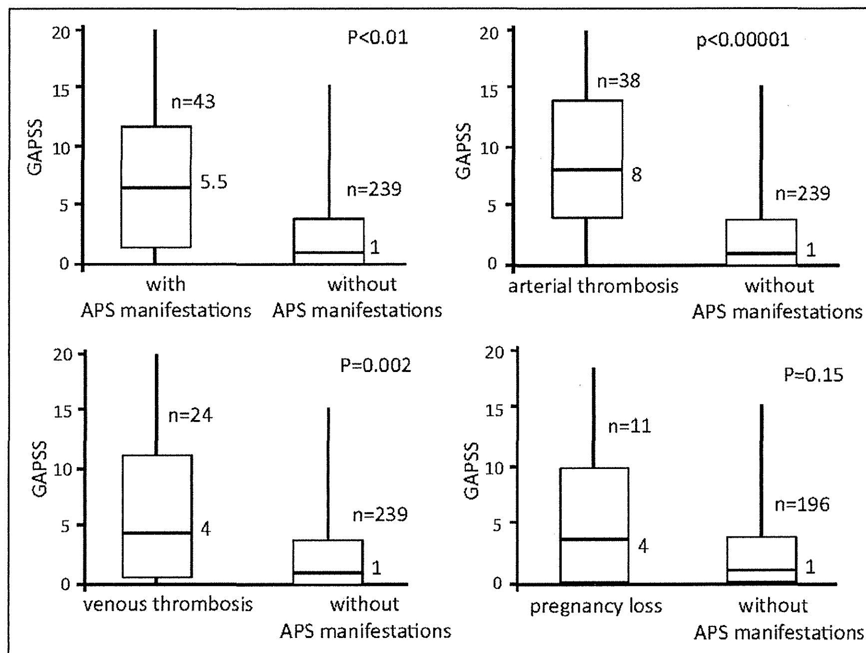

Figure 1 Global Anti-phospholipid Syndrome Score (GAPSS) in patients with autoimmune diseases. The GAPSS was calculated according to Sciscia et al. Data are shown as box plots, where each box represents the 25th to 75th percentiles: lines inside the box represent the median. The whiskers represent the 95% CI. Higher values of GAPSS were seen in patients who experienced APS manifestations when compared with those without APS manifestations ($p > 0.01$ by Mann–Whitney U test). When analysed separately, patients who experienced thrombosis showed higher GAPSS when compared with those without APS manifestations.

Table 1 Diagnostic accuracy including sensitivity, specificity, PPV, NPV, PLR and NLR for different cut-off values of GAPSS

Cut off	*AUC*	*Sensitivity*	*Specificity*	*PPPV*	*NPV*	*PLR*	*NLR*	*p value*
4	0.781	0.878	0.683	0.319	0.971	2.771	0.179	0.001>
6	0.857	0.805	0.912	0.600	0.965	8.890	0.215	0.001>
8	0.840	0.732	0.947	0.698	0.954	13.67	0.028	0.001>
10	0.752	0.537	0.967	0.733	0.925	16.30	0.479	0.001>
12	0.732	0.488	0.975	0.769	0.919	19.76	0.525	0.001>
15	0.693	0.390	0.996	0.941	0.906	94.83	0.612	0.001>

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Regular Article

THROMBOSIS AND HEMOSTASIS

β2-Glycoprotein I/HLA class II complexes are novel autoantigens in antiphospholipid syndrome

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Key Points

- β2GPI complexed with HLA class II molecules was found to be a target for autoantibodies in APS.
- More than 80% of patients with APS possess autoantibodies against β2GPI/HLA class II complexes.

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by thrombosis and/or pregnancy complications. β2-glycoprotein I (β2GPI) complexed with phospholipid is recognized as a major target for autoantibodies in APS; however, less than half the patients with clinical manifestations of APS possess autoantibodies against the complexes. Therefore, the range of autoantigens involved in APS remains unclear. Recently, we found that human leukocyte antigen (HLA) class II molecules transport misfolded cellular proteins to the cell surface via association with their peptide-binding grooves. Furthermore, immunoglobulin G heavy chain/HLA class II complexes were specific targets for autoantibodies in rheumatoid arthritis. Here, we demonstrate that intact β2GPI, not peptide, forms a complex with HLA class II molecules. Strikingly, 100 (83.3%) of the 120 APS patients analyzed, including those whose antiphospholipid antibody titers were within normal range, possessed autoantibodies that recognize β2GPI/HLA class II complexes in the absence of phospholipids. In situ association between β2GPI and HLA class II was observed in placental tissues of APS patients but not in healthy controls. Furthermore, autoantibodies against β2GPI/HLA class II complexes mediated complement-dependent cytotoxicity against cells expressing the complexes. These data suggest that β2GPI/HLA class II complexes are a target in APS that might be involved in the pathogenesis. (*Blood*. 2015;125(18):2835-2844)

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by arterial or venous thrombosis and pregnancy complications, including recurrent spontaneous abortion.[1,2] APS is associated with antiphospholipid (aPL) antibodies that bind to anionic phospholipid and serum protein complexes.[3-5] Interactions between aPL antibodies and vascular endothelial cells are thought to be involved in the pathogenesis of APS.[6-9] β2-glycoprotein I (β2GPI) is the main phospholipid-binding molecule recognized by aPL antibodies[5,10,11] and is produced predominantly by hepatocytes, although some endothelial cells of blood vessels and placental villous tissue also express it.[12,13] Plasma β2GPI circulates in a circular conformation with the aPL antibody epitopes being cryptic.[14] When β2GPI associates with anionic phospholipids such as cardiolipin (CL), the circular structure of plasma β2GPI is converted to a linear form, leading to exposure of the major epitope for aPL antibodies.[14-19] Therefore, β2GPI bound to negatively charged phospholipids or negatively charged plates is used clinically to detect antibodies.[20] However, autoantibodies against the β2GPI associated with phospholipids are detected in less than half the patients with clinical manifestations of APS,[21-23] suggesting the existence of additional targets of the autoantibodies. In addition, β2GPI is a secreted protein and is generally not present on the cell surface; therefore, how aPL antibodies bind vascular endothelial cells and induce thrombosis or pregnancy complications has remained unclear.

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Specific human leukocyte antigen (HLA) class II alleles are associated with susceptibility to APS, as in other autoimmune diseases.[24-27] Because peptide repertoires presented on different HLA class II alleles differ,[28,29] it has been proposed that specific peptide-HLA class II combinations affect T-cell development and/or tolerance, which may confer susceptibility or resistance to autoimmune diseases.[30] Nonetheless, the mechanisms by which HLA class II gene polymorphisms regulate susceptibility to autoimmune diseases are unknown.

Misfolded cellular proteins are generally eliminated by the process of endoplasmic reticulum-associated degradation[31] and would not be exposed to the immune system. Recently, however, we found that misfolded proteins are rescued from degradation and transported to the cell surface without processing to peptides when they associate with the peptide-binding groove of HLA class II molecules in the endoplasmic reticulum (ER).[32,33] Structural analyses of major histocompatibility complex (MHC) class II molecules have revealed that both ends of the MHC class II peptide-binding groove are open. Therefore, it is possible that MHC class II molecules might bind linear epitopes exposed on misfolded proteins. Indeed, several studies have suggested that MHC class II molecules have the capacity to associate with denatured proteins at the cell surface.[34-36] Furthermore, immunoglobulin G (IgG) heavy chains thus transported to the cell surface by HLA class II alleles associated with rheumatoid arthritis (RA) susceptibility were specifically recognized by autoantibodies from RA patients.[33] Because HLA class II expression on nonlymphoid cells, including endothelial cells, is frequently observed in various autoimmune diseased tissues,[37-41] we hypothesized that misfolded proteins rescued from protein degradation by HLA class II molecules might be targets for autoantibodies in autoimmune diseases. Here, we addressed whether structurally altered β2GPI is transported to the cell surface by HLA class II molecules and is recognized by autoantibodies in APS patients. Strikingly, 100 (83.3%) of the 120 APS patients, including those whose aPL antibody titers were within normal range, possessed autoantibodies against β2GPI/HLA class II complexes. Furthermore, autoantibodies from APS patients mediated complement-dependent cytotoxicity against cells expressing both β2GPI and HLA class II molecules. Our findings provide new insights into the pathogenesis of APS and also into an unexpected function of HLA class II molecules in autoimmune diseases.

Materials and methods

Sera and placental tissue samples

The collection and use of human sera and placental tissues was approved by the institutional review boards of Hokkaido University, Kobe University, Kyoto University, Dohgo Spa Hospital, and Osaka University. Written informed consent was obtained from all participants according to the relevant guidelines of the institutional review boards. The diagnosis of APS was based on the preliminary classification criteria for definite APS.[1,2] Sera from 63 of the 120 APS patients were derived from patients with secondary APS complicated by systemic lupus erythematosus. Sera from 50 healthy controls were purchased from George King Bio-Medical, Inc.

Measurement of anticardiolipin antibody, anti-β2GPI antibody, and lupus anticoagulant

Anticardiolipin (aCL) antibody was detected by using CL complexed with serum phospholipid-binding protein, and anti-β2GPI antibody was detected by using β2GPI bound to negatively charged plates as previously reported.[20,42] Normal ranges of aCL antibody-IgG (<18.5 IgG phospholipid) and anti-β2GPI antibody-IgG (<2.2 U) were established previously by using 132 healthy controls with 99th percentile cutoff values.[22] Lupus anticoagulant was measured by 3 clotting tests as previously reported.[43]

Plasmids

Complementary DNAs (cDNAs) prepared from pooled human peripheral blood mononuclear cells (3H Biomedical) were cloned into the pME18S or pCAGGS expression vectors. cDNA sequences for HLA class II were based on information contained in the Immunogenetics/HLA Database (http://www.ebi.ac.uk/imgt/hla/index.html). HLA-DRB1*04:04 containing a covalently attached HLA-Cw4 peptide (GSHSMRYFSTSVSWPGR) was generated as previously described.[44] Domain I-deleted β2GPI cDNA (acid residues 80-345) were cloned into the pCAGGS expression vector containing a human signaling lymphocytic activation molecule signal sequence. 293T cells were transiently transfected by using Polyethylenimine Max (Polyscience) and were analyzed 2 days after transfection.

