We previously showed that β₂-glycoprotein I (β₂GPI)–dependent lupus anticoagulants (LAs) form bivalent antigen-antibody complexes with high affinity for phospholipids; these complexes are responsible for their in vitro anticoagulant effect. We now studied the role of these bivalent complexes in arterial thrombosis in the hamster. Three monoclonal antibodies (mAbs) raised against human β₂GPI were selected on the basis of their cross-reactivity with hamster β₂GPI. Two of these, one with LA activity, 5H2, and one with only antcardiolipin properties, 11E8, were infused at 0 to 10 mg/kg prior to photochemically induced vessel damage.

5H2 promoted thrombus formation dose dependently, raising the thrombus size from 6.0 arbitrary units (AU) in controls (n = 9) to 65.0 AU in the high-dose group (10 mg/kg, n = 6, P = .007). The LA⁺ mAb 11E8 and mAb 27A8, reactive with human β₂GPI exclusively, did not significantly promote thrombus formation. In a second set of experiments, intact mAb 5H2 was compared to its fragments. Intact mAb 5H2 at 3.3 mg/kg and the equimolar dose of F(ab’₂) fragments (2.2 mg/kg) promoted thrombus formation equally well (55.8 AU, n = 8 and 62.5 AU, n = 7, respectively); mAb 5H2-derived Fab’ fragments were inactive. Immunohistochemical analysis showed platelet-rich thrombi, with 5H2 or its F(ab’₂) fragments mainly bound to individual platelets. Our results indicate that bivalent immune complex formation plays an important role in the genesis of arterial thrombosis by certain antiphospholipid antibodies. Cellular activation via the Fc portion of these immune complexes, however, is not essential, because F(ab’₂) fragments of 5H2 still promote thrombus formation.

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Introduction

Antiphospholipid antibodies (aPLs) are a heterogeneous group of immunoglobulins interacting with negatively charged phospholipids (PLs). They are found in serum or plasma of patients with rheumatic diseases, malignancies, or infections, but also in apparently healthy individuals. Persistently elevated aPL levels are associated with the occurrence of arterial and venous thrombosis, thrombocytopenia, and recurrent fetal loss.1 This clinical entity, referred to as the antiphospholipid syndrome (APS), is considered secondary or primary, respectively, in subjects with or without systemic autoimmune diseases, for example, systemic lupus erythematosus (SLE).2 Paradoxically, an important subset of aPLs, termed lupus anticoagulants (LAs), prolongs in vitro plasma clotting times.3,4

The first immunoassays for the detection of aPLs made use of cardiolipin.5,6 However, the so-called antithrombin antibodies (aCLs) also bind to other negatively charged PLs, such as phosphatidylserine.7 Affinity purification of aCLs revealed that aCL binding to cardiolipin depends on a plasma protein, β₂-glycoprotein I (β₂GPI).5,10 It is now generally accepted that autoimmune aPLs are directed against proteins binding to anionic PL surfaces rather than against PLs themselves, the major protein targets appearing to be β₂GPI and prothrombin.11,12 LA⁺ aPLs cross-link 2 β₂GPI molecules or 2 prothrombin molecules and thereby induce the correct spatial orientation of the PL-binding domains in these proteins required for optimal binding to PLs.13-16

The dimerized β₂GPI or prothrombin displays a marked gain in affinity for the PL surface and retards clotting in vitro by competing with clotting factors for the same PL surface.

Autoimmune aPLs are thought to be pathogenic because patients with aPLs not only have an increased risk for thrombosis but also show signs of a prothrombotic (hypercoagulable) state with elevated tissue factor (TF) expression17 and enhanced thrombin generation.18 The mechanisms by which these antibodies cause a prothrombotic state or promote thrombosis are still far from being elucidated. Several hypotheses have been proposed including a decreased prostacyclin formation by endothelium, inhibition of protein C activation or of activated protein C function, impairment of TF inhibition, interference with the function of antithrombin, impaired fibrinolytic potential, reduced anticoagulant potential of annexin V, and activation of platelets (for a review, see Rand19). However, none of these hypotheses explain why thrombosis can be venous as well as arterial and why LAs are more strongly associated with thrombosis than aCLs.20,21

