ARTICLE

New insight into antiphospholipid syndrome: antibodies to $\beta_2$glycoprotein I-domain 5 fail to induce thrombi in rats

Paolo Durigutto,[1] Claudia Grossi,[2] Maria Orietta Borghi,[2,3] Paolo Macor,[1] Francesca Pregnolato,[2] Elena Raschi,[2] Michael P. Myers,[4] Philip G. de Groot,[5] Pier Luigi Meroni[2] and Francesco Tedesco[2]

[1]Department of Life Sciences, University of Trieste, Italy; [2]Istituto Auxologico Italiano, IRCCS, Laboratory of Immuno-Rheumatology, Milan, Italy; [3]Department of Clinical Sciences and Community Health, University of Milan, Italy; [4]International Centre for Genetic Engineering and Biotechnology, Trieste, Italy and [5]Department of Clinical Chemistry and Haematology, University of Utrecht, University Medical Center Utrecht, the Netherlands

PD and CG contributed equally to this work. PLM and FT contributed equally to this work.

Ferrata Storti Foundation

Haematologica 2019
Volume 104(4):819-826

ABSTRACT

Clinical studies have reported different diagnostic/predictive values of antibodies to domain 1 or 4/5 of $\beta_2$glycoproteinI in terms of risk of thrombosis and pregnancy complications in patients with antiphospholipid syndrome. To obtain direct evidence for the pathogenic role of anti-domain 1 or anti-domain 4/5 antibodies, we analyzed the *in vivo* pro-coagulant effect of two groups of 5 sera IgG each reacting selectively with domain 1 or domain 5 in lipopolysaccharide (LPS)-treated rats. Antibody-induced thrombus formation in mesenteric vessels was followed by intravital microscopy, and vascular deposition of $\beta_2$glycoproteinI, human IgG and C3 was analyzed by immunofluorescence. Five serum IgG with undetectable anti-$\beta_2$glycoproteinI antibodies served as controls. All the anti-domain 1-positive IgG exhibited potent pro-coagulant activity while the anti-domain 5-positive and the negative control IgG failed to promote blood clot and vessel occlusion. A stronger granular deposit of IgG/C3 was found on the mesenteric endothelium of rats treated with anti-domain 1 antibodies, as opposed to a mild linear IgG staining and absence of C3 observed in rats receiving anti-domain 5 antibodies. Purified anti-domain 5 IgG, unlike anti-domain 1 IgG, did not recognize cardiolipin-bound $\beta_2$glycoproteinI while being able to interact with fluid-phase $\beta_2$glycoproteinI. These findings may explain the failure of anti-domain 5 antibodies to exhibit a thrombogenic effect *in vivo*, and the interaction of these antibodies with circulating $\beta_2$glycoproteinI suggests their potential competitive role with the pro-coagulant activity of anti-domain 1 antibodies. These data aim at better defining "really at risk" patients for more appropriate treatments to avoid recurrences and disability.

Introduction

Antiphospholipid syndrome (APS) is a chronic autoimmune disorder characterized by recurrent episodes of vascular thrombosis and adverse pregnancy outcomes in the presence of antibodies to phospholipid-binding proteins (aPL). It occurs either as a primary disease or concomitantly to other connective tissue diseases, particularly systemic lupus erythematosus (SLE).[1] Although thrombotic occlusion may affect the vessels of all organs and tissues, common presentations of the syndrome are: a) deep vein thrombosis in the legs often complicated by pulmonary embolism; and b) thrombotic occlusion of cerebral and coronary arteries leading to stroke and myocardial infarction.[2] This clinical condition is also associated with pregnancy morbidity, including fetal loss, pre-eclampsia, pre-term delivery, and 'small for gestational age' babies.[3] These are serious complications that

Correspondence:

FRANCESCO TEDESCO
tedesco@units.it

Received: May 22, 2018.

Accepted: November 14, 2018.

Pre-published: November 15, 2018.

doi:10.3324/haematol.2018.198119

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/104/4/819

particularly affect young people, and have both social and economic impacts. The disease may sometimes present as catastrophic syndrome, a more severe form of APS characterized by microthrombosis of small vessels in various organs resulting in multiple organ failure.[4] Anti-cardiolipin (aCL) and anti-$\beta_2$glycoprotein I (β_2GPI) antibodies and lupus anticoagulant (LA) activity are considered markers of APS and are included among the criteria currently proposed to classify the syndrome.[1] Clinical studies have revealed an increased risk of thrombosis and pregnancy complications in patients with medium to high levels of these antibodies and LA present in their plasma.[5] The triple positivity of these laboratory markers has also been shown to be associated with more severe forms of APS.[5] Conversely, the positivity for a single marker is often associated with a much lower risk of the clinical manifestations of APS.[5-9] It has been widely demonstrated that β_2GPI is the main antigen recognized by aPL, and the reactivity against the protein has been shown to be responsible for the positivity for aCL and anti-β_2GPI assays, and, in part, for the LA phenomenon strongly associated with the clinical manifestations of APS.[10] β_2GPI mainly circulates in blood in a circular form and is organized into four domains (D1-D4) composed of 60 amino acids with two disulfide bonds and a fifth domain (D5) containing an extra 24 amino acids that interact with anionic phospholipids on the target cells/tissues.[11] Besides the classical diagnostic assays measuring antibodies against whole molecule β_2GPI, new tests have recently been developed to detect anti-β_2GPI antibody subpopulations reacting with different domains of the protein, particularly the combined domains D4/5 and domain 1 (D1).[5-7,9,12-14]

