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Antibodies to β_2-glycoprotein I—a specific marker for the antiphospholipid syndrome

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SUMMARY

The antiphospholipid syndrome is a disorder characterized by recurrent thrombosis and the presence of antibodies specific to phospholipids. However, the diagnosis of this syndrome is hampered by the lack of a specific laboratory test. In this study an ELISA for the measurement of antibodies to solid-phase β_2-glycoprotein I (β_2-GPI) was established and compared with anticardiolipin antibodies for diagnosis of antiphospholipid syndrome. Significantly elevated levels of antibodies to β_2-GPI were found in all patients with definite antiphospholipid syndrome (median = 91 AU). Marginally elevated levels of antibodies to β_2-GPI were observed in 5% of patients with systemic lupus erythematosus (SLE; median = 4 AU), 1% with stroke (median = 3 AU), 13% with infectious mononucleosis (median = 3 AU), 10% with HIV infection (median = 3 AU) and 8% with VDRL false-positive serology for syphilis (median = 4 AU), but not in patients with rheumatoid factor, syphilis or carotid artery stenosis. In contrast, significantly raised levels of anticardiolipin antibodies were observed in 100% of patients with definite antiphospholipid syndrome, 30% with SLE, 88% with HIV infection, 94% with syphilis, 62% with infectious mononucleosis, 9% with rheumatoid factor-positive sera, 74% VDRL false-positive serology for syphilis, 47% with stroke and 0% with carotid artery stenosis. This solid-phase assay for antibodies to β_2-GPI is highly specific for the antiphospholipid syndrome and represents an advance in the laboratory diagnosis of this disorder.

Keywords β_2-glycoprotein I antiphospholipid syndrome ELISA cardiolipin thrombosis

INTRODUCTION

Antiphospholipid antibodies are a group of autoantibodies associated with increased risk of arterial or venous thrombosis, recurrent fetal loss and thrombocytopenia. The syndrome characterized by the presence of these clinical manifestations has been termed the antiphospholipid syndrome (APS), and may occur as a primary disorder or may be secondary to connective tissue disease, most commonly systemic lupus erythematosus (SLE) [1]. Patients with infections, notably syphilis, tuberculosis, endocarditis, infectious mononucleosis and HIV also develop antiphospholipid antibodies, but these antibodies are not associated with vascular complications [1–3]. In addition, patients with other inflammatory and connective tissue disease processes may also have antiphospholipid antibodies in the absence of clinical manifestations of the disorder [4].

The laboratory diagnosis of APS involves a co-ordinated group of tests for antiphospholipid antibodies. Additional tests such as platelet count and direct Coombs' test may also be useful. Antiphospholipid antibodies are identified in plasma by their ability to prolong phospholipid-dependent clotting times, i.e. lupus anticoagulant, and by their ability in serum to bind to solid-phase phospholipids, most commonly cardiolipin. However, there remains a great deal of controversy as to the most sensitive test(s) for the detection of lupus anticoagulant and the optimal conditions for identification of anticardiolipin antibodies (ACA) [5,6]. In addition, coagulation-based assays require fresh plasma for analysis, and such assays cannot easily be performed in the presence of anticoagulant therapy. Furthermore, neither ACA nor lupus anticoagulant are specific for APS [1–3]. Therefore the laboratory diagnosis of APS is particularly hampered by the lack of an appropriate specific test.

More recently, β_2-glycoprotein I (β_2-GPI), a 50-kD plasma protein, has been identified as a cofactor for the binding of antiphospholipid antibodies to phospholipids [7–9]. β_2-GPI has been shown to inhibit contact activation of the intrinsic blood coagulation pathway [10], ADP-mediated platelet aggregation [11], prothrombinase activity of activated platelets [12], and can

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bind negatively charged macromolecules such as DNA [13] and heparin [14].

Three different hypotheses have been proposed to explain cofactor activity. It is suggested that antibodies recognize (i) an epitope on β_2-GPI alone [7], (ii) an epitope on β_2-GPI–phospholipid complex formed on the interaction of β_2-GPI with phospholipid [8], and (iii) a cryptic epitope formed on β_2-GPI following interaction with phospholipids [15]. It has been suggested that the precise site on β_2-GPI recognized by anti-phospholipid antibodies is contained within a lysine-rich region on the fifth domain [16].