Analogous to heparin-induced thrombocytopenia (HIT), another syndrome of antibody-mediated thrombosis, a model of prothrombotic cellular activation was proposed. Limited damage or activation of blood cells or endothelium may cause exposure of anionic PLs on the cell surface. In the presence of aPLs with LA activity, bivalent antigen-antibody complexes may form on these cell membranes enriched in anionic PLs. These complexes may...
then bind to cellular Fcy receptors or activate the complement system leading to strong thrombosis-promoting cell activation via release of granule contents and of microvesicles, thromboxane A2 biosynthesis, tissue factor decetration, removal of endothelial heparan sulfate, and so forth.\textsuperscript{22,23}

In the present study, this hypothesis was tested in a hamster model of arterial thrombosis,\textsuperscript{24} adapted in our laboratory to study prothrombotic phenotypes.\textsuperscript{25} A murine monoclonal antibody (mAb) against human \(\beta_2\)GPI with clear LA activity and cross-reacting with hamster \(\beta_2\)GPI was selected. The consequences on photochemically induced platelet-rich thrombosis in the hamster were then studied after injection of intact antibody, or its F(ab\textsuperscript{'})\textsubscript{2}, respectively, Fab\textsuperscript{' }fragments. Our findings show that aPL-associated thrombosis, in contrast to HIT, can occur independently of Fc.

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**Materials and methods**

**mAbs against \(\beta_2\)GPI**

The mAbs against human \(\beta_2\)GPI, previously raised in Balb/c mice,\textsuperscript{15} were used in this study. These mAbs were purified from ascites by affinity chromatography on protein A–Sepharose.\textsuperscript{26}

**Preparation of F(ab\textsuperscript{'})\textsubscript{2} fragments**

Purified mAb 5H2 at 3 mg/mL was dialyzed overnight at 4°C against 100 mM sodium citrate buffer, pH 3.5. Digestion was performed by addition of 5 \(\mu\)g pepsin beads (Sigma, St. Louis, MO) per milligram mAb. After incubation for 60 minutes at 20°C, the beads were separated by centrifugation for 10 minutes at 4000 rpm and the pH of the supernatant was adjusted to 8 with 1 M Tris (tris(hydroxymethyl)aminomethane)–HCl buffer. Following dialysis against 100 mM Tris–HCl buffer, pH 8.1, intact Fc fragments and nondigested antibody were removed by protein A–Sepharose chromatography. The F(ab\textsuperscript{'})\textsubscript{2} preparation showed a single band at 110 kDa and a doublet of bands at 25 kDa in unreduced and reduced sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, respectively. The protein concentration was determined by absorbance (280 nm) measurements (extinction coefficient, 1.35 mL mg\textsuperscript{-1} cm\textsuperscript{-1}).

**Preparation of Fab\textsuperscript{' }fragments**

Purified mAb 5H2 at 3 mg/mL was dialyzed overnight at 4°C against 100 mM phosphate buffer, pH 7.0, after which 10 mM cysteine and 2 mM EDTA (ethylenediaminetetraacetic acid) were added. Digestion was performed by addition of 30 \(\mu\)g papain beads (Sigma) per milligram mAb. After incubation at 37°C for 60 minutes, the reaction was stopped by 75 mM iodoacetamide (Sigma). Following dialysis against 100 mM Tris–HCl buffer, pH 8.3, intact Fc fragments and nondigested antibody were removed by protein A–Sepharose chromatography. The Fab\textsuperscript{' }preparation showed a single band at 50 kDa and 25 kDa in unreduced and reduced SDS-PAGE and silver staining, respectively.

**Reactivity of mAbs with hamster \(\beta_2\)GPI**

To assess the cross-reactivity of the antihuman \(\beta_2\)GPI mAbs with hamster \(\beta_2\)GPI, microtiter plates (Costar no. 3590; Corning, NY) were incubated with 50 \(\mu\)L/well phosphatidylserine (Sigma) dissolved in absolute ethanol. The plates were then blocked with 5% hamster plasma in phosphate-buffered saline (PBS), as a source of \(\beta_2\)GPI, for 60 minutes at room temperature. Anti-\(\beta_2\)GPI mAbs dissolved in 1% hamster plasma in PBS (0.5–10 \(\mu\)g/mL) were added to the plate and incubated for 120 minutes at room temperature. Plates were washed 3 times with PBS and incubated for 120 minutes with 100 \(\mu\)L/well horseradish peroxidase–coupled goat antimouse immunoglobulins (GAM-HRP; Dako, Glostrup, Denmark) diluted 1:3000 in PBS, containing 1% hamster plasma. After washing, 160 \(\mu\)L 100 mM citrate/200 mM sodium phosphate buffer containing o-phenylenediamine (OPD; Fluka, Buchs, Switzerland) and 0.003% H\textsubscript{2}O\textsubscript{2} was added to each well. After approximately 30 minutes at room temperature, staining was stopped with 50 \(\mu\)L 4 M H\textsubscript{2}SO\textsubscript{4}. Absorbance (490 nm) was then measured with a multispec spectrophotometer (EL808; Bio-Tek Instruments, Winooski, VT).