In APS patients, a large proportion of anti-β_2GPI antibodies react with D1 and recognize a cryptic epitope (Arg39–Arg43) in the native molecule exposed after its interaction with anionic phospholipids[13,15] or oxidation.[16-18] Antibodies directed against D1 of β_2GPI with or without anti-D4/5 antibodies have frequently been found in APS patients associated with an increased risk of thrombosis and pregnancy complications.[7,9,19-24] In contrast, isolated high levels of anti-D4/5 antibodies have been reported in non-APS patients with leprosy, atopic dermatitis, atherosclerosis and in children born to mothers with systemic autoimmune diseases;[6] high levels have also been found in asymptomatic aPL carriers although these antibodies are not associated with either vascular or obstetric manifestations of the APS syndrome.[7,9] This finding prompted some authors to suggest that the ratio between anti-D1 and anti-D4/5 may be a useful parameter for identifying autoimmune APS and for ranking the patients according to their risk of developing the syndrome.[7]

An isolated positivity for anti-D4/5 is a rare condition and is usually associated with the absence of aCL and/or LA. In the majority of cases, there is some doubt as to the APS clinical profile and classification/diagnostic criteria are not fulfilled.[25] The finding that antibodies with this isolated specificity are observed mainly in the absence of clinical manifestations of hypercoagulable states has suggested that they may not be involved in thrombus formation.

The *in vivo* pathogenic role of aPL has been demonstrated for those directed against the whole molecule and against D1 of β_2GPI using animal models of thrombosis developed in rats and mice.[26-28] However, at present, there is no direct evidence that antibodies to D4/5 do not play an *in vivo* pathogenic role in blood clotting, nor is it clear whether they are able to interact with soluble or surface-bound β_2GPI. Data indicating that the antibodies are ineffective in causing blood clot due to their failure to recognize bound β_2GPI will be reported.

Methods

Serum source

Two groups of anti-β_2GPI positive sera[7,27] containing isolated antibodies to either D1 or D4/5 domains[6,7] and control sera with undetectable anti-β_2GPI antibodies were analyzed. All samples were also tested for aCL antibodies[7] and LA activity.[29] The anti-D1-positive sera were obtained from APS patients.[1] The sera were collected after obtaining informed consent and the IgG were purified by a Protein G column (HiTrap Protein G HP, GE Healthcare) as described.[27] The local Istituto Auxologico Italiano ethical committee approved the study.

Purification of $\beta_2$glycoprotein I and generation of recombinant domains D4 and D5

Methods of purification of human β_2GPI from pooled normal sera and the generation of D4 and D5 domains have been published previously.[12,27,30,31] Sequence analysis was performed as described[32] and compared to the published sequence of β_2GPI.[33] The fine specificity against D4 or D5 was investigated by ELISA.[27]

Animal model

An *in vivo* model of antibody-induced thrombus formation was established in male Wistar rats (270-300 g) kept under standard conditions in the Animal House of the University of Trieste, Italy, as previously reported in detail.[26] The *in vivo* procedures were performed in compliance with the guidelines of European (86/609/EEC) and Italian (Legislative Decree 116/92) laws and were approved by the Italian Ministry of University and Research and the Administration of the University Animal House. This study was conducted in accordance with the Declaration of Helsinki. Further details are available in the *Online Supplementary Methods.*

Immunofluorescence analysis

The mesenteric tissue was collected from rats at the end of the *in vivo* experiment.[26] Deposits of β_2GPI were analyzed using the biotinylated monoclonal antibody MBB2 and FITC-labeled streptavidin (Sigma-Aldrich).[27] IgG and C3 were detected using FITC-labeled goat anti-human IgG (Sigma-Aldrich) and goat anti-rat C3 (Cappel/MP Biomedicals) followed by FITC-labeled rabbit anti-goat IgG (Dako), respectively. The slides were examined using a DM2000 fluorescence microscope equipped with a DFC 490 photo camera and Application Suite software (Leica).

Antibody binding assays

Different concentrations of β_2GPI were added to CL-coated plates and the reactivity of IgG with CL-bound β_2GPI was measured.[7] The interaction of IgG with soluble β_2GPI was evaluated by incubating IgG with increasing concentrations of β_2GPI or bovine serum albumin (BSA) as unrelated antigen for one hour (h) at 37°C followed by overnight incubation at 4°C in a rotator. The samples were centrifuged at 3000 g for 5 minutes (min) at room temperature and the residual un-complexed antibodies were tested using β_2GPI-coated plates (Combiplate EB, Labsystems) as described.[7] Further details are available in the *Online Supplementary Methods.*