Antibodies

HL40 (EXBIO), L243 (American Type Culture Collection), FL-254 (Santa Cruz Biotechnology), and TAL.1B5 (Dako) were used to detect HLA-DR by flow cytometry, immunoprecipitation, western blotting, and immunohistochemistry, respectively. Anti-FLAG monoclonal antibody (mAb) (M2, Sigma-Aldrich), anti-His mAb (Wako), and rabbit anti-β2GPI antibody (specific to domains IV and V; HPA001654; Atlas Antibodies) were used for flow cytometry and western blotting. EY2C9, a human aPL mAb derived from an APS patient,[45] was purified from EY2C9-producing cells. Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described previously.[46] Briefly, cells were lysed in buffer containing 0.5% NP-40. The immunoprecipitates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and were blotted. Total cell lysates were also analyzed by immunoblotting.

Immunohistochemistry and in situ proximity-ligation assay (PLA)

Paraffin-embedded tissue sections from APS patients (n = 6) and individuals without APS (n = 6) were stained with anti-β2GPI and anti-HLA-DR antibodies, followed by Alexa 647- or Alexa 555-conjugated goat anti-rabbit IgG or mouse IgG antibodies (Molecular Probes). A Duolink was used for PLA according to the manufacturer's instructions (Olink Bioscience). The assayed tissue sections were analyzed by Axioplan 2 fluorescence microscopy (Zeiss).

Effect of exogenous β2GPI on EY2C9 mAb binding to HLA class II–expressing cells

Primary endothelial cells (human dermal microvascular endothelial cells; Lonza) were stimulated with interferon γ (IFN-γ; 500 U/mL; Miltenyi Biotec) and tumor necrosis factor α (TNF-α; 20 ng/mL; Miltenyi Biotec) for 24 hours. Thereafter, β2GPI (GenWay) was added to the medium at the concentration found in serum (200 μg/mL). HLA-DR7, invariant chain (Ii), and HLA-DM were transfected into 293T cells, and β2GPI (200 μg/mL) was added to the transfectants 24 hours later. Cells cultured in the presence of β2GPI for 48 hours were stained with EY2C9 mAb and anti-HLA-DR mAb (L243).

Competitive inhibition assay

β2GPI was purified from the culture supernatants of 293T cells transfected with FLAG-tagged β2GPI using anti-FLAG M2 affinity gels (Sigma-Aldrich). β2GPI bound to anti-FLAG M2 affinity gels was eluted by DYKDDDDK (FLAG) peptide (150 ng/μL; Wako). Purity of the β2GPI was more than 90% as determined by sodium dodecyl sulfate polyacrylamide

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gel electrophoresis. β2GPI and EY2C9 mAb (1 μg/mL) were incubated for 12 hours at 4°C in the presence or absence of CL (50 μg/mL; Sigma-Aldrich). 293T cells cotransfected with β2GPI, HLA-DR7, and green fluorescent protein (GFP) were stained with EY2C9 mAb preincubated with β2GPI and/or CL. Binding of EY2C9 mAb to GFP-expressing cells was measured by flow cytometry.

Determination of anti-β2GPI/HLA class II complex antibody titer

A serum in which aCL antibody titer was high (47 IgG phospholipid) and anti-β2GPI/HLA-DR7 complex antibody was detectable after 10^6-fold dilution was used as a standard throughout this study. The aβ2GPI/HLA-DR7 complex antibody titer of the standard serum was defined as 100 U. β2GPI, HLA-DR7, and GFP were transfected into 293T cells. Mean fluorescence intensities (MFIs) of IgG binding to GFP-positive and -negative cells in sequentially diluted standard sera (10^2- to ~10^6-fold dilution) were analyzed by flow cytometry. Specific IgG binding to the β2GPI/HLA-DR7 complexes was calculated by subtracting the MFI of IgG binding to GFP-negative cells from the MFI of IgG binding to GFP-positive cells. A standard curve was generated from the specific IgG binding to β2GPI/HLA-DR7 complexes in sequentially diluted standard sera. The anti-β2GPI/HLA-DR7 complex antibody titer of each serum was calculated from the standard curve. The normal range of anti-β2GPI/HLA-DR7 complex antibody titers (<1.8 U) was established by using 100 healthy controls with 99th percentile cutoff values.

Complement-mediated cytotoxicity of aPL antibody against cells expressing β2GPI and HLA-DR7

β2GPI, HLA-DR (*HLA-DRA*01:01* and *DRB1*07:01*), and GFP were cotransfected into 293T cells, and GFP-expressing cells were purified by using a cell sorter (FACSAria) 2 days after transfection. The purified transfectants were mixed with aPL (EY2C9) or control human IgM mAb (Calbiochem) on ice for 30 minutes followed by incubation with 1:10 diluted rabbit complement (Cedarlane) at 37°C for 30 minutes. Dead cells were stained with propidium iodide dye, and their proportions were determined by flow cytometry.

Statistics

To assess the significance of the correlation, Pearson's product-moment correlation coefficient was used and the correlation coefficient (r) and P value of the linear regression line were calculated. Student t test and Mann-Whitney U test were used to determine the significance of differences. P values of $<.05$ were regarded as statistically significant.

Results

β2GPI complexed with HLA class II molecules is recognized by aPL antibody

Free β2GPI has a circular conformation whereas phospholipid-bound β2GPI has a linear conformation that is accessible by aPL antibodies (Figure 1A).[14-18] Because β2GPI is a secreted serum protein, it is not generally detected on the surface of even the cells that produce it. In order to analyze whether β2GPI is transported to the cell surface by HLA class II molecules in a manner similar to IgG heavy chain,[33] β2GPI was cotransfected into 293T cells together with GFP and *HLA-DRA*01:01* and *DRB1*07:01* (HLA-DR7) or *HLA-DRA*01:01* and *DRB1*08:01* (HLA-DR8), and cell surface expression of β2GPI on GFP-positive cells was analyzed. β2GPI was not detected on the surface of cells transfected with β2GPI alone (Figure 1B). In contrast, it was found on the surface of cells cotransfected with HLA-DR7, an APS susceptibility allele.[24-27] Cells cotransfected with HLA-DR8, an allele not associated with APS susceptibility, expressed less surface β2GPI (Figure 1B). Similar results were obtained by using N-terminus FLAG-tagged β2GPI or C-terminus His-tagged β2GPI detected with anti-FLAG or anti-His mAb, respectively (supplemental Figure 1, available on the *Blood* Web site). Furthermore, β2GPI of the predicted size was coprecipitated together with HLA-DR7 from cells expressing both β2GPI and HLA-DR7 (Figure 1C). Similarly, HLA-DR was coprecipitated together with β2GPI from the transfectants. Because association of full-length IgG heavy chain with HLA-DR was detected in the ER,[33] full-length β2GPI, but not fragmented β2GPI, also seems to be associated with HLA-DR in the ER and transported to the cell surface by HLA-DR (Figure 1A).

APS patients possess autoantibodies that bind to cryptic epitopes on β2GPI revealed by conformational changes induced by association with phospholipids.[14-18] We tested the possibility that conformation of β2GPI bound to HLA-DR7 is similar to β2GPI complexed with phospholipids and thus is recognized by aPL antibodies from APS patients. EY2C9, a well-characterized human aPL mAb derived from an APS patient, represents binding and procoagulant properties of anti-β2GPI found in APS patients. EY2C9 mAb binds to β2GPI complexed with phospholipids but not to β2GPI or phospholipids alone.[45] We found that EY2C9 mAb bound well to cells expressing both β2GPI and HLA-DR7, but not β2GPI alone, in the absence of phospholipids. EY2C9 mAb bound weakly to cells expressing β2GPI together with HLA-DR8 (Figure 1B). Because EY2C9 recognizes β2GPI associated with phosphatidylserine, we analyzed cell surface phosphatidylserine on HLA-DR7 and HLA-DR8 transfectants by using annexin V, which binds to phosphatidylserine. There was no difference in annexin V binding to HLA-DR7 and HLA-DR8 transfectants, suggesting that preferential binding of EY2C9 to β2GPI and HLA-DR7 transfectants is not due to an increase of cell surface phosphatidylserine on HLA-DR7 transfectants (supplemental Figure 2). In addition, an HLA-Cw4 peptide (a peptide naturally bound to HLA-DR4[47]) covalently attached to HLA-DR4 significantly blocked transport of β2GPI to the cell surface and inhibited the binding of aPL antibody without affecting cell surface expression of HLA-DR (Figure 1D). These findings indicated that aPL antibody recognizes β2GPI bound to the peptide-binding groove of HLA-DR. Furthermore, the EY2C9 mAb binding to β2GPI complexed with HLA-DR7 was blocked by β2GPI complexed with CL, but not by β2GPI alone or CL alone (Figure 2A-B). These data indicate that EY2C9 mAb recognizes an epitope conserved between β2GPI/HLA-DR7 complexes and β2GPI/phospholipid complexes. It has been suggested that domains I, IV, and V of β2GPI are involved in EY2C9 mAb binding.[48-50] Indeed, when domain I-deleted β2GPI and HLA-DR were cotransfected, EY2C9 mAb failed to recognize the domain I-deleted β2GPI complexed with HLA-DR7, although the mutant β2GPI complexed with HLA-DR7 was well recognized by anti-β2GPI domain IV-V antibody (supplemental Figure 3). These data suggested that domain I plays a critical role for EY2C9 mAb recognition of the β2GPI/HLA-DR7 complexes and that domain I is not involved in the association of β2GPI with HLA-DR7.