**LA activity of anti-\(\beta_2\)GPI mAbs in hamster plasma**

Determination of LA activity was based on its effect on the dilute prothrombin time (dPT).\textsuperscript{27} Platelet-poor plasma (PPP) was prepared by double centrifugation from hamster blood collected on trisodium citrate. The anti-\(\beta_2\)GPI mAbs were 10-fold diluted in this PPP to achieve final concentrations between 0 and 150 \(\mu\)g/mL and incubated for 10 minutes at 37°C prior to testing. The dPT was determined by incubating 50 \(\mu\)L Innovin (DADE, BEHRING, Liederbach, Germany) diluted 1:200 in Owren Veronal buffer, with 50 \(\mu\)L PPP containing mAb for 7 minutes at 37°C after which coagulation was initiated by adding 50 \(\mu\)L CaCl\textsubscript{2} (25 mM). Coagulation times were measured using a SYSMEX CA 6000 coagulometer (TOA Medical Instruments, Kobe, Japan).
lamp (L4887; Hamamatsu Photonics, Hamamatsu, Japan) equipped with a heat-absorbing filter and a green filter. Irradiation was directed via a 3-mm–diameter optic fiber attached to a manipulator. All tested reagents were administered via an intravenous (slow) bolus injection prior to rose-bengal injection. Intact mAbs and F(ab’)2 fragments were given 15 minutes before photochemical vessel injury, whereas Fab’ and buffer injections just preceded rose-bengal injection.

Quantification of mural thrombi in the hamster carotid artery was performed as described with minor modifications. Thrombus formation in the injured transilluminated vessel was constantly monitored for 40 minutes via a camera (CV-M70; JAI, Yokohama, Japan) mounted on a microscope. The images were digitized with an image processing software (Optimas 6.5 for Windows 95/98 and NT 4.0; Media Cybernetics, Silver Spring, MD), with a specific extension from IP Consult, Breda, The Netherlands) and constantly recorded. The transmitted light intensity versus time curve was established and thrombus formation was measured by comparing the area under the curve, expressed in arbitrary light units (AU).

Determination of the concentration of intact 5H2 and its F(ab’)2 fragments in hamster plasma

Plasma was prepared from blood drawn via the intravenous catheter just after completion of the in vivo experiments. Plasma concentrations of 5H2 or its F(ab’)2 fragments were measured by enzyme-linked immunosorbent assay (ELISA) as follows. Microtiter plates were coated overnight at 4°C with 200 μL/well polyclonal rabbit antimouse IgG (5 μg/mL), blocked with 1% BSA and washed 3 times with PBS containing 0.1% Tween 20. Hamster plasma samples, 1:2000 and 1:1000 diluted in PBS for measurement of 1% BSA and washed 3 times with PBS containing 0.1% Tween 20. Hamster plasma samples, 1:2000 and 1:1000 diluted in PBS for measurement of intact 5H2 and its F(ab’)2 fragments, respectively, were added and incubated for 120 minutes at 20°C. Standard curves were constructed using 5H2 or 5H2-derived fragment solutions (0-200 μg/mL in hamster plasma), diluted in the same way as the ex vivo samples. After washing, bound mAbs were detected by GAM-HRP diluted 1:3000 in PBS, containing hamster and rabbit plasma (1:300) to adsorb goat antimouse antibodies cross-reacting with hamster and rabbit antibodies. The HRP activity was determined with OPD as a substrate.

Immunohistochemistry

Carotid arteries containing thrombi were carefully dissected, fixed over-night at 4°C in 4% formaldehyde in PBS, pH 7.0, and transferred to PBS containing 20% sucrose for 24 hours. Arteries were embedded in OCT compound (Tissue-Tec; Miles, Elkhart, IN), snap-frozen in precooled 2-methyl butane and stored at −70°C until further analysis. Then, 7-μm-thick sections were made through the whole thrombosed artery for hematoxylin-eosin staining. Immunohistochemical staining for the presence of 5H2 and its F(ab’)2 fragments was done with a GAM-HRP diluted 1:250 in TRIS-buffered saline (TBS) containing 2% BSA and preincubated for 30 minutes with 10% hamster plasma to adsorb nonspecific antibodies. Peroxidase staining was performed in 50 mM Tris-HCl buffer, pH 7.0, containing 0.06% 3,3-diaminobenzidine and 0.01% H2O2. Tissue sections were counterstained with hematoxylin.