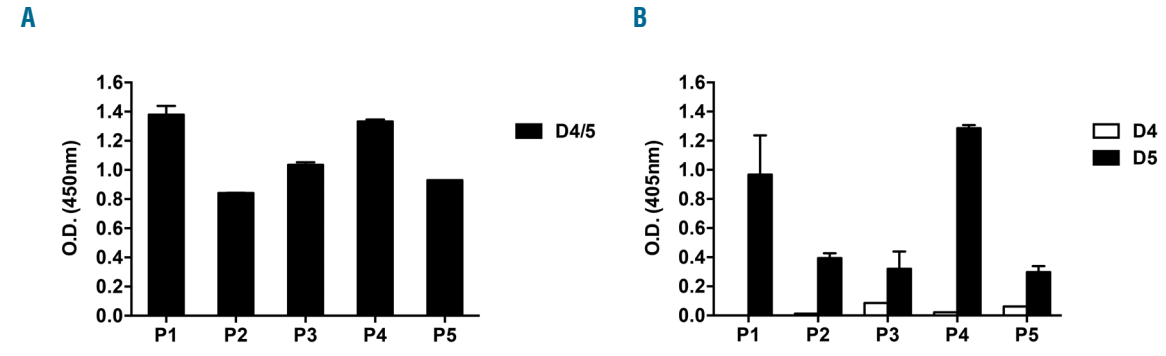

Figure 1. Anti-domain (D) 4/5 antibodies specifically react against domain (D) 5 of $\beta_2$glycoprotein I (β_2GPI). Reactivity of 5 anti-D4/5-positive patient sera (P1-P5) against different recombinant human β_2GPI domains. (A) Reactivity against the combined D4/5 peptides (▬), in an assay produced for research use (QUANTA Lite β_2GPI D4/5 ELISA, INOVA Diagnostics). (B) Reactivity against the recombinant domain D4 (▭) or D5 (▬) antigens separately immobilized on the wells of γ-irradiated polystyrene plates in in-house ELISA. Optical Density (O.D.) values are expressed as mean±Standard Deviation. Data were analyzed with the Student *t*-test for paired data. The average reactivity against D5 is significantly higher than that against D4 (P=0.0428).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 for Windows. The domain reactivity of the anti-β_2GPI D4/5 positive sera was expressed as mean+Standard Deviation (SD) and analyzed with the paired Student *t*-test. Data from *in vivo* thrombus formation were compared by Dunnett test. The interaction between IgG and β_2GPI bound to CL was analyzed with the Kruskall-Wallis with Dunn *post-hoc* test. The interaction between IgG and soluble β_2GPI was expressed as median and interquartile range and analyzed with the two-way repeated measure ANOVA with Sidak *post-hoc* test. Probabilities of <0.05 were considered statistically significant.

Results

Antibody to phospholipid-binding protein profile of the serum samples

Anti-β_2GPI IgG titers were comparable in the anti-D4/5- and anti-D1-positive samples [1.04±0.26 Optical Density (OD) and 1.46±0.48 OD, mean+SD, respectively]. The isolated anti-D4/5-positive samples displayed anti-D4/5 levels of 50.67±9.86 arbitrary units (AU) (mean±SD) while they were negative for aCL (<10 GPL) and LA. The isolated anti-D1-positive samples showed anti-D1 levels of 75.36±17.15 AU (mean±SD), high titers of IgG aCL (124.4±46.9 GPL, mean±SD), and displayed LA activity. Control samples were negative in all the assays. The purified IgG fractions maintained the antigen specificity shown in the whole serum. Clinical and serological data of all the subjects/patients included in the study are reported in *Online Supplementary Table S1*.

Fine epitope-specificity of antibodies to domains 4/5

The IgG against D4/5 used in this study were selected for their ability to react with the combined domains obtained from INOVA Diagnostics, but it was unclear whether they recognized one or the other domain or both. To clarify this point, we assessed the reactivity of serum IgG towards recombinant D4 and D5. The amino acid sequences of the two domains are reported in *Online Supplementary Figure S1*. The results presented in Figure 1 clearly show that all the anti-D4/5 reacted with D5 and did not recognize D4. The difference in the reactivity of the various sera IgG towards D4/5 is essentially similar to that observed in their reaction with D5.

Antibodies to domain 5 fail to cause thrombus formation *in vivo*

To evaluate the pro-coagulant activity of sera containing antibodies to different domains of β_2GPI, two groups of serum IgG positive for either D1 or D5 domains were analyzed for their ability to induce thrombus formation followed *in vivo* by intravital microscopy. IgG from sera negative for antibodies to β_2GPI served as a control group. All anti-D1-positive IgG induced blood clots that could be seen from 15 min after serum infusion (Figure 2). Their number progressively increased to reach the highest value after 1 h and was maintained thereafter for up to 90 min. Thrombus formation was associated with vascular occlusion that resulted in a marked decrease, and, in some vessels, in a complete blockage of blood flow. Conversely, the anti-D5-positive IgG did not exhibit pro-coagulant activity and failed to cause reduced blood flow. The latter results were not statistically different from those of anti-β_2GPI-negative blood donors at each time point. On the contrary, the data of anti-D1 IgG were statistically different from those of anti-β_2GPI-negative samples at all times starting from 15 min of analysis (P<0.05).