In this study we describe an ELISA for the measurement of antibodies to solid-phase β_2-GPI. A range of sera was examined for the presence of antibodies to β_2-GPI, notably patients with infections, stroke, carotid artery stenosis, rheumatoid factor (RF), systemic lupus erythematosus (SLE) and subjects with a putative clinical and laboratory diagnosis of APS. The level of anti-β_2-GPI antibodies was correlated with the clinical manifestations and compared with the level of anticardiolipin antibody in all patient groups.

PATIENTS AND METHODS

Patients

Sera from the following patient groups were examined for antibodies to β_2-GPI and cardiolipin: putative APS ($n = 29$), SLE ($n = 20$), RF^+ ($n = 22$), stroke ($n = 85$), carotid artery stenosis ($n = 24$), syphilis ($n = 16$), VDRL false-positive serology for syphilis ($n = 24$), infectious mononucleosis ($n = 30$), HIV ($n = 30$) and normal controls ($n = 48$). Patients with putative APS were identified as a group of patients with ACA where there was also a working clinical diagnosis of APS.

Clinical patient details

The charts of patients with a putative diagnosis of APS, SLE, stroke and carotid artery stenosis were retrieved and examined for clinical and laboratory features of the APS according to the criteria for classification of the primary APS outlined by Alarcon-Segovia & Sanchez-Guerrero in 1989 [17].

Anti-β_2-GP1 ELISA

β_2-GPI (Crystal Chem, Chicago, IL) was coated overnight onto a microtitre plate (Nunc Maxisorp, Roskilde, Denmark) at a concentration of 2 μg/well in 0·012 M disodium carbonate, 0·035 M sodium hydrogen carbonate buffer, pH 9·6. Checkerboard experiments were performed to determine the optimum concentration of β_2-GPI with which to coat plates. After incubation, β_2-GPI-coated wells were washed three times in 0·1 M sodium phosphate, 0·15 M sodium chloride pH 7·2, 0·05% (v/v) Tween 20 (PBS–T). Wells were incubated with 100 μl of a 1:25 dilution of serum in PBS-T for 1 h at 37°C. Wells were washed as before and incubated with 100 μl peroxidase-conjugated rabbit immunoglobulins to human IgG (Dakopatts, Glostrup, Denmark) at a 1:1000 dilution in PBS–T for 1 h at 37°C. The wells were again washed and 100 μl of 1,2-phenylenediamine (Dakopatts) substrate were added. The reaction was allowed to proceed at room temperature for 3 min and the absorbance measured at 492 nm using a microplate reader (Titertek Multiscan plus MKII; Flow Labs Int. SA, Switzerland). A standard curve was established using a known positive serum. A reference range was established in a normal control group ($n = 48$) from a mean value in arbitrary units (AU) ± 5 s.d. All test sera were assayed in triplicate, PBS–T was used as a control blank (mean optical density (OD) = 0·07) and a quality control serum was included on each plate. The intra-assay and interassay variations which were established for the assay were 10% and 14%, respectively.

Inhibition experiments

The ability of both fluid and solid-phase β_2-GPI to absorb specifically anti-β_2-GPI antibody reactivity was examined. To measure the inhibition of anti-β_2-GPI antibodies by fluid-phase β_2-GPI, antibodies were first diluted to a dilution previously found to be midway along the linear portion of the standard curve, and incubated with β_2-GPI (2–64 μg/well) in PBS–T for 2 h at 37°C and overnight at 4°C. To measure inhibition by solid-phase β_2-GPI, β_2-GPI (2–64 μg/well) was also coated overnight onto a microtitre plate. After washing, 100 μl of the appropriate serum dilution were added and incubated as described. The serum was recovered and retested on the anti-β_2-GPI ELISA. Ovalbumin (Sigma, St Louis, MO; 2–64 μg/well) was used as an irrelevant control antigen in all experiments. To ensure that β_2-GPI did not non-specifically absorb antibody, fluid and solid-phase β_2-GPI were also incubated with serum known to contain antibodies to the protein alpha-gliadin, and the sera were then retested for antibody levels to alpha-gliadin.