Platelet aggregation studies

Blood for platelet aggregation studies was freshly drawn from healthy donors on 109 mM trisodium citrate and centrifuged at 150g for 15 minutes. The platelet-rich plasma (PRP) was collected and the platelet counts were adjusted to 2.5 × 105 platelets/μL with autologous PPP. Light transmission during adenosine 5′-diphosphate (ADP)–induced platelet aggregation was recorded on a 4-channel aggregometer (Chrono-log, Haverton, PA). Four minutes before stimulation with a subthreshold concentration of ADP, the PRP was incubated during 3 minutes at 37°C either with 75 μg/mL intact mAb 5H2 or 50 μg/mL of its F(ab’)2 fragments or its Fab’ fragments.

Statistical analysis

Intergroup comparison was performed with the Mann-Whitney U test and potential correlations were evaluated using the Spearman rank order test. P < .05 were considered significant.
around 1.1 mg/kg (median 24.5 AU, n = 6; Figure 2). The LA− mAb 11E8 promoted thrombus formation only marginally (median AU at a dose of 10 mg/kg: 14.3, n = 8, P = .18; not shown). The influence of mAb 27A8 on thrombus formation was negligible (median AU: 9.7, n = 6; not shown).

In a second set of experiments, 8 hamsters treated with intact 5H2 at a dose of 3.3 mg/kg were compared with animals treated with 5H2-derived Fab′ fragments at a dose of 2.2 mg/kg (n = 7) and 4.5 mg/kg (n = 8) and control animals (n = 16). Fab′ fragments promoted thrombus formation similarly to the intact antibody both at an equimolar dose (2.2 mg/kg) and a double equimolar dose (4.5 mg/kg; Figure 2B). The median AU for the control group and for the animals receiving intact 5H2 and its Fab′ fragments promoted thrombus formation similarly to the intact antibody both at an equimolar dose (2.2 mg/kg) and a double equimolar dose were 17.9, 55.8, 62.3, and 43.2 AU, respectively. The differences between the treated groups and the control group were all statistically significant. No statistical differences were found among the 3 treated groups.

A last series of animal experiments revealed lack of thrombogenicity of the 5H2-derived Fab′ fragments administered at a dose of 2.2 mg/kg (median thrombus light intensity 3.3 AU [n = 6] versus 6.1 AU [n = 9] in the control group, P = .556; Figure 2C).

The median antibody levels measured in the plasma collected just after completion of the experiments were 37 µg/mL for the group having received intact 5H2, and 15 and 35 µg/mL for the animals treated with the lower or higher Fab′ dose. No significant correlation was found between the antibody or antibody fragment concentrations and the thrombus light intensity (Spearman rank order correlations: R = 0.405, 0, and 0.309; P = .320, 1, and .456, respectively). Immunohistochemical analysis of carotid artery thrombi showed that intact 5H2 (Figure 3) and its Fab′ fragments (data not shown) were mainly found in association with platelets within the platelet-rich thrombus and were to a much lesser extent bound to vascular endothelium.

Because 5H2 and its Fab′ fragments both promoted platelet-rich thrombus formation in vivo, we studied the effect of the intact mAb and its fragments on platelet aggregation in vitro using optical aggregometry. 5H2 by itself did not induce platelet aggregation, even when used at concentrations up to 200 µg/mL. However, when subthreshold concentrations of ADP, by themselves only inducing a first wave of aggregation, were added to PRP preincubated with 75 µg/mL 5H2, strong aggregation responses were observed (Figure 4). Equimolar concentrations of the Fab′ fragments (50 µg/mL) also promoted ADP-induced aggregation, whereas Fab′ fragments did not.

Discussion

The association between the presence of aPL and thrombosis affecting both veins and arteries is well established.1 In addition, prospective studies, showing that elevated aPL levels are a risk factor for future thrombosis, suggest that aPLs may be involved in
thrombogenesis. More direct evidence for the thrombogenicity of aPLs was provided by animal models using vessel wall injury to induce thrombosis11-15 (and present study). Thus, after limited mechanical injury to the femoral vein in CD-1 mice, enhanced thrombosis was observed at the site of injury as well as slower thrombus disappearance after injection of immunoglobulin, affinity-purified acCL, and even a monoclonal IgG acCL, all from patients with APS.11-13 Similar observations were reported after active immunization with human β2-GPI. In this experimental setting, murine monoclonal aCLs possessing LA properties were thrombogenic, whereas an anti-β2-GPI antibody without LA activity had no effect.13 These studies implicate aPLs in venous thrombus formation but no direct support is available for the notion that aPLs may be involved in arterial thrombosis.

