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Original article

Evaluation of platelet aggregation in the presence of antiphospholipid antibodies: anti-β2GP1 and anticardiolipin

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ABSTRACT

Introduction: The antiphospholipid syndrome (APS) is an autoimmune condition characterized by recurrent arterial and venous thrombosis, besides obstetric complications. The pathogenesis is associated with the presence of antiphospholipid and/or anti-b2-glicoprotein I (anti-b2GPI) antibodies that appear to change the anticoagulant activity of b2GPI. Antibody-induced dimerization of b2GPI seems to be related to the induction of platelet aggregation, contributing to the development of thrombosis in APS.
Objectives: The objective of the present study is to demonstrate the influence of antiphospholipid antibodies in platelet aggregation tests with different agonists (ADP, collagen, and adrenaline).
Methods: We analyzed platelet aggregation tests with different agonists (ADP, collagen, adrenalin) when normal platelets were exposed to serum with different concentrations of antiphospholipid antibodies.
Results: Results demonstrated a significant inhibition in adrenalin- and ADP-induced platelet aggregation curves ($P < 0.05$) in all antibody concentrations tested when compared to the control. The paradox between the prothrombotic state and the presence of autoantibodies that show anticoagulant activity *in vitro* was demonstrated in the literature, making it difficult to understand the pathophysiologic mechanism of the antiphospholipid syndrome.
Conclusion: Results showed that anticardiolipin and anti-b2GPI antibodies-rich serum, both of which belonging to the IgG class, can interfere with platelet aggregation curves.

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Avaliação da agregação plaquetária em presença de anticorpos antifosfolípides: anti-β2GP1 e anticardiolipina

Palavras-chave:
Síndrome antifosfolípide
Anticorpos antifosfolípides
Agregação plaquetária

RESUMO

Introdução: A síndrome antifosfolípide (SAF) é uma condição autoimune que apresenta fenômenos trombóticos arteriais e venosos de repetição além de complicações obstétricas. Sua patogênese está associada à presença de anticorpos antifosfolípides e/ou anti-β2 glicoproteína I (β2GPI) que aparentemente modificam o efeito anticoagulante da β2GPI. A dimerização da β2GPI induzida por anticorpos parece estar relacionada à indução da agregação plaquetária contribuindo para o estado trombofílico na SAF.
Objetivos: O presente trabalho objetiva demonstrar a influencia dos anticorpos antifosfolípides em testes de agregação plaquetária com diferentes agonistas (ADP, colágeno e adrenalina).
Métodos: Foram analisados testes de agregação de plaquetas normais com diferentes agonistas (ADP, colágeno, adrenalina) na presença de soro contendo anticorpos antifosfolípides em diferentes concentrações.
Resultados: As análises obtidas mostraram uma inibição significativa ($P < 0,05$) nas curvas de agregação plaquetária induzidas por ADP e adrenalina quando comparadas ao controle. O paradoxo entre o estado protrombótico e a presença de autoanticorpos que *in vitro* apresentam atividade anticoagulante foi demonstrado na literatura, dificultando o entendimento patofisiológico da síndrome antifosfolípide.
Conclusão: Os resultados obtidos demonstraram que o soro rico em anticorpos anticardiolipina e anti-β2GPI, ambas da classe IgG, interferem em testes de curvas de agregação plaquetária.

Introduction

Primary antiphospholipid syndrome (APS) is a clinical condition characterized by recurrent venous and arterial thrombosis and fetal mortality with recurrent miscarriage associated with the presence of antiphospholipid antibodies (aPL).[1,2]

Laboratorial tests to characterize APS include immunoassay for anticardiolipin (aCL) IgM and IgG antibodies, anti-β2-glycoprotein-I antibodies, and coagulation tests to determine the presence of lupus anticoagulant (LA). Confirmation of the presence of these antibodies 12 weeks after they are first identified in the patient's serum allows the exclusion of transitory cases and the classification of patients according to the criteria of the XI International Congress on antiphospholipid antibodies in Sydney, Australia, 2006.[3,4,5]