Anticardiolipin ELISA

ACA were assayed in the patient groups using a commercial assay for ACA (Diastat; Shield Diagnostics Ltd, Dundee, UK) according to the manufacturer's instructions. A reference range was established as for the anti-β_2-GPI ELISA.

RESULTS

Anti-β_2-GPI levels in the different patient populations

Figure 1 illustrates the levels of antibodies to β_2-GPI in the patient groups and Table 1 represents the percentage positivity using 9 AU as the positive cut-off value as determined from a normal control group (mean 2·9 AU + 5 s.d.). Significantly elevated levels of β_2-GPI antibodies were only detected in patients with putative APS (76%, median = 54 AU). Marginally elevated levels of antibodies

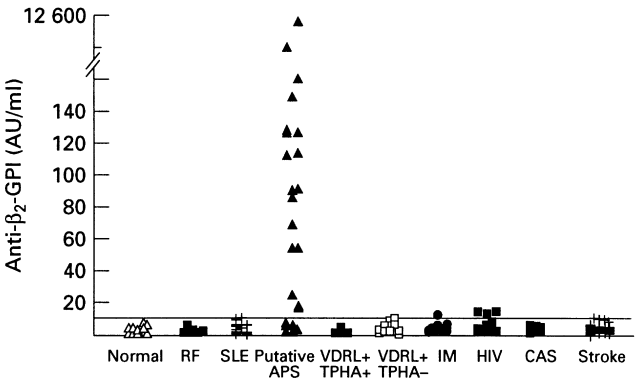

Fig. 1. The levels of antibodies to β_2-glycoprotein I (β_2-GPI) in the patient groups. Levels of antibodies to β_2-GPI in the different populations tested using 9 AU as the positive cut-off value (horizontal line). These subjects consisted of normal controls, rheumatoid factor-positive (RF), systemic lupus erythematosus (SLE), putative antiphospholipid syndrome (APS), syphilitic (VDRL+, TPHA+), VDRL false-positive serology for syphilis (VDRL+, TPHA–), infectious mononucleosis (IM), HIV, carotid artery stenosis (CAS) and stroke.

Table 1. The percentage positivity and median levels of antibodies to β_2-glycoprotein I (β_2-GPI), using 9·0 AU as the positive cut-off value

Number	Patient group	Percent positivity	Range (AU)	Median
29	Putative APS	76	1–12 400	54
20	SLE	5	1–11	3
22	Rheumatoid factor	0	1–7	1
24	Carotid artery stenosis	0	1–6	3
85	Stroke	2	1–10	3
16	VDRL+, TPHA+	0	1–5	1
24	VDRL+, TPHA−	8	1–11	4
30	Infectious mononucleosis	13	1–13	3
30	HIV	10	1–15	3

Abbreviations as in Fig. 1.

to β_2-GPI were observed in patients with SLE (5%, median = 4 AU), stroke (1%), infectious mononucleosis (13%), HIV (10%) and VDRL false-positive serology for syphilis (8%). The median value of antibodies to β_2-GPI in all of these groups was 2–4 AU (Table 1). Elevated antibody levels to β_2-GPI were not seen in patients with RF, syphilis or carotid artery stenosis.

Patient clinical details

The medical histories of patients with putative APS and SLE were independently reviewed for clinical and laboratory features of APS (Table 2). These patients were assigned to one of three groups: definite, probable and not APS, according to the criteria for classification of primary APS outlined by Alarcon-Segovia & Sanchez-Guerrero in 1989 [17]. A fourth group designated possible APS ($n = 6$) was defined for either: patients with ACA and a clinical manifestation of APS which could clearly be explained on the basis of alternate pathology ($n = 5$); or patients with a clinical manifestation of APS who had positive lupus anticoagulant and negative ACA on repeat testing ($n = 1$).

Seventeen patients were identified as definite APS, five with probable, six with possible and one as not APS (Table 2). All patients with definite APS ($n = 17$) had significantly elevated levels of antibodies to β_2-GPI. Two of five patients with probable and three of six with possible, had raised levels of antibodies to β_2-GPI. Of 20 patients with SLE, 19 had no clinical features of the APS and were categorized as not APS, and one patient was considered to have probable APS.