Autoantibodies against β2GPI complexed with HLA class II molecules are present in most APS patients

We examined whether β2GPI complexed with HLA-DR is recognized by autoantibodies from APS patients whose clinical characteristics are provided in Table 1. We found that sera from APS patients, including aCL-IgG antibody–negative and anti-β2GPI-IgG antibody–negative patients, contained IgG autoantibodies against

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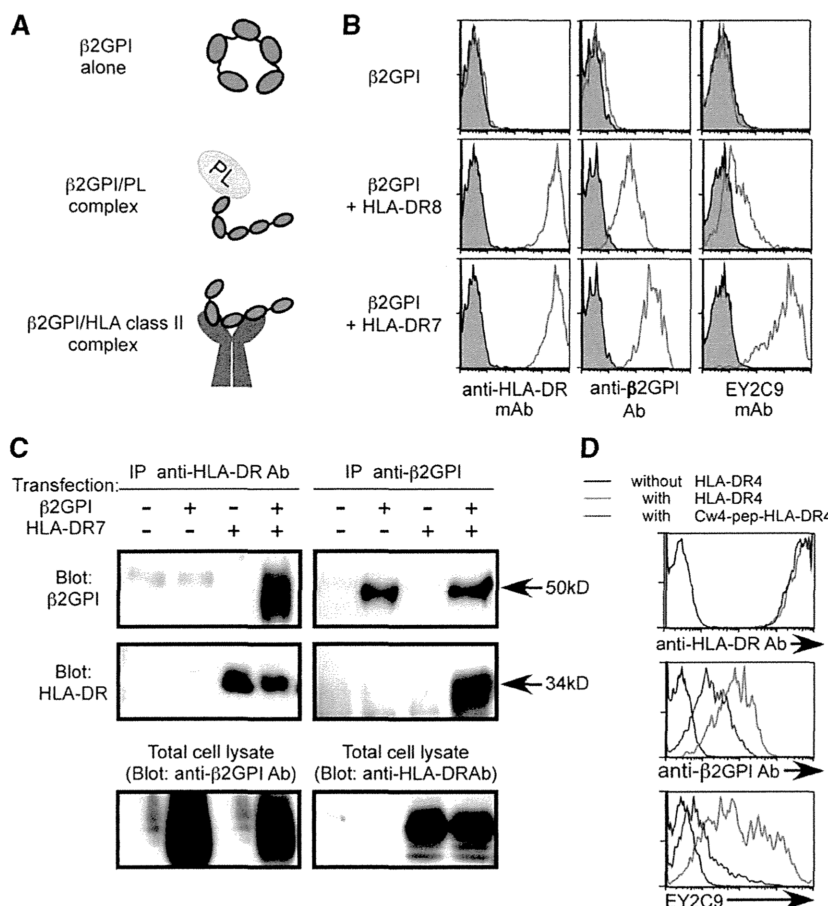

Figure 1. β2GPI complexed with HLA class II molecules is recognized by aPL antibody. (A) Possible conformations of β2GPI. Free β2GPI in serum shows a closed circular conformation, whereas β2GPI associated with phospholipids (PLs) shows a linear conformation. Because cellular misfolded proteins are presented by HLA class II molecules,[32] β2GPI with a unique conformation might also be presented by them. (B) β2GPI is displayed on the cell surface in the presence of HLA-DR. β2GPI was transfected into 293T cells together with GFP in the presence or absence of HLA-DR7 or HLA-DR8, and the transfectants were stained with anti-β2GPI, anti-HLA-DR, or aPL antibody (EY2C9) (red line). Antibody (Ab) binding to GFP-expressing cells is shown. Cells transfected with GFP alone were stained as a control (shaded histogram). (C) Direct association of β2GPI with HLA-DR. β2GPI and HLA-DR were cotransfected, and HLA-DR or β2GPI was precipitated. β2GPI and HLA-DR in the precipitates were detected by western blotting. HLA-DR or β2GPI in total cell lysates was also detected. (D) HLA-DR4 containing a covalently attached Cw4 peptide (blue lines) or wild-type HLA-DR4 (red lines) was cotransfected into 293T cells together with β2GPI and GFP. Cells transfected with β2GPI and GFP alone were used as a control (black line). β2GPI expression and aPL antibody binding to GFP-expressing cells were analyzed. Data are representative of at least 3 independent experiments.

β2GPI bound to HLA-DR7, whereas sera from almost all healthy individuals did not (Figure 3). Cells transfected with HLA-DR7 alone were not recognized by serum IgG from either APS patients or healthy individuals (supplemental Figure 4). In addition, autoantibodies from APS patients bound more effectively to cells cotransfected with β2GPI and HLA-DR7 than to those with β2GPI and HLA-DR8, similar to EY2C9 mAb (supplemental Figure 5). Strikingly, anti-β2GPI/HLA-DR7 complex IgG antibody titers in 100 (83.3%) of the 120 APS patients examined were above the normal range (<1.8 U) established by using 100 healthy controls with 99th percentile cutoff values (Figure 4A-B). Anti-β2GPI/HLA-DR7 complex antibody titers between APS patients and healthy controls were significantly different ($P = 3.3 \times 10^{-33}$). It is noteworthy that 60 (51.3%) of the 117 APS patients possessed autoantibodies that bind to β2GPI/HLA-DR7 complexes but not β2GPI bound to negatively charged plates (Figure 4C). Similarly, 60 APS patients (50%) whose aCL-IgG antibody titers were within normal range possessed autoantibodies to β2GPI/HLA-DR7 complexes (Figure 4D). Therefore, β2GPI/HLA-DR7 complexes appear to possess unique epitopes that are frequently recognized by autoantibodies in APS, but such epitopes are not present on plate-bound β2GPI or β2GPI/CL complexes. Conversely, a significant correlation between anti-β2GPI/HLA-DR7 complex antibody titers and anti-β2GPI-IgG antibody titers was observed when 52 APS

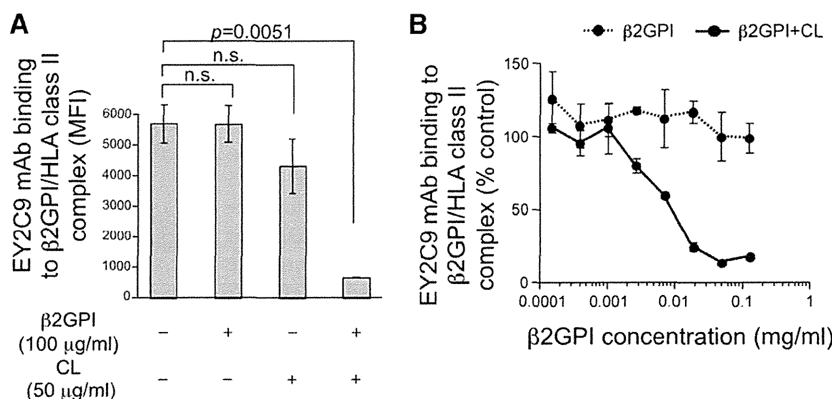

Figure 2. β2GPI complexed with HLA class II molecules shares aPL antibody epitopes with β2GPI complexed with CL. EY2C9 mAb was incubated with β2GPI in the presence or absence of CL at the concentrations indicated and was used for staining cells transfected with β2GPI and HLA-DR7. (A) MFI of the stained cells and (B) relative MFI compared with staining with EY2C9 mAb alone is shown as mean + standard deviation (SD) of triplicates. Data are representative of at least 3 independent experiments.

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Table 1. Patient characteristics

Patient	Age, y	Sex	Primary or secondary APS (complicated by)	Clinical manifestation
APS1	68	F	Secondary (SLE)	Lacunar infarction
APS2	77	M	Secondary (RA)	Lacunar infarction
APS3	33	F	Secondary (SLE)	Deep venous thrombosis
APS4	39	F	Secondary (SLE)	Lacunar infarction
APS5	69	F	Secondary (SLE)	Deep venous thrombosis, pulmonary embolism
APS6	42	F	Primary	Recurrent spontaneous abortion

F, female; M, male; SLE, systemic lupus erythematosus.

patients (44.4%) with detectable anti-β2GPI-IgG antibody titers were analyzed ($r = 0.57$; $P = 1.13 \times 10^{-5}$) (Figure 4C). A similar significant correlation between anti-β2GPI/HLA-DR7 complex antibody titers and aCL-IgG antibody titers was also observed ($r = 0.44$; $P = .330 \times 10^{-6}$) (Figure 4D). This suggests that β2GPI/HLA-DR complexes also possess autoantibody epitopes shared by β2GPI bound to negatively charged plates or β2GPI/CL complexes.

HLA-DR allele differences influence aPL antibody binding to β2GPI/HLA-DR complexes

Specific HLA-DR alleles are associated with susceptibility to APS.[24-27] We analyzed the ability of different HLA-DR alleles to transport β2GPI to the cell surface (Figure 5). In addition to HLA-DR7, HLA-DR4 (*HLA-DRA*01:01* and *DRB1*04:02*), another APS susceptibility allele, was found to be particularly effective at transporting high levels of β2GPI to the cell surface, as recognized by the EY2C9 aPL mAb. In contrast, very little β2GPI was transported to the cell surface by several other HLA-DR alleles. The Ii associates with nascent HLA class II molecules and blocks their association with other ER proteins. Similarly, we found that Ii blocked β2GPI binding by most HLA-DR alleles, whereas HLA-DR7 and HLA-DR4 still bound significant amounts of β2GPI, even in the presence of Ii (supplemental Figure 6). These data suggested that interactions between β2GPI or Ii and HLA-DR might differ among different HLA-DR alleles. APS susceptibility conferred by certain HLA-DR alleles might be partially explained by these differences.

β2GPI is complexed with HLA-DR on endothelial cells of vessels in uterine decidual tissues from APS patients

Because β2GPI bound to HLA-DR is a target for autoantibodies in APS patients, we tested the hypothesis that β2GPI is bound to HLA-DR in diseased tissues of APS patients. Accordingly, we analyzed β2GPI and HLA-DR expression on placental tissues obtained from APS patients with spontaneous abortion (n = 6) by immunofluorescence staining and PLAs, which detect close proximity (less than 40 nm) between two molecules.[51] β2GPI and HLA-DR were found to colocalize in endothelial cells of vessels and in stromal cells in the placental decidua of 4 of the 6 APS patients examined (Figure 6A-C). Furthermore, PLA signals between β2GPI and HLA-DR were detected in endothelial cells of vessels in decidua, but not in stromal cells (Figure 6D). In villous tissues, β2GPI was expressed in syncytiotrophoblasts (Figure 6F) and stromal cells around placental stem vessels (Figure 6J), but no colocalization of β2GPI and HLA-DR was observed (Figure 6G-H, K-L). Conversely, β2GPI, but not HLA-DR, was present in endothelial cells of vessels in decidua of placental tissues from patients without APS (n = 6) (Figure 6M-O), and no PLA signal was detected (Figure 6P). These results suggest that HLA class II expression is induced in uterine decidua of APS patients, and thus β2GPI forms complexes with HLA-DR, which can be targeted by autoantibodies in APS patients.

