained by the anti-β_2-GPI ELISA using the irradiated plates with 100 kGy of γ-ray radiation and those by the β_2-GPI–dependent aCL ELISA.

rene plates with 100 kGy of γ-ray radiation was carried out. The ELISA was assayed under the simultaneous addition of SLE-1, WB-CAL-1, or Cof-18 antibody with various inhibitors. As shown in Fig. 3, binding of either SLE-1 or WB-CAL-1 antibody in the ELISA was competitively inhibited by the addition of CL-coated latex beads at varying CL concentration from 0 to 4.0 μg/ml mixed together with 10 μg/ml of β_2-GPI. 50% inhibition of the SLE-1 and WB-CAL-1 binding was obtained by addition of the β_2-GPI–CL complex composed of 0.66 and 0.71 μg/ml of CL, respectively. There was no or slight inhibitory effect with either high excess of CL micelles (500 μg/ml) or β_2-GPI (500 μg/ml) alone. In contrast, the binding of Cof-18 antibody in the ELISA was inhibited by the addition of β_2-GPI (0–500 μg/ml) in a dose-dependent manner but was not inhibited by the addition of 500 μg/ml CL micelles. Similar phenomena were observed in the ELISA system using the β_2-GPI–coated plates that were previously irradiated with 50-kGy electron radiation.

Significance of Anti-β_2-GPI Antibodies Detected by Using β_2-GPI Coated on the Irradiated Polystyrene Plates. To evaluate the prevalence of anti-β_2-GPI antibodies that recognize the protein associated with the oxidized surface, 40 sera of APS patients associated with both of SLE and APS were tested in the two ELISA systems. As shown in Fig. 4, there was a high correlation (r = 0.89, $p < 0.01$) between binding values (OD) of aCL in sera from 40 APS patients detected by the anti-β_2-GPI ELISA using the irradiated plates with 100 kGy of γ-ray radiation and those by the β_2-GPI–dependent aCL ELISA.

Discussion

APA such as aCL have been frequently detected in sera from patients with SLE or related connective tissue disorders (8–14). Recent studies (1–5) have shown that β_2-GPI is involved in aCL binding to solid phase CL but aCL do not recognize the CL structure directly. In the present study, we further characterized the binding properties of aCL by ELISA using irradiated polystyrene plates.

It has been demonstrated that β_2-GPI can bind to a variety of negatively charged molecules, including phospholipids (PL) (5) and heparin (26), whereas the aCL from APS

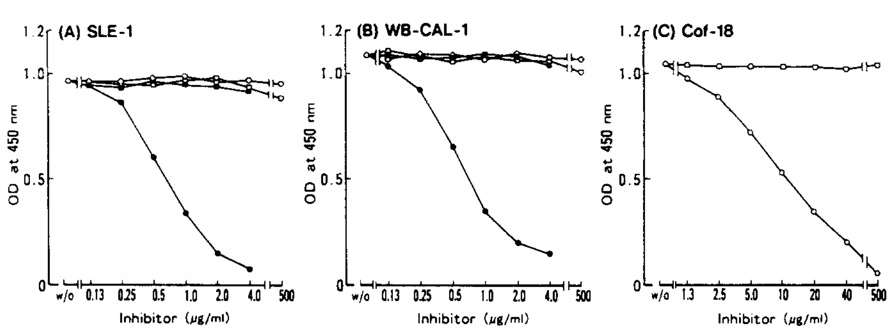

Figure 3. A simultaneous competitive inhibition assay for antibody binding in ELISA using β_2-GPI coated on the irradiated plates with 100 kGy of γ-ray radiation. SLE-1 serum (200-fold dilution), WB-CAL-1 antibody (0.5 μg/ml), or Cof-18 antibody (0.5 μg/ml) was incubated into β_2-GPI–coated wells of the plates. β_2-GPI (0–500 μg/ml, ○), CL micelles (0–500 μg/ml, □), CL-coated latex beads (0–4.0 μg/ml of CL, ■), or CL-coated latex beads (0–4.0 μg/ml of CL, ●) mixed together with 10 μg/ml of β_2-GPI was added to the wells as an inhibitor. Antibody binding was determined as described in Materials and Methods.

bound only to β_2-GPI complexed with PL, such as CL coated on polystyrene plates (1, 3–5), or polyacrylamide gels in which liposomes composed of CL or phosphatidylserine were entrapped (1, 5, 27).

The present data show that the aCL bound directly to β_2-GPI coated on an irradiated polystyrene surface in the absence of CL. The XPS analyses showed that a significant amount of oxygen was covalently introduced onto the surface of the irradiated plates but any other atomic compositions were not changed. The extent of aCL binding was dependent upon the radiation dose applied to the surface (Fig. 2). The maximal binding of either SLE-1 or WB-CAL-1 antibody was observed with 100 kGy of γ-ray radiation and their bindings were 16.6- and 25.3-fold greater as compared with controls, respectively. Moreover, the aCL binding was inhibited by simultaneous addition of CL-coated latex beads mixed together with β_2-GPI but not by β_2-GPI, CL micelles, or CL-coated latex beads alone (Fig. 3).

Arvicux et al. (18) have shown that antibody binding directed to β_2-GPI coated on the polystyrene plates was inhibited by prior incubation with liposomes composed of negatively charged PL (CL or phosphatidylserine) and cholesterol, depending on the PL dose. We have further confirmed evidence that inhibitory effect on antibody binding to β_2-GPI coated on the irradiated polystyrene plates with 100 kGy of γ-ray radiation was provided by prior incubation with a mixture of β_2-GPI and CL micelles but was not with CL micelles or β_2-GPI alone (our unpublished data). This evidence strongly suggests that aCL derived from APS patients that react to the complex of β_2-GPI and CL in aCL ELISA (1, 5) recognize an epitope appearing on a conformationally altered β_2-GPI structure resulting from interactions with an oxygenated solid phase surface. Our results also support a model in which β_2-GPI binds to a surface containing anionic PLs (either micelles, liposomes, or biological membranes) and reveals an epitope(s) that is recognized by the aCL.

Galli et al. (2), Arvicux et al. (18), and Viard et al. (19) have individually reported that aCL derived from autoimmune patients (i.e., SLE and/or APS) were directed to the β_2-GPI molecule coated on polystyrene plates. However, we obtained no evidence that autoimmune aCL bound directly to the structure of β_2-GPI either in the fluid phase or on plain polystyrene surfaces (solid phase). We have also noted that some commercially available plates that are irradiated by the manufacturer, such as EB plates (Labsystems, Finland), Immulon 2, and 4 (Dynatech, Chantilly, VA), and Sumilon H type (Sumitomo Bakelite Co., Ltd.), showed β_2-GPI binding of aCL in the absence of CL (our unpublished data). They did not state the surface conditions on the plates they had used.

Hunt et al. (28) reported that β_2-GPI could be proteolytically cleaved between Lys-317 and Thr-318 (as a potential thrombin cleavage site) and the cleavage resulted in loosing lipid binding and activity to provide antibody binding in aCL ELISA. Thus, a neoepitope for aCL might be exposed on the region located near the COOH terminus (on the fifth sushi domain) of β_2-GPI when β_2-GPI interacts with an oxidized surface or anionic PLs.

Finally, the definition of aCL specificity reinforces arguments that immune response could be addressed to lipid-bound β_2-GPI (and resulted in alteration of own structure) rather than lipid or β_2-GPI alone and that thrombotic complications could be associated with β_2-GPI and aCL circulating in blood stream.

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