β2-glycoprotein-I, also known as apolipoprotein H, is a phospholipid cofactor with anticoagulant characteristics. It has an important role in the preservation of vascular endothelial surface, forming a complex with phospholipids and prothrombin. It inhibits the activation of coagulation factor XII and it seems to contribute with the activation of protein C. On platelet surfaces, β2GPI inhibits the generation of factor Xa, besides blocking platelet aggregation and conversion of prothrombin to thrombin.[6]

The pathogenesis of APS is uncertain, but it is believed that the interaction of autoantibodies against anionic phospholipids or against β2GPI present on platelets would be associated with the release of thrombogenic components. Antiphospholipid antibodies would occupy spaces on the β2GPI-prothrombin complex, with the consequent endothelial and platelet activation.[7]

To understand the pathophysiology of this syndrome, one should analyze the different clinical types of APS and the elements considered to participate of the pathophysiological process of the disease, such as platelets, thrombin, and β2GPI. The search for the main reasons of this phenomenon will provide better therapeutic choices for these patients in the future. Therefore, the study of laboratory tests used in the diagnosis of this syndrome is paramount.

Material and methods

Female patient with APS

A female patient with APS, according to the criteria of the XI International Congress on antiphospholipid antibodies in Sydney, Australia, 2006,[3] was selected to obtain the serum with aPL. The study was approved by the ethics on research committee of the Universidade Santo Amaro with registry number 069/2011, and the patient signed an informed consent.

A 24-year old female patient who lived in Volta Redonda, RJ, experienced a miscarriage in November 2000, in the 12th week of pregnancy, and again in March 2002, in her second pregnancy, approximately in the 18th week. The histopathology of the second miscarriage showed partial fetal and placental autolysis due to extensive ischemic infarctions, suggestive of deep venous thrombosis of maternal origin. Soon after the second miscarriage (May 2002), the patient developed hypertension and episodes of absence that were related to probable cerebral ischemia, which made her clinician suspect of prothrombotic and circulatory disorders.

Laboratory tests requested by her clinician in May 2002 showed: aPTT 28 sec. with normal values ranging from 25-35 sec., and INR 1.0; antinuclear factor (ANF) and rheumatoid factor non reactive; positive VDRL titers 1:4; FTA-ABS IgM and IgG negative; positive direct Coombs; IgG aCL antibodies 65 GPL (negative < 10 GPL); IgM aCL antibodies 15 MPL (normal < 10 MPL). Complete blood count showed 6,500 leukocytes/mm^3 (normal = 4,500-11,000) without left shift; mild anemia with hematocrit of 35% (normal = 37-47%); hemoglobin 11.8 g/dL (normal = 12-16 g/dL); RBC = 3.5 million/mm^3 (normal = 4-5.5 million/mm^3), and mild thrombocytopenia, with 135,000 platelets/mm^3 (normal = 150,00-400,000/mm^3). Biochemistry showed normal glucose and liver function tests (AST, ALT, and bilirubin), but the patient had mild change in creatinine clearance, at 85 mL/min (normal = 97-130 mL/min).

Anticardiolipin antibodies were positive two other times in routine tests, in September 2002 (IgG aCL 73 GPL and IgM aCL 13.5 MPL) and October 2002 (IgG aCL 63 GPL and IgM aCL 11.5 MPL). Positive aCL at intervals greater than 12 weeks (May, September, and October), associated with clinical criteria of recurrent miscarriages allowed her characterization as having primary antiphospholipid syndrome, according to the criteria established in Sydney, in 2006.[3] Treatment with anticoagulants was instituted with periodic PT and INR.