We analyzed whether β2GPI is complexed with HLA class II molecules on primary endothelial cells (supplemental Figure 7A). Because it has been reported that endothelial cells express β2GPI,[12,13] and that IFN-γ and TNF-α upregulate HLA class II expression,[52,53] we stimulated primary endothelial cell lines (human dermal microvascular endothelial cells) with these cytokines and analyzed EY2C9 mAb binding. HLA class II expression was induced on endothelial cells upon stimulation with IFN-γ and TNF-α. However, endothelial cells stimulated with IFN-γ and TNF-α were not recognized by EY2C9 mAb. Conversely, when endothelial cells were stimulated with IFN-γ and TNF-α in the presence of exogenous β2GPI at the concentration found in serum (200 μg/mL), EY2C9 mAb bound to the endothelial cells. To analyze the role of HLA class II molecules on EY2C9 mAb binding, we examined 293T cells transfected with HLA-DR (supplemental Figure 7B). HLA-DR 293T transfectants were also recognized by EY2C9 mAb in the presence of exogenous β2GPI. These results suggested that not only intracellular β2GPI but also extracellular β2GPI can be a target for aPL antibodies upon association with HLA class II molecules.

Complement-mediated cytotoxicity of aPL antibody against cells expressing β2GPI and HLA class II molecules

Because β2GPI associated with HLA class II molecules is a target for autoantibodies in APS patients, we analyzed whether aPL antibodies are cytotoxic for cells expressing β2GPI and HLA-DR (Figure 7A-B). aPL mAb EY2C9, but not control mAb, exhibited complement-mediated cytotoxicity against cells expressing β2GPI together with the APS susceptibility allele HLA-DR7, but not HLA-DR8. Cells expressing β2GPI alone were not killed. These results suggest that expression of HLA class II on β2GPI–expressing cells might play a crucial role in the pathogenesis of APS (supplemental Figure 8).

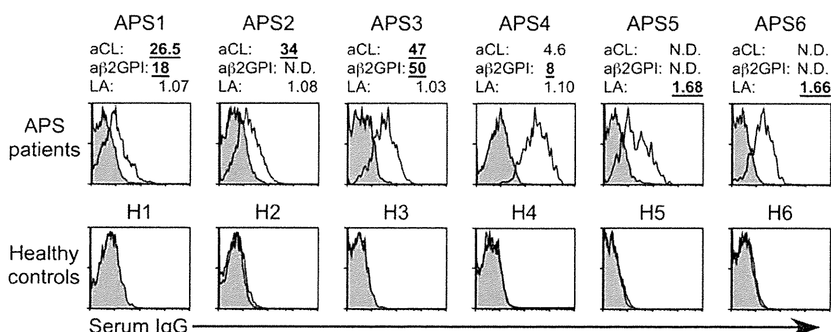

Figure 3. β2GPI complexed with HLA class II molecules is recognized by autoantibodies in APS patients. Autoantibody binding to β2GPI bound to HLA class II in aPL antibody-positive and -negative APS patients and healthy controls. Diluted sera were mixed with cells transfected with β2GPI and HLA-DR7, and IgG Ab binding to the cells was assessed (open histogram). Cells transfected with GFP alone were stained as a control (shaded histogram). Levels of aCL antibody-IgG, anti-β2GPI antibody-IgG, and lupus anticoagulant (LA) of each sample are indicated. Levels above the normal ranges (aCL antibody-IgG, 18.5 IgG phospholipid; anti-β2GPI antibody-IgG, 2.2 U; LA, 1.3) are indicated in bold underlined numbers. N.D., not detected. Data are representative of at least 3 independent experiments.

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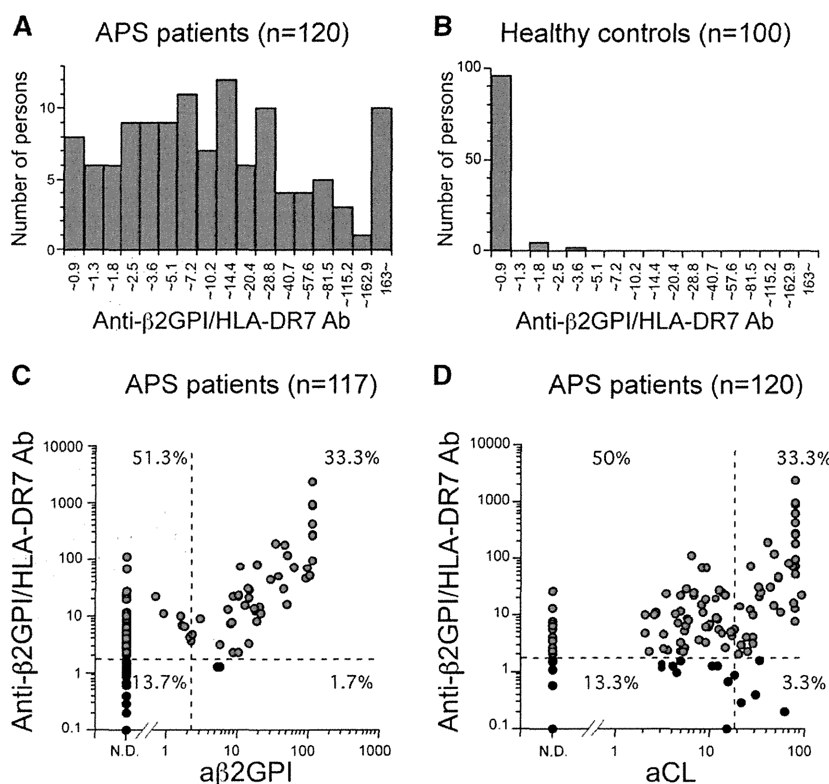

Figure 4. Autoantibodies against β2GPI/HLA class II complex are detected in most APS patients. (A-B) Distribution of serum anti-β2GPI/HLA-DR7 complex antibody titers in APS patients and healthy controls. Anti-β2GPI/HLA-DR7 complex antibody titers higher than the normal upper limit for anti-β2GPI/HLA-DR7 complex antibody titers established by using 100 healthy controls (1.8 U) are indicated as red bars. (C-D) Correlations between serum anti-β2GPI/HLA-DR7 complex antibody titers and serum anti-β2GPI antibody or aCL antibody titers in APS patients. The normal upper limits for anti-β2GPI antibody, aCL antibody, and anti-β2GPI/HLA-DR7 complex antibody titers are shown as dashed lines. Patients whose anti-β2GPI/HLA-DR7 complex antibody titers are higher than the normal upper limit are indicated as red circles. Data are representative of at least 3 independent experiments.

Discussion

Although HLA class II molecules are well recognized to present peptide antigens to T cells, recently we found that ER misfolded proteins are transported to the cell surface by HLA class II molecules when they are associated with the peptide-binding groove of HLA class II molecules.[32] Furthermore, intact IgG heavy chain is transported to the cell surface by HLA class II molecules via association with the peptide-binding groove, and IgG heavy chain/HLA class II complexes are recognized by autoantibodies in rheumatoid factor–positive sera from RA patients.[33] In contrast, autoantibodies in rheumatoid factor–positive sera from non-RA individuals did not bind to IgG heavy chain/HLA class II complexes, suggesting that IgG heavy chain complexed with HLA-DR is a specific target for autoantibodies from RA patients. Of note, a strong correlation between autoantibody binding to IgG complexed with certain HLA-DR alleles and the odds ratio for association of these alleles with RA was observed.[33] These findings suggested that misfolded ER proteins complexed with certain HLA class II alleles might affect susceptibility to other autoimmune diseases as a specific target for autoantibodies.

Here, we found that intact β2GPI protein, not peptide, is also transported to the cell surface by HLA class II molecules. Because both ends of the peptide-binding groove of HLA class II molecules are open, it is structurally possible that large proteins, including

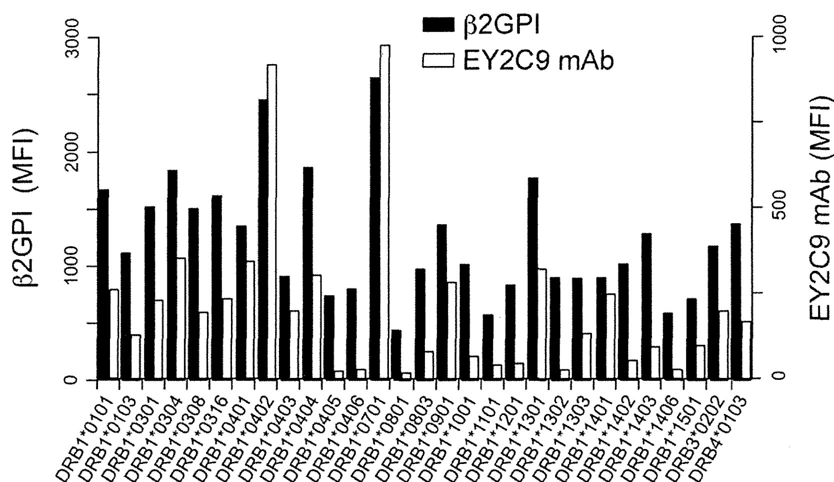

Figure 5. aPL antibody binds to β2GPI complexed with different HLA-DR alleles. N-terminal His-tagged β2GPI and GFP were cotransfected into 293T cells together with different HLA-DR alleles. The transfectants were stained with anti-His mAb (β2GPI, solid bars) or aPL antibody (EY2C9 mAb, open bars), followed by APC-labeled anti-mouse IgG or anti-human IgM Ab, respectively. MFIs of APC on GFP-positive cells are shown. Data are representative of 3 independent experiments.