Antibodies to domain 5 fail to interact with surface-bound $\beta_2$glycoprotein I

Having observed an absence of intravascular coagulation in rats that had received anti-D5-positive IgG, we decided to investigate whether this was due to the inability of the antibodies to interact with endothelium-bound β_2GPI. To this end, samples of ileal mesentery were analyzed for the presence of β_2GPI, human IgG and C3. As expected from our previous findings,[30] β_2GPI was detected on the vessel endothelium of rats primed with LPS (Figure 3), while it was totally absent in unprimed animals (*data not shown*). A search for IgG and C3 revealed marked granular deposits of both proteins on endothelial cells of rats treated with anti-D1 IgG, while a milder linear staining for IgG and absence of C3 were observed in rats receiving anti-D5 IgG (Figure 3). The animals treated with anti-β_2GPI-negative sera showed negligible staining for IgG and undetectable C3 (Figure 3). Since several molecules

other than β_2GPI are expressed on the endothelial cell surface and represent potential targets for human IgG, we set out to determine whether the fluorescence was due to the IgG specifically directed against β_2GPI. To do this, we set up a β_2GPI-dependent CL assay in which the β_2GPI supplementation was carried out by adding human purified β_2GPI at increasing concentrations instead of fetal calf serum. The system allowed us to test the IgG reactivity with β_2GPI added at different concentrations to the CL-plates. The anti-D1 IgG reacted with the β_2GPI molecule most likely by recognizing the D1 epitope exposed on the β_2GPI molecule following its binding to CL (Figure 4). The IgG level detected in the assay varied in different patients and was related to the concentration of β_2GPI used to coat CL. In contrast, anti-D5 IgG failed to interact with CL-bound β_2GPI even at the highest concentration of β_2GPI, suggesting that D5 domains were not accessible to the antibodies under these experimental conditions. Like the anti-D5 antibodies, in the assay, the IgG from control sera were negative.

Antibodies to domain 5 interact with soluble $\beta_2$glycoprotein I

Electron microscopy studies have revealed that β_2GPI adopts a circular form in plasma and that this is maintained by the interaction of D1 with D5.[34] This special conformation prevents the access of autoantibodies to hidden epitopes on D1[19] and predicts the presence of cryptic epitopes on D5, though this has not been formally proven.[35] We first decided to examine the *in vivo* interaction of the antibodies with circulating β_2GPI and the effect of this interaction on β_2GPI bound to vascular endothelium. To this purpose, the *in vivo* model was slightly modified administering IgG intraperitoneally followed 15 h later by LPS given by the same route; this approach would allow sufficient time for the antibodies to react with the target antigen prior to the binding of β_2GPI to vascular endothelium promoted by LPS. The IgG from two sera with relatively high levels of antibodies to D1 and D5, respectively, and from an anti-β_2GPI-negative serum were tested and the amount of vascular deposits of β_2GPI and IgG was evaluated. As expected, the rat treated with anti-D1 developed endovascular thrombi associated with deposition of IgG, both of which were undetectable in animals that received anti-D5-positive or anti-β_2GPI-negative IgG (Figure 5). Analysis of the ileal mesentery showed that β_2GPI was present on the vascular endothelium of the animals that received the three IgG fractions with no clear difference in the staining intensity observed in the rats treated with anti-D5 and anti-D1 IgG (Figure 5).

Since the *in vivo* data did not provide convincing evidence of the ability of anti-D5 to prevent binding of circulating β_2GPI to vascular endothelium, we decided to further investigate this issue using an *in vitro* inhibition assay. IgG purified from anti-D5-positive, anti-D1-positive or anti-β_2GPI-negative sera were incubated with increasing