aPL antibodies

During three months, one blood sample a month was collected from the patient in a tube without anticoagulant. Blood samples were centrifuged at 3,000 rotations per minute (rpms) for 10 minutes, and the serum was labeled sample 1 (A1), sample 2 (A2), and sample 3 (A3), as it was collected. Since the patient was on oral anticoagulant, under close supervision of her clinician and strict evaluation of possible risks, those medications were discontinued 7 days prior to the collection date to avoid interference with the procedure.[8]

Lupus anticoagulant testing, performed through the Russell viper venom test (Dade Behring®) was negative (integrated automated test which includes the screening and confirmation testing) and confirmed by aPTT testing with silica activator.[9]

Quantification of aPL for aCL and anti-β_2GPI antibodies was done using the IgG/IgM EliA immunoassay (ImmunoCAP 250, Phadia, Pharmacia Diagnostics®), with sensitivity and specificity similar to other immunoassay testing, such as ELISA, for antiphospholipid antibodies.[10] Results were reported in IgG phospholipid units (GPL) and IgM phospholipid units (MPL) where 1 unit is equal to 1 mg/mL of IgG or IgM (Tables 1 and 2).

Platelet aggregation test

The pool of platelet-rich plasma (PRP) was obtained from 20 healthy volunteers, ages 28-43 years, without a history of coagulation disorders, and with normal PT and aPTT. After the pool was obtained, it underwent aPL testing (IgM and IgG anticardiolipin antibodies and IgM and IgG anti-β_2GPI antibodies), using the same method used in the aPL-rich serum, with negative results (less than 10 MPL, for IgM aCL, and less than 10 GPL, for IgG aCL). Blood samples were collected in tubes with sodium citrate at a rate of 1:9 and centrifuged at 1,000 rpm for 15 minutes. The pool of plasma obtained was used as control for aggregation curves. Platelet-poor plasma (PPP) was obtained from these patients for calibration of the aggregometer after centrifugation at 2,500 rpm for 10 minutes. Agonists used included: ADP (1 µL-1nM), collagen (0,5 µL-1 mg/mL), and adrenaline (0,5 µL-1 mg/mL) (Chrono-par®, Chrono-log Corporation, USA) and platelet aggregation tests were performed in a Chrono-log aggregometer model 530 (Biomédica S/C Ltda).

aPL-containing serum samples, A1, A2, and A3, were added to the PRP with a final volume of 500 µL (Table 3) and underwent aggregation. The number of platelets in the samples was verified and standardized in 300,000/mm^3 in all samples, an adequate number for the aggregation tests performed in the equipment used.[11] The Mann-Whitney test was used to analyze the results, adopting a significance level of $P < 0.05$.

Results

Tables 4, 5, and 6 show the mean and standard deviation (M ± SD) of the results of platelet aggregation expressed in % after 5 minutes using the pool of control PRP and after the addition of the aPL in different concentrations, A1, A2, and A3.

Table 1 – Results of aPL antibodies in samples A1, A2, and A3.

	ACL, IgM antibodies	ACL, IgG antibodies
A1	12.3 MPL	71.1 GPL
A2	10.5 MPL	71.9 GPL
A3	10.8 MPL	72.4 GPL
Reference values*	IgM: Negative → Lower than 10 MPL Undetermined → 10-19 MPL Moderate reactivity →20-80 MPL Strong reactivity → Above 80 MPL	IgG: Negative → Lower than 10 GPL Undetermined → 10-19 GPL Moderate reactivity → 20-80 GPL Strong reactivity → Above 80 GPL

*Reference value for EliA IgG/IgM (ImmunoCAP 250, Phadia, Pharmacia Diagnostics®).

Table 2 – Results of anti-β2GPI antibodies in samples A1, A2, and A3.