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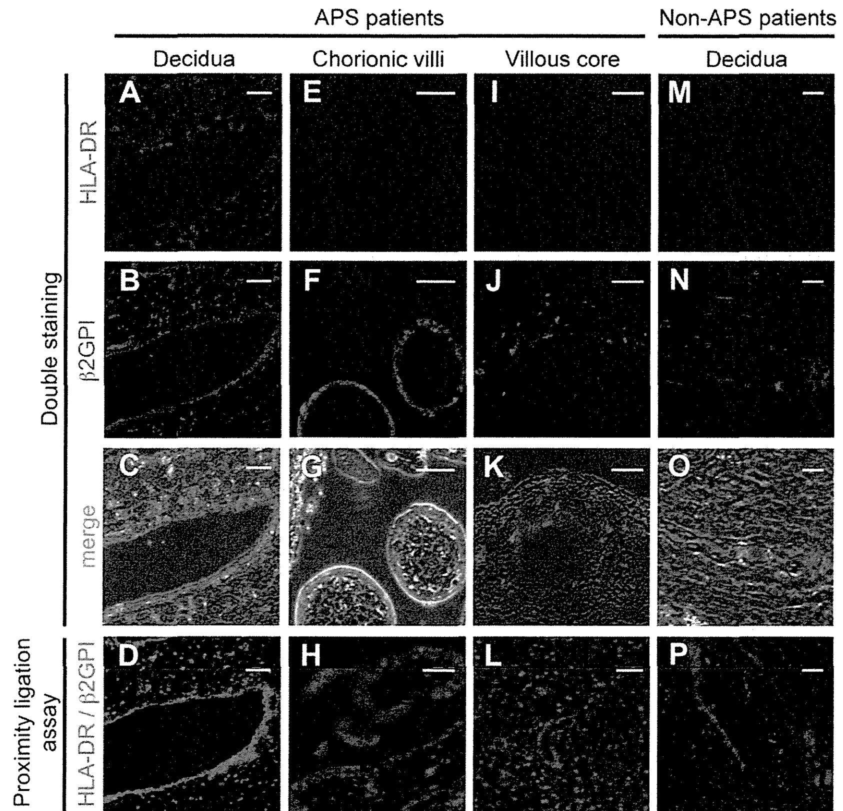

Figure 6. β2GPI is complexed with HLA-DR in endothelial cells of vessels in uterine decidual tissues obtained from APS patients. (A-C, E-G, I-K, M-O) β2GPI and HLA-DR are coexpressed in endothelial cells of vessels in uterine decidual tissues of APS patients, but not patients without APS. Tissue sections of (A-D) uterine decidua, (E-H) chorionic villi, and (I-L) villous core from APS patients and (M-P) decidua from APS-free patients were costained with anti-HLA-DR Ab (red, A, E, I, M) and anti-β2GPI Ab (green, B, F J, N). The images were merged to show colocalization of β2GPI and HLA-DR (C, G, K, O). PLA signals (red) between HLA-DR and β2GPI were analyzed in tissues of APS patients and those without APS (D, H, L, P). Scale bars, 50 μm. Data are representative of 3 independent experiments.

β2GPI, associate with the peptide-binding groove of HLA class II molecules. Although the structure of β2GPI complexed with HLA class II molecules has not yet been determined, it is likely that linear epitopes exposed on misfolded or structurally altered β2GPI proteins associate with HLA class II molecules, because all of the proteins that we have shown to associate with HLA class II molecules are not correctly folded proteins.[32,33] It is noteworthy that 83.3% of APS patients, including aCL-IgG antibody–negative and anti-β2GPI-IgG antibody–negative patients, were found to possess autoantibodies against β2GPI complexed with HLA class II molecules in the absence of phospholipids. In contrast, autoantibodies against β2GPI complexed with HLA class II molecules were rarely detected in healthy individuals. These results suggest that β2GPI/HLA-DR complexes are a major target in APS and that anti-β2GPI/HLA-DR complex autoantibodies might be a novel and useful diagnostic marker for APS.

β2GPI is a serum lipoprotein produced mainly by hepatocytes, although some endothelial cells of blood vessels and placental villous tissue also express it.[12,13] A circular conformation of β2GPI in plasma is changed to linear conformation by association with negatively charged phospholipids or negatively charged plates, resulting in the exposure of an epitope visible to aPL antibodies.[14-18] Because misfolded cellular proteins, but not correctly folded proteins, are transported to the cell surface by HLA class II molecules,[32] β2GPI complexed with HLA class II molecules appears to exhibit a similar linear conformation and is thus recognizable by autoantibodies from APS patients. The significant correlation between anti-β2GPI/HLA-DR antibody titers and anti-CL antibody or anti-β2GPI

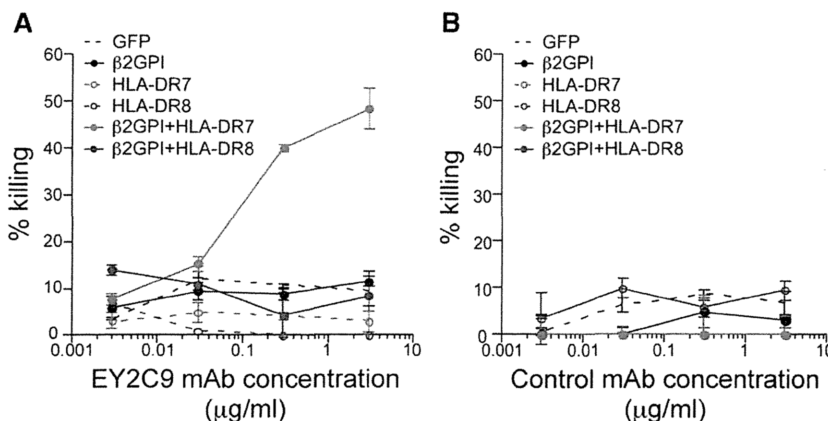

Figure 7. APL antibodies exert complement-mediated cytotoxicity against cells expressing β2GPI/HLA-DR7 complexes. Complement-mediated cytotoxicity by (A) aPL antibody (EY2C9 mAb) or (B) control mAb against cells transfected with β2GPI and HLA-DR7 or HLA-DR8. Percentage specific cytotoxicity is shown as mean + SD of triplicates. Data are representative of 3 independent experiments.

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antibody titers suggests that there are common autoantibody epitopes shared between β2GPI/HLA-DR complexes and β2GPI bound to phospholipid on negatively charged plates. However, about half of APS patients (whose autoantibody titers against β2GPI bound to phospholipid or negatively charged plates were within normal range) possess autoantibodies that bind to β2GPI/HLA-DR7 complexes. These results indicate that β2GPI/HLA-DR7 complexes also possess unique autoantibody epitopes for APS that are not present on phospholipid-bound β2GPI or plate-bound β2GPI. It has been reported that domains IV and V of β2GPI are involved in EY2C9 mAb binding.[48,49] Conversely, mutational analyses of domain I suggested that domain I is also involved in EY2C9 mAb recognition of β2GPI.[50] Our analyses of domain I deletion mutants also suggest that domain I plays an important role in recognition of β2GPI/HLA-DR complexes by EY2C9 mAb. These observations support our hypothesis that pathogenic epitopes on β2GPI that are recognized by autoantibodies in APS are exposed by association with HLA-DR.

Specific HLA-DR alleles are associated with susceptibility to APS[24-27] and *DQB1*06:04/5/6/7/9-DRB1*13:02* haplotypes are also reported to be involved in APS susceptibility.[54] However, *DRA1*01:01/DRB1*13:02* transported β2GPI to the cell surface less efficiently than HLA-DR7 and HLA-DR4. Therefore, certain HLA-DP or HLA-DQ alleles that are closely linked to the *DRB1*13:02* haplotype or certain autoantigens for APS other than β2GPI, such as prothrombin,[54] might be involved in APS susceptibility caused by the *DQB1*06:04/5/6/7/9-DRB1*13:02* haplotypes.

The mechanisms of thrombosis production in patients with APS are not completely defined. aPL antibodies are thought to induce perturbation and/or damage in endothelial cells, which results in a prothrombotic and proinflammatory response and subsequently thrombosis.[7,8] It has been suggested that anti-β2GPI autoantibodies mediate nuclear factor κB–dependent activation of endothelial cells via annexin A2 and toll-like receptor 4, which form multiprotein complexes with β2GPI.[55,56] Conversely, a recent study suggested that activation of platelets by aPL antibodies is associated with thrombosis formation.[57] In these ways, aPL antibodies seem to be a major contributor to the pathogenesis of APS.[6-9] HLA class II expression is induced on endothelial cells after exposure to cytokines such as IFN-γ and TNF-α.[40,41] Although expression of β2GPI on endothelial cells has remained controversial, EY2C9 mAb bound to IFN-γ and TNF-α stimulated endothelial cells in the presence of exogenous β2GPI. Similarly, HLA-DR transfected cells were recognized by EY2C9 mAb in the presence of exogenous β2GPI. Therefore, cytokines produced in response to inflammatory stimuli, such as those resulting from viral infection, might induce the formation of β2GPI and HLA class II complexes on endothelial cells. Complement deposition on decidual endothelial cells is detected in APS patients and thus complement activation is suggested to be involved in the production of thrombosis and pregnancy morbidity in patients with aPL antibodies.[58-60] Therefore, complement-mediated cytotoxicity of endothelial cells expressing both β2GPI and HLA class II by autoantibodies, in addition to nuclear factor κB–dependent activation of endothelial cells, might target tissues affected by APS. This may explain why symptoms in some patients are mainly thrombosis, whereas others mainly suffer recurrent spontaneous abortion despite the presence of aPL antibodies in both pathologies.

Because misfolded proteins associated with MHC class II molecules efficiently stimulate antigen-specific B cells,[32] β2GPI complexed with HLA-DR might be involved in autoantibody production in APS patients. Furthermore, autoantibodies against β2GPI/HLA class II complexes are detectable in some patients with unexplained recurrent pregnancy loss who are negative for both aPL antibodies and lupus anticoagulant (K. Tanimura, H.Y., and H.A., unpublished observation). Therefore, the presence of autoantibodies against β2GPI/HLA-DR complexes might help to understand as yet uncharacterized immune disorders. Conversely, it is well known that aPL antibodies are often detected in patients with leprosy regardless of the absence of clinical manifestations of APS. Further studies are needed to determine whether these aPL antibodies are different from autoantibodies against β2GPI/HLA-DR complexes. In addition to those in APS and RA, autoantibodies in Graves' disease and Hashimoto thyroiditis also recognize self-antigens complexed with disease-susceptible HLA class II molecules (H.J., L.L.L., and H.A. unpublished observation), suggesting that self-antigens complexed with HLA class II molecules might be general targets for autoantibodies produced in many autoimmune diseases. Further analyses of misfolded protein transport by HLA class II molecules will help us better understand autoimmune diseases.

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Authorship

Contribution: K. Tanimura, H.J., N.A., S.M., and K.K. performed experiments and analyzed data; K.H. performed statistical analysis; Y.E., S.Y., T.H., K. Takasugi, K.O., I.K., T.A., and H.Y. collected and analyzed clinical samples; T. Suenaga, N.A., K.H., M.K., I.K., K.Y., T. Sasazuki, T.A., H.Y., and L.L.L. helped design the study and write the manuscript; H.A. designed the study and wrote the manuscript; and all authors discussed data.