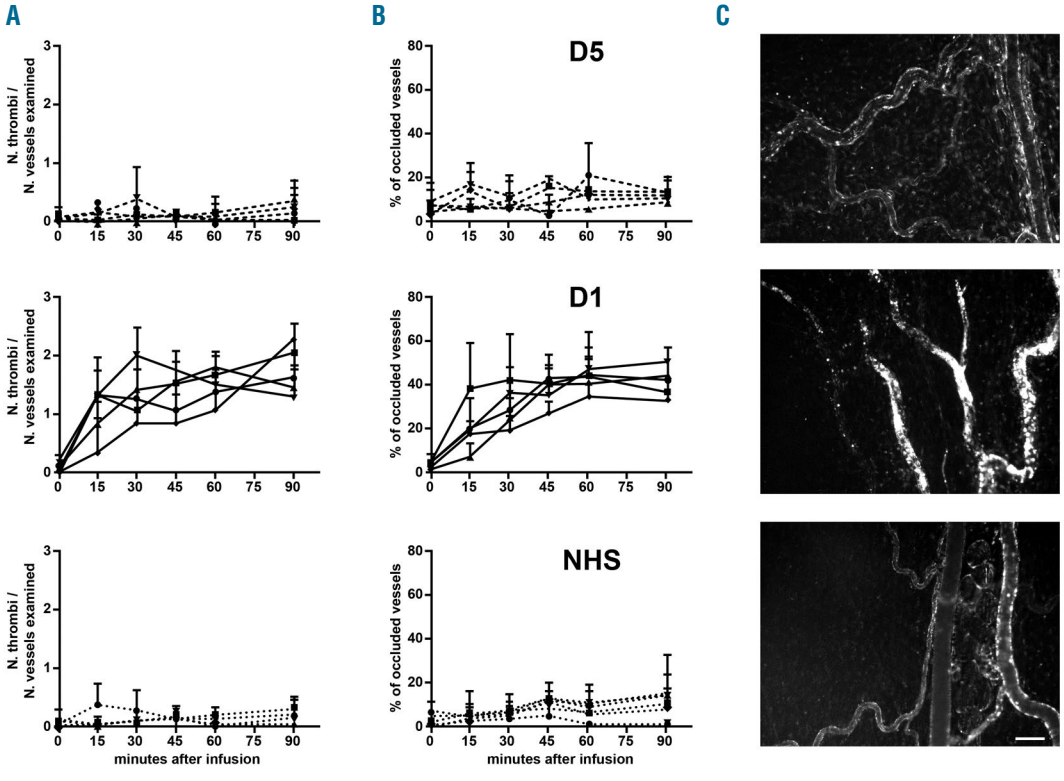

Figure 2. Anti-domain (D) 5 antibodies fail to induce thrombi in rats. Thrombus formation and vascular occlusion visualized by intravital microscopy in the ileal mesentery of rats that received an intraperitoneal injection of lipopolysaccharide (LPS) (2.5 mg/kg body weight) followed by the injection into the carotid artery of antibodies (10 mg/rat) directed against domain 5 (D5), domain 1 (D1), or anti-$\beta_2$glycoprotein I (β_2GPI)-negative (NHS). The number of thrombi (A) and vessel occlusions (B) were evaluated at various time intervals on 3 rats per each serum. The results are expressed as a ratio between the number of thrombi and the number of microvessels examined and as a percentage of occluded microvessels. Data are reported as mean±Standard Deviation. (C) Sections of the ileal mesentery showing endovascular thrombi in anti-D1-treated rat and undetectable in the vessels of animals receiving anti-D4/5-positive or anti-β_2GPI-negative sera. Original magnification 100x. Scale bar 50 μm.

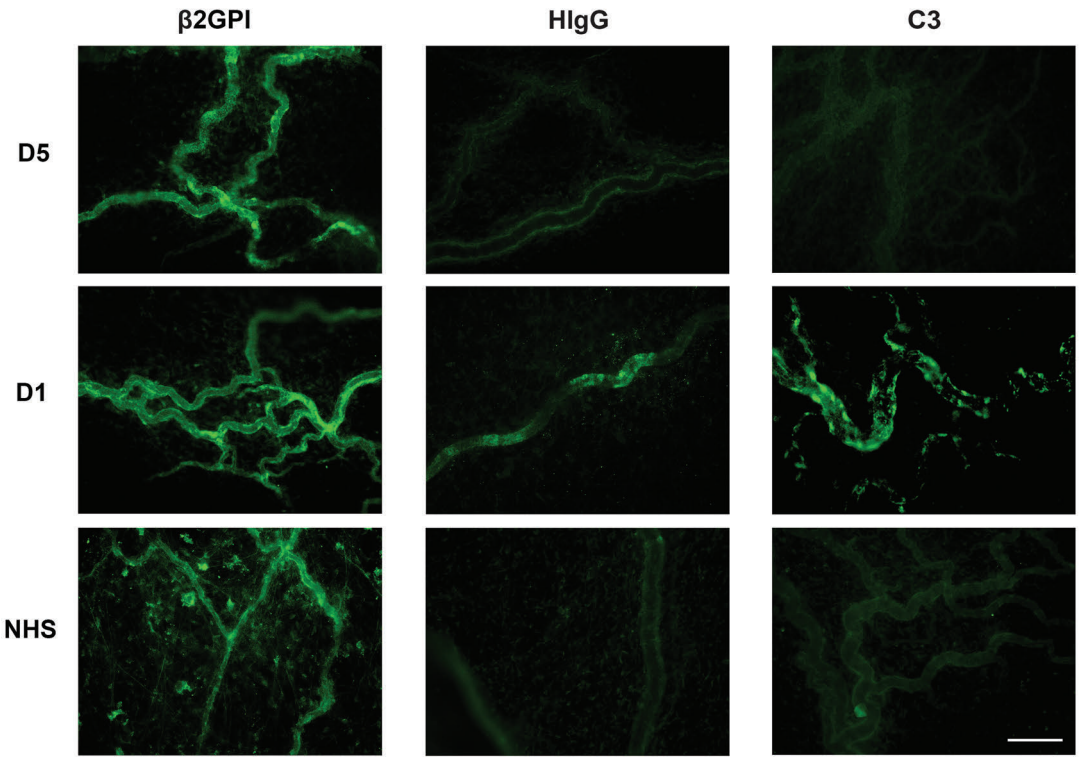

Figure 3. Deposition of $\beta_2$glycoprotein I (β_2GPI), human IgG and C3 on mesenteric vessels of rats treated with antibodies to domain 5 (D5) or domain 1 (D1) of β_2GPI. The animals were treated with lipopolysaccharide (LPS) followed by the injection of antibodies directed against domain 5 (D5), domain 1 (D1), or negative for anti-β_2GPI (NHS). Mesenteric tissue samples after 90 minutes analyzed for vascular deposition of β_2GPI, human IgG and C3 by immunofluorescence. Original magnification 200x. Scale bar 50 μm.