	β2 GPI, IgM antibodies	β2 GPI, IgG antibodies
A1	Lower than 5 U/mL	Greater than 100 U/mL
A2	Lower than 5 U/mL	Greater than 100 U/mL
A3	Lower than 5 U/mL	Greater than 100 U/mL
Reference values	IgM: Negative → Lower than 5 U/mL Undetermined → 5-8 U/mL Positive → Above 8 U mL	IgG: Negative → Lower than 5 U/mL Undetermined → 5-8 U/mL Positive → Above 8 U/mL

Table 3 – Volume of addition of samples A1, A2, and A3 in platelet-rich plasma for platelet aggregation.

Sample	Volume added	Volume of PRP	Volume and concentration of the aggregant added
A1/A2/A3	50 μL	450 μL	ADP (1 μL – 1 nM)
A1/A2/A3	100 μL	400 μL	ADP (1 μL – 1 nM)
A1/A2/A3	150 μL	350 μL	ADP (1 μL – 1 nM)
A1/A2/A3	50 μL	450 μL	Colagen (0.5 μL – 1 mg/mL)
A1/A2/A3	100 μL	400 μL	Colagen (0.5 μL – 1 mg/mL)
A1/A2/A3	150 μL	350 μL	Colagen (0.5 μL – 1 mg/mL)
A1/A2/A3	50 μL	450	Adrenaline (1 μL – 5 nM)
A1/A2/A3	100 μL	400	Adrenaline (1 μL – 5 nM)
A1/A2/A3	150 μL	350	Adrenaline (1 μL – 5 nM)

Table 4, for the aggregant adrenaline, shows a statistically significant fall ($P < 0.05$) in the values of platelet aggregation in the presence of anti-β2GPI IgG- and aCL IgG-rich serum.

Regarding the agonist ADP, shown in Table 5, a statistically significant fall was also observed with the two higher concentrations of anti-β2GPI IgG and aCL IgG ($P < 0.05$).

As for the agonist collagen, whose values are shown in table 6, a statistically significant influence was not observed with anti-β2GPI IgG and aCL IgG ($P > 0.05$).

Discussion

The characteristic of APS is the association of several clinical aspects, including arterial or venous thrombosis and morbidity during pregnancy (recurring fetal loss, preeclampsia, eclampsia, and spontaneous miscarriages) associated with the presence of aPL.[12]

The name APS seems to be incorrect due to the discovery that some forms of aPL are not directed against phospholipids, but against proteins with phospholipid complexes, such as β_2GPI, prothrombin, and annexin V.[5,13-15] Prior studies suggest that anti-β_2GPI in complexes with β_2GPI activates platelets, potentially contributing for the thrombotic tendency of patients with APS. However, the presence of aPL does not implicates in the development of APS, as they can be present in up to 1% of the normal population and in 3% of elderly individuals.[16-18]

To verify whether evidence of *in vivo* platelet hyperactivity would be reproduced *in vitro*, analysis of platelet aggregation curves with different agonists in the presence of aPL-rich human serum of a patient with primary APS diagnosed according to the criteria of the XI International Congress on antiphospholipid antibodies held in Sydney, in 2006, was performed. Samples collected from the patient at varying intervals showed elevated IgG aCL, which the literature has indicated to be more relevant than the IgM aCL regarding thrombotic phenomena.[19] The titers of anti-β_2GPI IgG antibodies were also elevated in all three samples (> 100 U/mL). These antibodies are fundamental for the clinical aspects of APS, since the β_2GPI plasma protein to which they bind has a great affinity for anionic phospholipids, working as a cofactor for the development of APS.[20]

The results showed a statistically significant ($P < 0.05$) inhibition of platelet aggregation for adrenaline, in all antibody concentrations, and ADP, in the two highest antibody concentrations. As for collagen, the inhibition was non-significant.