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β2-Glycoprotein I/HLA class II complexes are novel autoantigens in antiphospholipid syndrome

Kenji Tanimura, Hui Jin, Tadahiro Suenaga, Satoko Morikami, Noriko Arase, Kazuki Kishida, Kouyuki Hirayasu, Masako Kohyama, Yasuhiko Ebina, Shinsuke Yasuda, Tetsuya Horita, Kiyoshi Takasugi, Koichiro Ohmura, Ken Yamamoto, Ichiro Katayama, Takehiko Sasazuki, Lewis L. Lanier, Tatsuya Atsumi, Hideto Yamada and Hisashi Arase

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ORIGINAL ARTICLE

Real-world practice of obstetricians in respect of assays for antiphospholipid antibodies

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Abstract

Objective. The international classification criteria (CC) for definite antiphospholipid syndrome (APS) recommend confirmation of the sustained presence, for at least 12 weeks, of both lupus anticoagulant (LA), as determined by aPTT and RVVT, and anti β2glycoprotein I (β2GPI) or anticardiolipin (aCL) IgG and/or IgM. However, it remains unclear whether obstetricians comply with the aforementioned CC for the diagnosis of APS in daily clinical practice. We performed a nationwide survey to examine the attitudes of Japanese obstetricians toward the use of assays for antiphospholipid antibodies (aPLs).
Methods. A questionnaire was sent to 2,700 obstetric facilities where maternity checkups are carried out. The types of assays conducted for aPLs, ascertainment of persistence of the antibodies for at least 12 weeks, and the cutoff points used for the assays were examined.
Results. Of the facilities surveyed, 61.5% carried out the assay(s) only once. In regard to the type of assay performed, 97.1% carried out the assay for aCL IgG and/or β2GPI-dependent aCL, while 67.9% performed the LA-aPTT and/or LA-RVVT assay. Only 8.8% carried out assays for both LA. As for the cutoff points used, 98% of the facilities used lower cutoff points described in the manufacturers' manuals rather than the cutoff values recommended in the CC.
Conclusion. Thus, only a limited number of facilities adhered precisely to the CC for the diagnosis of APS. Inappropriate treatment and unnecessary expense are potentially major concerns when facilities overdiagnose APS using lower cutoff points or without ascertaining the persistence of the antibodies for at least 12 weeks. On the other hand, some patients miss the opportunity to be treated for APS because of the absence of testing for LA.

Keywords

Antiphospholipid antibody, lupus anticoagulant, obstetric antiphospholipid syndrome, recurrent pregnancy loss, survey

History

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Introduction

Established causes of recurrent pregnancy loss (RPL) include the presence of antiphospholipid antibodies (aPLs), uterine anomalies, abnormal chromosomes, particularly translocations in either partner, and abnormal embryonic karyotype [1–5]. Antiphospholipid syndrome (APS) is the most important treatable etiology. The reported live birth rate in women with APS treated with low-dose aspirin plus heparin is 70–80% [6–8].

According to the International classification criteria (CC) for definite APS by the International congress on aPLs, patients with two positive test results, spaced at least 12 weeks apart, for lupus anticoagulant (LA) as determined by activated partial thromboplastin time (LA-aPTT) and dilute Russell viper venom time (LA-RVVT), and anti β2glycoprotein I (β2GPI) and anticardiolipin (aCL) IgG and/or IgM can be diagnosed as having APS [9]. No definite recommendation can be given on the assays of choice for LA testing. However, while both LA-aPTT and LA-RVVT are suitable for the assay of LA, the CC for the diagnosis of APS recommend the use of two tests with different principles, because no single test is 100% sensitive for LA [10]. Thus, we currently measure LA by 5x diluted aPTT established in our laboratory, commercially available LA-RVVT, and β2GPI-dependent aCL (β2GPI aCL) assay as our standard aPL tests in daily clinical practice. In our previous study carried out in 1676 patients with RPL, the estimated prevalence of use of all the three assays for the diagnosis of APS was only 4.5% [3].

Few facilities measure aPLs in their own laboratories in Japan, with most facilities sending their samples to some central laboratories. There are many kinds of commercially available assays for aPLs, including antiphosphatidylethanolamine antibody (aPE) and antiphosphatidylserine antibody (aPS) assays, which are not included in the CC. Furthermore, the central laboratories use a lower cutoff value of 10 IU/ml for aCL IgG and IgM stated in the manufacturer's manual rather than the 99th percentile of the values in healthy controls recommended in the CC. It is speculated that physicians use the reports from these central laboratories for clinical decision-making without referring to the CC.

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There has been no survey conducted to determine the attitudes of obstetricians for the examination of aPLs in clinical practice. Therefore, we conducted the present survey to examine what types of assays for aPLs and what cutoff values for these assays obstetricians use in clinical practice. This is the first survey to examine real-world practice vis-à-vis the CC for definite APS.

Methods

Participant recruitment

We conducted a nationwide survey between August and September 2013. The primary questionnaire was sent to the heads of 2700 obstetric facilities in Japan where regular maternity checkups are carried out, and whose addresses are available with the Japan Society of Obstetrics and Gynecology.

Questionnaire

The real questionnaire consisted of the six items and the classification criteria for definite antiphospholipid syndrome (Table 1).

A: The type of facilities.
B: The number of pregnant women with a history of two or more pregnancy losses.
C: The number of patients with APS.
D: The assays for aPLs in patients with a history of recurrent pregnancy loss, and the cutoff values.
E: The tests to determine the presence of thrombophilia in patients with a history of recurrent pregnancy loss, and the cutoff points.
F: The confirmation of persistence of positive aPLs.

Table 1. The questionnaire consisted of the following items and the classification criteria for definite antiphospholipid syndrome.

Survey for the management of pregnancy complicated with antiphospholipid syndrome

A: What is the type of place you practice at?
University hospital, general hospital, clinic handling deliveries, clinic not handling deliveries.
B: How many pregnant women with a history of two or more pregnancy losses do you treat per year at your facility?
C: How many patients with APS do you treat?
D: Do you perform the following assays for aPLs in patients with a history of recurrent pregnancy loss, and what cutoff values do you use?

Anticardiolipin antibody IgG (Mesacup)	Yes, using the cutoff values provided by the company (10.0IU)	Yes, using other cutoff points ()	No
Anticardiolipin antibody IgM	Yes (10.0IU)	Yes ()	No
β2 glycoprotein I-dependent anticardiolipin IgG (Yamasa)	Yes (3.5IU)	Yes ()	No
LA-RVVT (Gradipore)	Yes (1.3)	Yes ()	No
LA-aPTT (StaClot LA)	Yes (6.3)	Yes ()	No
PTT-LA test	Yes (55.5)	Yes ()	No
Antiphosphatidylethanolamine (aPE) IgG	Yes (0.32)	Yes ()	No
aPE IgM	Yes (0.44)	Yes ()	No
Antiphosphatidylserine antibody	Yes	Yes ()	No
Phosphatidylserine-dependent antiprothrombin IgG	Yes (1.2)	Yes ()	No
Others	Yes ()		No

E: Do you perform the following tests to determine the presence of thrombophilia in patients with a history of recurrent pregnancy loss, and what cutoff points do you use?

Protein S antigen	Yes, using the cutoff values provided by the company (65%)	Yes, using other cutoff points	No
Protein S activity	Yes (60%)	Yes ()	No
Antithrombin antigen	Yes (23.6 mg/dl)	Yes ()	No
Antithrombin activity	Yes (80%)	Yes ()	No
Protein C antigen	Yes (70%)	Yes ()	No
Protein C activity	Yes (64%)	Yes ()	No
Factor XII activity	Yes (50%)	Yes ()	No

F: Do you carry out the assay(s) for antiphospholipid antibodies at two time-points spaced 12 weeks apart according to the guideline of the International Congress on Antiphospholipid Antibodies?

- Yes, test at two time-points12 weeks apart.
- Test twice, but at two time-points less than 12 weeks apart.
- No, test only once.

The classification criteria for definite antiphospholipid syndrome

Clinical criteria

1. Vascular thrombosis
2. Pregnancy morbidity

a. One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, with normal fetal morphology documented by ultrasound or by direct examination of the fetus, or
b. One or more premature births of a morphologically normal neonate before the 34th week of gestation because of: (i) eclampsia or severe pre-eclampsia defined according to standard definitions, or (ii) recognized features of placental insufficiency, or
c. Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.

Laboratory criteria

1. Anticardiolipin (aCL) antibody of IgG and/or IgM isotype, present in medium or high titer (i.e., >40 GPL or MPL, or >the 99th percentile), measured by a standardized ELISA.
2. Anti-β_2 glycoprotein-I antibody of IgG and/or IgM isotype (in titer > the 99th percentile), measured by a standardized ELISA.
3. Lupus anticoagulant (LA) present in plasma.

On two or more occasions, at least 12 weeks apart

Anticardiolipin antibody IgG, IgM (Mesacup, Medical & Biological laboratories co., LTD, Nagano, Japan) [11], β2glycoprotein I-dependent anticardiolipin IgG (β2GPI aCL, Yamasa Corp., Choshi, Japan) [12], LA-RVVT (Gradipore Ltd, Pyrmont, Australia), LA by diluted aPTT with the mixing test and neutralizing test using the StaClot LA kit (Diagnostica Stago), LA by diluted aPTT (PTT-LA test; Diagnostica Stago), aPE IgG, IgM [13], aPS, and phosphatidylserine-dependent antiprothrombin (aPS/PT) assay for IgG, IgM [14] were included in the questionnaire because these tests were carried out in the central laboratories.

The analysis was carried out using the SPSS for Windows, Version 19.0 (SPSS 19.0; IBM, Chicago, IL, USA). The study was conducted with the approval of the Research Ethics Committee at Nagoya City University Medical School.

Results

A total of 829 facilities (829/2700; 30.7%) returned their responses to the questionnaire, including 59 University hospitals, 369 general hospitals, 374 clinics handling deliveries, and 29 clinics not handling deliveries.

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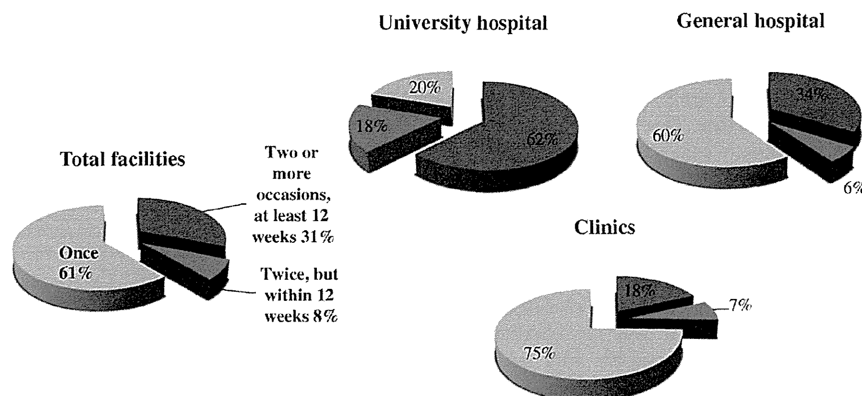

Figure 1. The distribution of facilities to ascertain persistence of aPLs, twice at least 12 weeks apart, twice but within 12 weeks, and only once in 559 facilities which answered to examine assays at least once.