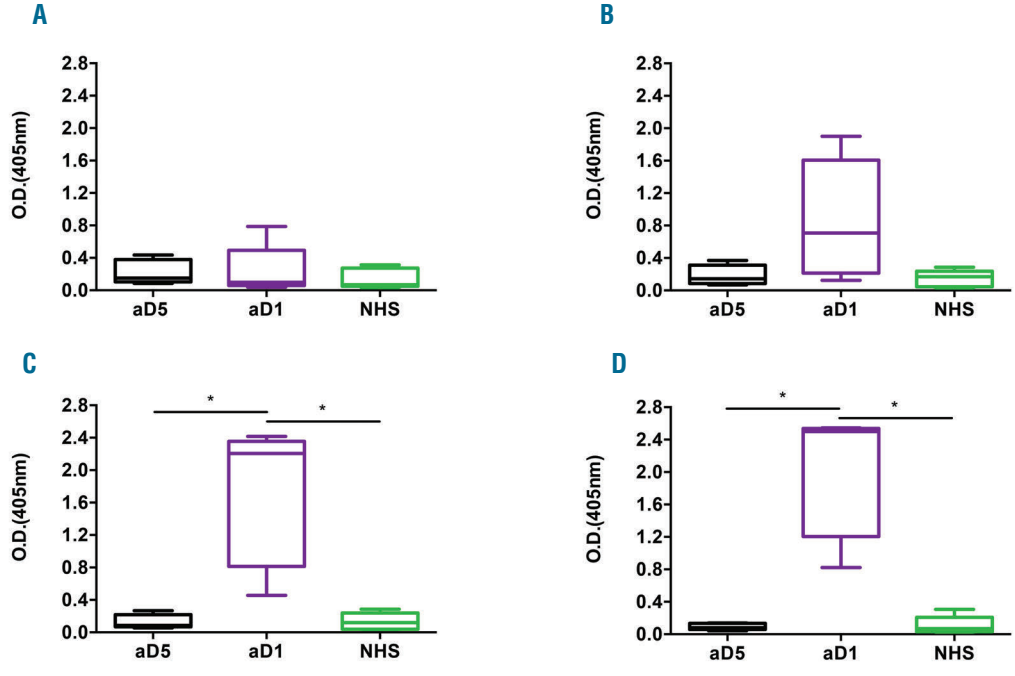

Figure 4. Anti-domain 5 (D5) antibodies fail to interact with $\beta_2$glycoprotein I (β_2GPI) bound to cardiolipin (CL). Reactivity of anti-D5 (aD5) (▭), anti-domain 1 (aD1) (▭) or anti-β_2GPI negative (NHS) (▭) antibodies (50 μg/mL) against different concentrations of β_2GPI bound to cardiolipin. Binding of IgG to: (A) CL alone, (B) 1 μg/mL CL-bound β2GPI, (C) 5 μg/mL CL-bound β_2GPI, (D) 75 μg/mL CL-bound β_2GPI. Optical Density (OD) values are expressed as median and interquartile range, and presented as box plots. *P<0.05.