This apparent paradox between aPL and the inhibition of platelet aggregation was also demonstrated in a recent study in which elevated levels of anti-β_2GPI antibodies reduced significantly platelet aggregation (this inhibition was proportional to the concentration of said antibodies) in the presence of the aggregants, ADP and collagen.[21]

The inhibition of platelet aggregation after addition of aCL- and anti-β_2GPI antibodies-containing serum *in vitro* suggests that other factors are implied in the thrombotic phenomena that occur *in vivo*. Activation of endothelial cells, endothelial lesion mediated by oxidants, interference with proteins that bind anionic phospholipids responsible for hemostasis, interactions of these antibodies with monocytes or natural anticoagulants, such as proteins S and C, can also justify the vaso-occlusive phenomena.[22] Another possibility would be

Table 4 – Mean aggregation % of samples A1, A2, and A3 after 5 minutes of aggregation with adrenaline.

Control	50 μL	100 μL	150 μL
A1 = 70%	A1 = 59%	A1 = 56%	A1 = 53%
A2 = 70%	A2 = 61%	A2 = 60%	A2 = 57%
A3 = 70%	A3 = 56 %	A3 = 52%	A3 = 50%
Mean = 70%	Mean = 59%	Mean = 56%	Mean = 53%
	P = 0.016	P = 0.026	P = 0.014

According to the Mann-Whitney test, a significant inhibition ($P < 0.05$) was observed in samples A1, A2, and A3 for the agonist adrenaline.

Table 5 – Mean aggregation % of samples A1, A2, and A3 after 5 minutes of aggregation with ADP.

Control	50 μL	100 μL	150 μL
A1 = 74%	A1 = 74%	A1 = 59%	A1 = 58%
A2 = 74%	A2 = 58%	A2 = 63%	A2 = 56%
A3 = 74%	A3 = 58%	A3 = 58%	A3 = 51%
Mean = 74%	Mean = 63%	Mean = 60%	Mean = 55%
	P = 0.184	P = 0.012	P = 0.012

According to the Mann-Whitney test, a significant inhibition ($P < 0.05$) was observed for samples A1, A2, and A3 for the agonist ADP in the volumes of 100 μL and 150 μL.

Table 6 – Mean aggregation % of samples A1, A2, and A3 after 5 minutes of aggregation with collagen.

Control	50 μL	100 μL	150 μL
A1 = 70%	A1 = 69%	A1 = 69%	A1 = 68%
A2 = 67%	A2 = 65%	A2 = 63%	A2 = 62%
A3 = 67%	A3 = 65%	A3 = 60%	A3 = 53%
Mean = 68%	Mean = 66%	Mean = 64%	Mean = 61%
	P = 0.378	P = 0.267	P = 0.246

According to the Mann-Whitney, test, significant inhibition was not observed ($P > 0.05$) for samples A1, A2, and A3 for the agonist collagen.

that anti-β_2GPI antibodies may inhibit the release of platelet dense granules and inhibit the arachidonic acid metabolic pathway.[21]

In a selection of clinical studies performed with aPL, only LA was consistently related as a risk factor for clinical thrombotic phenomena. However, studies with aCL and anti-β_2GPI antibodies were inconclusive and partly controversial. aCL IgG and anti-β_2GPI IgG demonstrated greater relationship with thrombotic processes (aCL related to more specific situations of stroke and myocardial lesions) than the respective IgM antibodies, but they did not show the close relationship that LA has with those phenomena.[23]

The aforementioned data seem important for the interpretation of our results of inhibition of platelet aggregation, since the level of LA of the patient with primary APS was negative in the samples used for the testing, and these antibodies are more closely related to clinical thrombosis.

Besides, other authors have demonstrated, in patients with thromboembolic complications, the presence of autoantibodies that, *in vitro*, showed to have anticoagulant activity and caused prolongation of coagulation tests.[20]

The results of the present study suggest that the *in vivo* thrombotic effect of aPL can be associated to the type of antibodies present in the patient with APS and other factors that were not detected in the *in vitro* aggregation test. The use of these aggregation tests for diagnosis can cause misinterpretation and failure to refer the patient to test for the presence of aPL even in the presence of symptomatology compatible with APS.

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Conflicts of interest

The authors declare no conflicts of interest.

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