In regard to testing for the persistence of aPLs for at least 12 weeks apart, only 30.8% (172 of 559) of the facilities reported carrying out the assays for aPLs at two time-points spaced at least 12 weeks apart, in compliance with the CC (Figure 1), 7.7% (43) carried out the assays at two time-points, but less than 12 weeks apart, and 61.5% (344) carried out the assay(s) at only one time-point.

When classified by the type of facility, 62.5% of university hospitals, 34.0% of general hospitals, and 18.3% of clinics carried out the assays for aPLs at two time-points more than 12 weeks apart, in compliance with the CC. Significantly more university hospitals complied with the CC than general hospitals and clinics ($p < 0.001$), and significantly more general hospitals complied with the CC than clinics ($p < 0.001$).

Only 33.5% of the facilities (182/544) used aPTT for the assay of LA, 17.8% (97) performed only PTT-LA, which is the mixing test, and 11.8% (64) used only LA-aPTT StaClot, which is the confirmatory test. Of 544 facilities, 43.2% (235/544) used LA-RVVT, while only 9.4% (51/544) used both aPTT and RVVT for LA assay.

The frequency of use of each assay for aPLs at the 559 facilities that had carried out the assays at least once is shown in Figure 2. Of the total, 97.1% of the facilities (529/545) carried out the test for aCL IgG and/or β2GPI aCL; 63.3% (345/545) used both assays even though the two are based on the similar principle, 11.7% (64) carried out only the test for aCL IgG, and 22.0% (120) carried out only the test for β2GPI aCL. 2.9% (16) did not conduct either of the assays, even though these are included in the CC.

The following are the percentages of institutions that used different cutoff points from those recommended by the respective manufacturers: 2.0% (8/410) for aCL IgG, 11.8% (28/237) for aCL IgM, 1.9% (9/467) for β2GPI-dependent aCL, 2.4% (2/85) for LA-aPTT StaClot, and 1.7% (2/119) for PTT-LA.

In regard to the test(s) for thrombophilia, 33.7% (164/487), 43.9% (214/487), 8.6% (42/489), 40.4% (197/488), 26.0% (127/488), 43.7% (213/488), and 41.6% (204/490) of the facilities carried out assays for the PS antigen, PS activity, AT antigen, AT activity, PC antigen, PC activity, and factor XII activity, respectively (Figure 3).

Discussion

The clinical guideline of the Japan Society of Obstetrics and Gynecology and Japan Association of Obstetricians and Gynecologists has been demonstrating the guideline by the CC [15]. Thus, all obstetricians can have access to the CC. However, it was found that only a limited number of facilities complied with the CC for the diagnosis of APS.

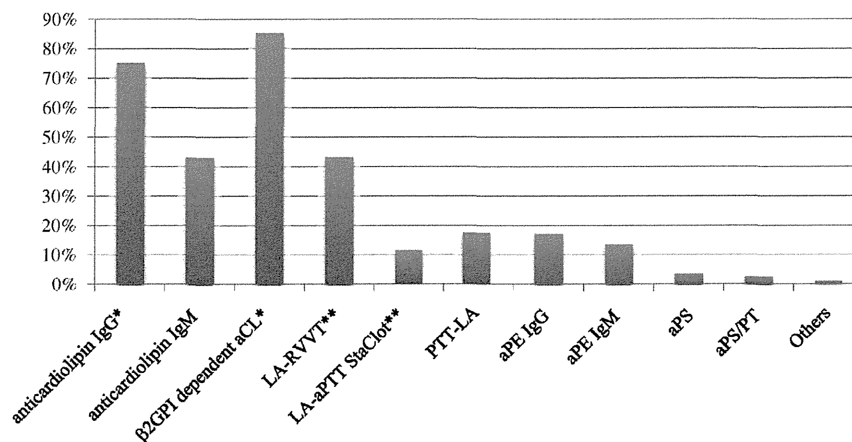

Figure 2. The frequency of use each of the assays for antiphospholipid antibodies at 559 facilities that carried out the assay(s) at least once. *The national health insurance covers any one of aCL IgG or β2GPI-dependent aCL. **The national health insurance covers any one of LA-RVVT or LA-aPTT StaClot.

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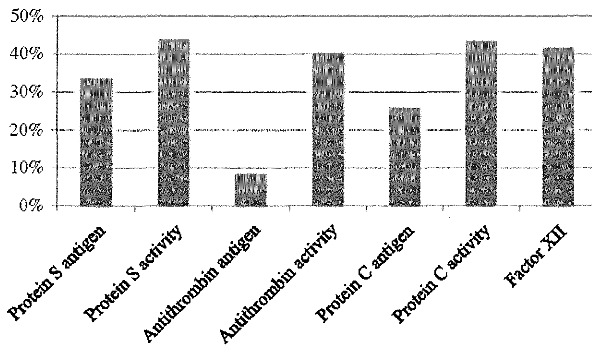

Figure 3. The frequency of assays for thrombophilia examined in 561 facilities which answered to examine assays for antiphospholipid antibody at least once.

Of the facilities surveyed, 61.5% carried out the assay(s) for aPLs only once. Significantly more general hospitals and clinics than university hospitals failed to comply with the CC. The maternal age has been increasing year by year in Japan, and it would appear that it is stressful for relatively elderly patients and clinicians to wait for more than 12 weeks to confirm persistence of the antibodies. Both patients and clinicians might wish for any chance of treatment. It can therefore be speculated that many patients receive medication at once after a positive test result for aPLs. Furthermore, clinicians might prefer the assays with relatively higher prevalence such as aCL or aPE. However, informed consent is important, because patients might not have the knowledge that it is necessary to ascertain persistence of the antibodies because of possible false-positives, and 70% of patients with RPL of unexplained etiology have live births without treatment [16,17].

Most of facilities used not the 99th percentile of the values in healthy controls recommended by the CC, but lower cutoff values either described in the kits or established by the central laboratories. The approved aCL kit and Gradipore kit used in one major laboratory recommend cutoff values of 10 IU/ml and 1.1 for CL IgG and LA-RVVT, respectively, which are lower than the 99th percentile [15].

Only 33.5% of facilities carried out testing for LA using aPTT, 11.8% used only LA-aPTT StaClot, which is a confirmatory test, and only 8.8% used both aPTT and RVVT for the LA assay. LA is well-known to be better correlated with pregnancy morbidity than aCL [10]. The PROMISS study concluded that LA, but not classical aCL, was a predictor of adverse pregnancy outcomes [18]. Harris et al. also confirmed that classical CL IgG and IgM were rarely associated with adverse pregnancy outcomes [19]. Both aPTT and RVVT are suitable for assay of LA, and two tests with different assay principles is recommended [9,10]. Therefore, a combination of aPTT-based LA and dRVVT-based LA could be used in daily clinical practice.

This might be influenced by the national health insurance system in the Ministry of Health, Labour and Welfare of Japan. The national health insurance covers all people in Japan. Either of LA-aPTT StaClot or LA-RVVT can be covered by the insurance. All expense for examination of RPL cannot be covered by the insurance if both tests were performed. It is important that we appeal the Ministry of Health, Labour and Welfare to realize the CC.

Of the facilities surveyed, 97.1% carried out the assay for aCL IgG or β2GPI aCL, which is in line with the recommendations in the CC. However, 63.3% carried out both the assays, even though the two are similar in principle. The assay for β2GPI aCL was the most frequently carried out, because this assay was approved first and because our earlier study demonstrating the predictive value of β2GPI aCL for adverse pregnancy outcome was widely known in Japan (Figure 2) [12,20]. The prevalence of β2GPI aCL is, however, relatively small (2.3%) [21], and clinicians might prefer assays for antibodies occurring at a higher prevalence, such as aCL IgG.

The prevalence of aPE IgG and IgM has been reported to be relatively high, being about 20% in patients with RPL [22]. Our previous cohort study showed that the distribution of aPE IgG was distinct from that of the conventional aPLs and that treatment could not improve the live birth rate in patients with a single positive test result [21].

In regard to assays for thrombophilia, our survey revealed that many facilities carry out assays for thrombophilia. Heritable thrombophilia has been reported to be associated with RM [23,24]. However, the clinical significance of examination for thrombophilia in patients with RPL is unclear, because no treatment has been established yet [5]. Examination for thrombophilia in women with RPL is not covered by the national health insurance in Japan. The health insurance covers one of the assays for LA-aPTT StaClot or LA-RVVT, and one of the assays for aCL IgG or β2GPI aCL (Figure 2).

In the present study, only a limited number of facilities were found to comply with the CC. It should be noted that both the patients and society would pay inappropriate expenses for unnecessary treatment in case of improper diagnosis of APS. In addition, some patients may miss the opportunity for treatment of APS as a result of failure to perform the assay(s) for LA. LA is the primary predictor of adverse pregnancy outcomes. In contrast, neither aCL nor anti-β GPI IgG/M predicts adverse pregnancy outcomes unless LA is proven to co-exist [18]. Live birth rate can be improved by combined therapy with low dose aspirin and heparin [7]. Patients as well as clinicians need to become more aware of the CC.

Acknowledgments

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Conflict of interest

None.

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PAPER

Primary prophylaxis to prevent obstetric complications in asymptomatic women with antiphospholipid antibodies: a systematic review

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Objective: Obstetric complications are common in patients with antiphospholipid syndrome. However, the impact of antiphosholipid antibodies (aPL) in the pregnancy outcomes of asymptomatic aPL carriers is uncertain. The aim of this systematic review is to assess whether primary prophylaxis is beneficial to prevent obstetric complications during pregnancy in asymptomatic women positive for aPL who have no history of recurrent pregnancy loss or intrauterine fetal death. **Methods:** Studies evaluating the effect of prophylactic treatment versus no treatment in asymptomatic pregnant aPL carriers were identified in an electronic database search. Design, population and outcome homogeneity of studies was assessed and meta-analysis was performed. The pooled Mantel–Haenszel relative risk of specific pregnancy outcomes was obtained using random effects models. Heterogeneity was measured with the I^2 statistic. All analyses were conducted using Review Manager 5.3. **Results:** Data from five studies involving 154 pregnancies were included and three studies were meta-analysed. The risk ratio and 95% confidence interval (CI) of live birth rates, preterm birth, low birth weight and overall pregnancy complications in treated and untreated pregnancies were 1.14 (0.18–7.31); 1.71 (0.32–8.98); 0.98 (0.07–13.54) and 2.15 (0.63–7.33),respectively. Results from the meta-analysis revealed that prophylactic treatment with aspirin is not superior to placebo to prevent pregnancy complications in asymptomatic aPL carriers. **Conclusion:** This systematic review did not find evidence of the superiority of prophylactic treatment with aspirin compared to placebo or usual care to prevent unfavourable obstetric outcomes in otherwise healthy women with aPL during the first pregnancy. *Lupus* (2015) **24**, 1135–1142.