Therefore, in the present study the impact of aPLs was investigated in a model of carotid artery thrombosis in the hamster. This animal model complies with the concept of thrombosis as a “double-hit” phenomenon. Very mild thrombosis is provoked by limited photochemically induced injury to the vessel wall (“first hit”). This injury affects the entire area of the irradiated vascular segment but is confined to the endothelium. In this model, factors promoting platelet activation16 or coagulation17 enhance thrombus formation (“second hit”). mAbs previously raised against human β2-GPI18 were selected on the basis of their cross-reactivity with hamster β2-GPI and their LA and acCL properties in hamster plasma. We found that the LA+ and acCL+ mAb 5H2, which cross-reacts with hamster β2-GPI, dose-dependently promoted thrombus formation (Figure 2A). This finding provides the first direct evidence that aPLs may indeed be implicated in arterial thrombosis. mAb 27A8, non-cross-reacting with hamster β2-GPI and chosen as a negative control, had no clear effect. The thrombogenic effect of the acCL+ but LA− anti-β2-GPI mAb 11E8 was not significant, a finding that is in line with the clinical observation that LAs are more strongly associated with thrombosis than aCLs.20

The availability of sufficient quantities of mAb 5H2 enabled us to prepare F(ab)2 and Fab’ fragments from this antibody and to evaluate whether bivalent hamster β2-GPI-antibody complex formation on PL surfaces and possible FC involvement constitute the basis for the development of thrombotic complications. The F(ab)2 and Fab’ fragments of 5H2 had similar affinity for β2-GPI as the intact mAb (data not shown). The 5H2-derived F(ab)’2 fragments and the intact mAb 5H2 promoted binding of hamster β2-GPI to phospholipid surfaces equally well (Figure 1). As shown previ-ously with other LA+ anti-β2-GPI mAbs, this binding was concentration-dependent and inhibited by their Fab’ fragment that prevents bivalent β2-GPI-antibody complex formation.15

An important and novel finding of this study is that F(ab)’2 fragments derived from an LA+ mAb enhance arterial thrombosis in vivo. This somewhat unexpected finding strengthens our hypothesis that the thrombogenicity of aPLs relies on cellular activation by surface-bound bivalent antigen-antibody complexes, but weakens the suggested involvement of cellular FcγR receptors or the complement system. Animal models of venous thrombosis have provided evidence for Fc receptor–independent thrombotic mechanisms.17 In venous thrombosis, bivalent β2-GPI-antibody complexes may reduce the anticoagulant effects of protein C and protein S by competition for the PL surface on which they function.19 Immunohistochemical analysis of the arterial thrombus formed (Figure 3) localized 5H2 and its F(ab)’2 fragments mainly to platelets in certain areas of the thrombus. This allows us to propose the following scenario: following mild endothelial damage, a small platelet thrombus develops (first hit); the slightly activated platelets expose negatively charged PL; this leads to patchy deposition of bivalent β2-GPI-antibody complexes; these complexes cause further platelet activation and thrombus growth (second hit). In contrast to a previous suggestion,38 binding to endothelial cells seems less involved. The possibility that bivalent β2-GPI-antibody complexes might promote platelet activation in an Fc-independent manner was tested in vitro. Intact 5H2 further stimulated platelet aggregation induced by subthreshold concentrations of ADP. At least part of this proaggregatory effect was Fc independent because equimolar concentrations of F(ab)’2 also potentiated ADP-induced aggregation, whereas Fab’ fragments did not. Recent work by others further strengthens the concept of platelet activation by β2-GPI dimers. A chimeric recombinant protein consisting of 2 β2-GPI molecules linked together through the dimerization domain (apple4) of factor XI at the amino-terminal ends of β2-GPI domain I also has LA properties and enhances thrombus formation when added to whole blood perfused over a collagen surface.40 How β2-GPI dimers increase platelet deposition in vitro and thrombus formation in vivo (this study) is subject to ongoing research. In conclusion, the present study has revealed that certain aPLs enhance arterial thrombosis by forming bivalent β2-GPI-antibody complexes with affinity for PL and that this prothrombotic action is largely Fc independent.

References