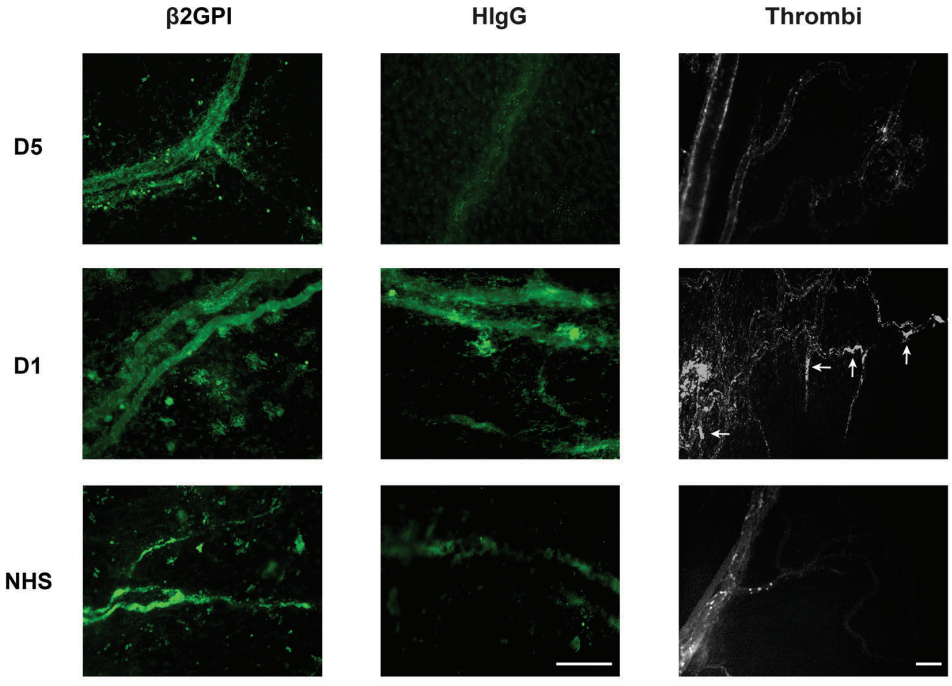

Figure 5. Deposition of β$_2$glycoprotein I (β$_2$GPI) and IgG on mesenteric vessels of rats treated with patients' and controls' serum IgG prior to lipopolysaccharide (LPS) challenge. The animals were treated with antibodies directed against domain 5 (D5), domain 1 (D1), or anti-β$_2$GPI-negative (NHS) (10 mg/rat) before LPS administration (2.5 mg/kg body weight). Mesenteric tissue samples were analyzed for vascular deposition of β$_2$GPI (left) and human IgG (center). Original magnification for immunofluorescence analysis 200x. Scale bar 50 μm. Thrombus formation in mesenteric vessels was monitored by intravital microscopy for 90 minutes and mesenteric tissue was collected at the end of the experiment. Thrombi formed in the vessels are indicated with arrows (right). Original magnification 100x. Scale bar 50 μm.

concentrations of soluble β$_2$GPI and the residual IgG interacting with β$_2$GPI directly bound to the plate wells were measured. The amount of IgG anti-D5 free to bind to solid-phase β$_2$GPI after incubation with the soluble molecule decreased compared to that of the IgG incubated with BSA, particularly at a higher concentration of soluble β$_2$GPI (Figure 6). In contrast, the level of IgG anti-D1 bound to solid-phase β$_2$GPI following incubation with soluble β$_2$GPI was slightly lower, but not significantly different from that of the IgG incubated with BSA.

Discussion

Antiphospholipid syndrome is now recognized as an antibody-dependent and complement-mediated syndrome and antibodies to β$_2$GPI have been identified as important players in thrombus formation in APS patients.[10] Efforts are being made to determine the clinical relevance of antibodies to D1 and D4/5 domains of the molecule detected in these patients. Clinical studies have suggested that antibodies to D4/5, unlike those directed against D1, do not represent a risk factor for thrombosis and pregnancy complications.[7,9,14] The *in vivo* data presented here focused on the thrombotic aspect of the syndrome and support the clinical observation that the anti-D4/5 antibodies are pathologically irrelevant.

The animal model used in this and in previous studies proved to be an invaluable tool to investigate the ability of the anti-β$_2$GPI antibodies to induce blood clots in rats primed with LPS that provides the first hit, followed by the infusion of the antibodies acting as a second hit.[10] As expected, all anti-D1 IgG promoted thrombus formation and vascular occlusion, confirming the pathogenicity of these antibodies suggested by clinical observations. It is possible that LA detected in the plasma of these patients may have also contributed to anti-β$_2$GPI-induced blood clots. However, although β$_2$GPI antibody-dependent LA has been shown to correlate with the increased risk of thrombosis,[13,14,36] evidence supporting the *in vivo* prothrombotic activity of LA independently of anti-β$_2$GPI antibody has not yet been provided. Instead, there is good evidence that the antibodies recognizing the D1 domain of β$_2$GPI are directly involved in thrombus formation and vessel occlusion. We have previously shown that a human monoclonal antibody that recognizes D1 induces blood clots and that a CH2-deleted non-complement fixing variant molecule competes with anti-β$_2$GPI antibodies from APS patients and prevents their pro-coagulant activity.[27] A similar inhibitory effect was obtained using recombinant D1 to control the thrombus enhancement activity of aPL in mice.[37]

The *in vivo* experiments showed that none of the anti-D5 IgG exhibited a prothrombotic activity supporting the observations made in clinical studies that these antibodies are pathologically irrelevant.[7,14] A possible explanation for this finding is the inability of these antibodies to interact with cell-bound β$_2$GPI. In line with this hypothesis, we showed that anti-D5-positive IgG fractions were unable to react with β$_2$GPI bound to CL-coated plates *in vitro* because of the shielding of D5 in the β$_2$GPI molecule bound to the CL-coated plate. However, in rats treated with LPS (used to promote binding of β$_2$GPI) and anti-D5 IgG, the mild staining for IgG observed on the endothelium of mesenteric vessels did not allow any definite conclusions to be