Key words: Pregnancy morbidity; obstetric outcome; aspirin; lupus anticoagulant; anticardiolipin antibodies

Introduction

Antiphospholipid antibodies (aPL) are autoantibodies directed against phospholipid binding proteins including lupus anticoagulant, detected by clotting assays and anticardiolipin or anti-β2glycoprotein I antibodies, measured by enzyme linked immunosorbent assays. The occurrence of recurrent thrombotic events and/or pregnancy morbidity in conjunction with persistent presence of aPL, at least 12 weeks apart, defined the antiphospholipid syndrome (APS).[1]

Antiphospholipid antibodies can be found in otherwise healthy subjects with an estimated prevalence ranging from 1% to 5%.[2] In patients with systemic lupus erythematosus (SLE), aPL can be detected in up to 40% of patients, but only one third develop clinical manifestations of APS.[3] In addition, aPL have been observed during infections, after vaccination and as a reaction to several drugs; in these cases, the antibodies are usually transient, at low titre, and without clear association with clinical manifestations of APS.[4,5]

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Poor obstetric outcome is one of the manifestations of APS. According to the international criteria, obstetric APS includes three or more early miscarriages, one or more intrauterine fetal deaths and preterm births caused by pre-eclampsia or placental insufficiency.[1] Furthermore, intrauterine growth restriction and HELLP syndrome (haemolytic anaemia, elevated liver enzymes and low platelet count) have been observed in aPL-positive women.[6–8] A meta-analysis demonstrated the superiority of the heparin and aspirin combination with aspirin alone in achieving more live births in patients with aPL and recurrent pregnancy loss.[9]

Positive aPL can be recognized through the screening of otherwise asymptomatic pregnancies.[10] The impact of the type of aPL and the evolution of aPL titres during the pregnancy is, however, poorly understood.

The presence of aPL in high-risk pregnancies may be associated with elevated probability of further pregnancy loss in untreated patients.[11] On the other hand, it has yet to be established whether asymptomatic aPL carriers are at higher risk of pregnancy complications during the first pregnancy.

Clinicians encountering asymptomatic pregnant women with aPL have to consider whether prophylactic therapy should be used to prevent obstetric complications. The answer to this question is a matter of controversy, which largely stems from expert opinions. If no clear benefit exists from prophylactic therapy for these women, the screening of aPL should be avoided.

The aim of this systematic review is to assess whether prophylaxis with aspirin and/or heparin would be beneficial to prevent obstetric complications during the first pregnancy in women with aPL without a history of clinical manifestations of APS.

Methods

We performed a systematic review and meta-analysis of studies in which any prophylactic therapy was tested in terms of pregnancy outcomes in pregnant women with positive aPL and no previous diagnosis of APS. The literature search was performed in the major databases: Medline from 1950 via PubMed, Embase and the Cochrane Central Register of Controlled Trials (search completed 28 February 2014). The search strategy combined free text search, exploded medical subject headings (MESH/EMTREE) terms and all synonyms of the following MESH terms to identify relevant published articles: 'antiphospholipid syndrome', 'antiphospholipid antibodies,' 'anticardiolipin' antibodies', 'lupus anticoagulant', 'lupus coagulation inhibitor', 'pregnancy', 'pregnant', 'prophylaxis', 'prevention', 'heparin', 'aspirin', 'low dose aspirin', 'antithrombocytic agent', 'platelet aggregation inhibitor', 'anticoagulant' and 'fibrinolytic agent' (see Appendix 1, online supplementary material, for full details of the search strategy). In addition, we searched conference abstracts from January 2012 to July 2014 on the official webpages of the American College of Rheumatology (ACR), the European League against Rheumatism (EULAR), the International Society of Thrombosis and Haemostasis (ISTH), the American College of Obstetricians and Gynaecologists (ACOG), the European Congress of Obstetrics and Gynaecology (EBCOG) and the Royal College of Obstetricians and Gynaecologists World Congress (RCOG).

Studies were selected for the review if they met the following criteria: (1) at least one group of subjects were pregnant women with aPL without APS; (2) pregnancy outcomes were reported (live birth, preterm birth, pre-eclampsia, fetal death, low birth weight, and miscarriage); (3) prophylactic treatment was evaluated; (4) the study was a clinical trial or a cohort study of low risk of bias. Studies with abstracts in languages other than English, Spanish or Japanese were excluded.

Two investigators (OA and DF) independently screened titles and abstracts identified in the database search against the selection criteria. All discrepancies were resolved by consensus. When consensus could not be reached, OA made the final judgement for study eligibility. The full text of the selected studies was then retrieved and data were extracted in ad hoc data extraction forms.

Risk of bias for two observational studies was examined using a pre-specified scale based on the Newcastle–Ottawa scale[12] containing the following items: (1) selection of patients: low if patients were very representative of patients with aPL without previous pregnancy; high if patients were selected (such as those who did not have a poor outcome, as can be found in a case report); (2) case definition: low if aPL definition is clear; (3) randomization: low only if patients were randomly assigned to prophylactic alternatives; (4) adjustment: low if analyses were adjusted for important prognostic and confounding factors, or if groups were randomly assigned at the start of trial; and (5) detection: low if follow-up was sufficiently long and complete to assure that all outcomes were registered and blinded to the group assignment in

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trials. Risk of bias assessment for three randomized controlled trials (RCTs) was performed using the Cochrane risk of bias tool as described in the Cochrane Handbook[13] and assessed the following domains: (a) random sequence generation; (b) allocation concealment; (c) blinding of participants and personnel; (d) blinding of outcome assessment; (e) incomplete outcome data; (f) selective outcome reporting, and (g) other bias.

Studies were compiled into an evidence table. Meta-analysis was performed after assessment of the homogeneity of designs, populations and outcomes. The grouped effect size selected was the pooled Mantel–Haenszel relative risk of specific pregnancy outcomes obtained from random effects models. Heterogeneity was measured with the I^2 statistic. Subgroup analysis was conducted by study design (RCTs versus retrospective cohort study) to assess interaction between study designs. All analyses were conducted using Review Manager version 5.3.

Results

The search strategy identified 3328 studies (Figure 1). After initial screening, 58 articles were selected by consensus for detailed assessment. Manuscripts were further assessed for eligibility and 53 were excluded, as they did not meet the inclusion criteria (see Appendix 2, online supplementary material, for a list of studies with reasons for exclusion). Finally, five articles were included for qualitative synthesis.[14–18] Table 1 gives a brief overview of these studies. Risk of bias in all studies was moderate to good (Figure 2). Risk of bias assessment for three RCTs was performed using the Cochrane risk of bias tool and showed low to unclear risk of bias (Appendix 3).

A total of 154 pregnancies were evaluated. Prophylactic treatment was administered in 92 pregnancies; in 98% of cases, women were treated with a low dose of aspirin. Only two treated pregnancies did not include aspirin; a woman received prednisone and another woman received prednisone plus heparin.[15] In 62 pregnancies, women did not receive any prophylactic treatment. Some pregnant women included in this review did not have a history of previous pregnancy,[15,18] some women had a history of full-term deliveries,[15] and for other women a clearly detailed obstetric history was lacking.[14,16–18]

In four out of the five selected studies, differences in obstetric outcomes between treated and

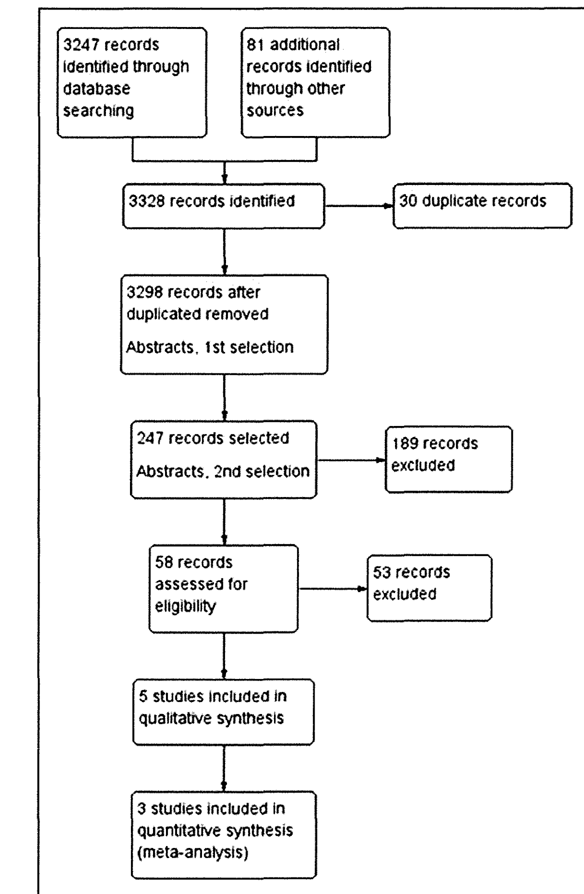

Figure 1 Flow chart of literature search. Other sources include results of the abstracts and guideline search of the official conference webpages, described in the Methods section.

untreated pregnancies were not statistically significant (Table 2).[14,16–18] In the study by Julkunen et al.,[15] which evaluated 16 pregnancies in aPL carriers with SLE, obstetric complications were observed in 100% of the untreated pregnancies in comparison with 25% in the treated group (Table 3).

Three studies including aPL-positive asymptomatic women without SLE were meta-analysed.[16–18] Although two of the studies were randomized trials and one was observational, we considered that there was enough similarity and the outcomes were hard enough not to be influenced by the design. Pregnancy outcomes were evaluated in a total of 132 pregnancies, and the results are shown in Figure 3. The risk ratio (RR) of live birth rates in treated and untreated pregnancies was 1.14 (95% confidence interval (CI) 0.18 to 7.31, 132 women, I^2=0%) (Figure 3(a)). The outcome of overall pregnancy complications was not

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