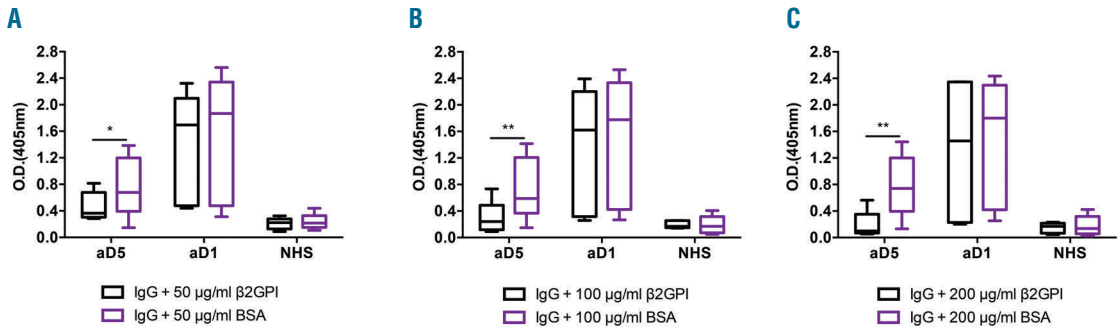

Figure 6. Anti-domain 5 (D5) antibodies interact with $\beta_2$glycoprotein I (β_2GPI) in fluid phase. Reactivity of anti-D5 (aD5), anti-D1 (aD1), or anti-β_2GPI-negative (NHS) antibodies (50 µg/mL) against purified β_2GPI directly coated on ELISA plates, measured after their incubation with (A) 50 µg/mL, (B) 100 µg/mL, and (C) 200 µg/mL of purified β_2GPI (▭) or BSA (▭) in fluid phase. Optical Density (OD) values are expressed as median and interquartile range, and presented as box plots. *P<0.05; **P<0.01.

drawn on this issue. It must be emphasized, however, that the staining intensity varied among different sera and was not related to the level of antibodies. The linear deposition of IgG on the mesenteric endothelium from rats treated with anti-D5-positive IgG suggests their interaction with antigens constitutively expressed on endothelial cells. This distribution pattern differs from the irregular staining for IgG seen with the anti-D1-positive IgG most likely explained by their reaction with a plasma-derived molecule, such as β_2GPI, bound to the endothelial cell surface. The different distribution of anti-D1 and anti-D5 IgG resembles the well-known difference in the granular and linear distribution patterns of IgG observed in the kidney of patients with SLE and Goodpasture syndrome, respectively. The linear pattern of IgG in Goodpasture is the result of the interaction of the antibodies with their target antigen constitutively expressed on the glomerular basement membrane. In contrast, the granular distribution of IgG in SLE is caused by irregular deposition of circulating immune complexes.[38,39] The finding that C3 deposition was undetectable on the vascular endothelium of rats treated with anti-D5 IgG is consistent with the failure of these antibodies to induce thrombus formation. We and others have provided convincing evidence that complement activation is critically involved in the coagulation process induced by anti-β_2GPI IgG and in this study by antibodies to the D1 domain.[26,27,40-43]

The anti-D4/D5 antibodies present in the sera analyzed in this study selectively recognized the recombinant D5 domain and are likely to inhibit deposition of β_2GPI on the endothelium by shielding its binding site for the anionic phospholipid on endothelial cells.[44] Our attempt to document *ex vivo* reduced binding of circulating β_2GPI to vascular endothelium of the anti-D5-treated rats was unsatisfactory; this was most likely due to a much higher level of serum β_2GPI compared to that of injected antibodies *in vivo*. The *in vitro* data obtained under more controlled conditions of IgG and β_2GPI concentrations showed a fluid phase interaction between anti-D5 IgG and soluble β_2GPI, resulting in a significantly reduced reactivity of these antibodies against surface-bound β_2GPI (when the molecule was bound to a plate).

The finding that anti-D5 IgG have no pro-coagulant effect in our *in vivo* model has important clinical implications suggesting that individuals with isolated presence of these antibodies should not be considered to be at risk of thrombosis. It should be pointed out, however, that anti-D1 and anti-D5 IgG often co-exist in a large proportion of APS patients, and that they are likely to be susceptible to anti-D1-dependent thrombus formation. In view of the ability of the anti-D5 IgG to interact with soluble β_2GPI, thus preventing its binding to the target cells, it is tempting to speculate that the anti-D5 IgG may antagonize the pro-coagulant activity of anti-D1 antibodies, according to antibody levels. In accordance with this, we recently published data indicating that patients positive for anti-D1 and anti-D4/5 antibodies have a lower risk of thrombosis if the levels of anti-D4/5 are higher than those of anti-D1 antibodies.[7,9] Overall, our experimental findings fit with the clinical observation and offer new tools for stratifying patients into different risk categories. This would help in better preventing recurrences of the clinical manifestations and avoiding overtreatment, thus ultimately improving the patients' quality of life and sparing them treatment side-effects.

In conclusion, the data presented in this work indicate that, unlike the anti-D1 positive sera, those containing antibodies against D5 are unable to induce clot formation and vascular occlusion. The failure of the anti-D5 antibodies to promote coagulation is due mainly to their inability to interact with the target epitopes hidden on the surface-bound molecule, and possibly to the recognition of native β_2GPI in plasma that may, to some extent, potentially prevent its binding to the surface of activated endothelial cells. The detection of anti-D5 antibodies in patients with a doubtful APS clinical profile and a single positivity for anti-β_2GPI in the absence of a positive aCL assay may offer a valuable tool for ruling out a definite APS diagnosis and for identifying subjects at lower risk of clinical manifestations.

Acknowledgments

The authors would like to thank Linda Vuch, Luca De Maso and Paola A. Lonati for their valuable technical contribution; Michael Mahler, Gary Norman and Filippo Sarra (INOVA Diagnostics and Werfen Italia) for their support.

Funding

This work was partially supported by Istituto Auxologico Italiano, Ricerca Corrente 2016 (PLM).

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