The medical histories of patients with carotid artery stenosis and stroke were also reviewed for evidence of APS according to published criteria for classification of APS [17]. None of these patients had definite or probable APS, nor did they have elevated levels of antibodies to β_2-GPI. Although the charts of HIV, infectious mononucleosis, syphilis, VDRL false-positive serology for syphilis or RF^+ patients were not reviewed, there was no additional clinical or laboratory evidence to suggest APS in these patients.

Lupus anticoagulant results were not available on all the patients reviewed. Of those patients on whom a result was available, lupus anticoagulant positivity was noted in 12/12 patients classified as definite APS, 3/5 patients with probable and 2/2 patients classified as possible APS. One patient classified as not APS was negative for lupus anticoagulant. In the group of 20 SLE patients, lupus anticoagulant was positive in one of 13 tested.

Absorption studies

To examine the specificity of positive sera for β_2-GPI, serum was absorbed with β_2-GPI in both fluid and solid phases. For all sera tested, antibody activity could be absorbed specifically with solid-phase β_2-GP1 (mean percentage inhibition = 40%). It was found that fluid-phase β_2-GPI did not absorb antibody reactivity to β_2-GPI at 64 μg/well (mean percentage inhibition = 2%). When a control antigen, ovalbumin, was used, no change in antibody level was noted (mean percentage inhibition = 1%). β_2-GPI did not non-specifically absorb alpha-gliadin antibody which was used as an irrelevant antibody control (mean percentage inhibition < 1%).

Anticardiolipin antibody levels in patient groups

Figure 2 illustrates the levels of ACA in the different patient groups tested using 15·1 anti-cardiolipin IgG (GPL) units/ml as the positive cut-off value as determined from a normal control group (mean 5·16 GPL units/ml + 5 s.d.). Elevated levels of ACA were seen in the following patients: putative APS (100%), SLE (30%), HIV (88%), syphilis (94%), infectious mononucleosis (62%), RF^+

Table 2. Clinical and laboratory evidence of the antiphospholipid syndrome (APS)

Patient group	Clinical features	Anti-β_2-GPI antibody	Anti-cardiolipin
Definite APS ($n = 17$)	Thrombosis, 13/17 (76%) Thrombocytopenia, 12/17 (71%) Miscarriage, 7/17 (41%)	17/17	17/17
Probable APS ($n = 5$)	Thrombosis, 2/5 (40%) Thrombocytopenia, 2/5 (40%) Miscarriage, 2/5 (40%)	2/5	5/5
Possible APS ($n = 6$)	Thrombosis, 3/6 (50%) Thrombocytopenia, 3/6 (50%) Miscarriage, 1/6 (14%)	3/6	5/6
Not APS ($n = 20$)	No relevant clinical features, 1/20 SLE 19/20	0/20	6/20

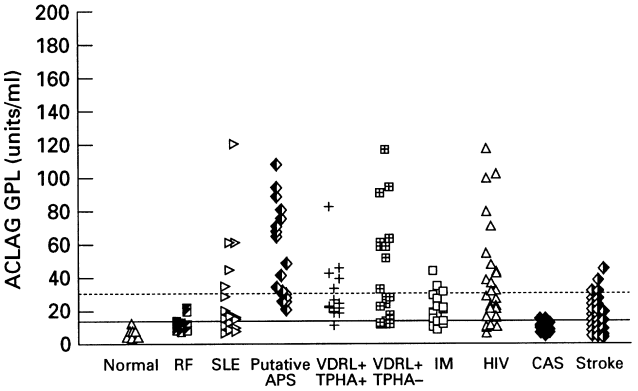

Fig. 2. The levels of anticardiolipin antibodies in the patient groups. Levels of anticardiolipin antibodies in the different groups tested using 15·1 GPL units/ml (—) or 30 GPL units/ml (manufacturer's recommendations) (– – –) as the positive cut-off values. Abbreviations as in Fig. 1.

(9%), VDRL false-positive serology for syphilis (74%), carotid artery stenosis (0%) and stroke (47%).

Specificity, sensitivity, positive and negative predictive values for the anti-β_2-GPI ELISA and anticardiolipin ELISA

Anti-β_2-GPI ELISA. Using 9 AU (mean + 5 s.d.) as the positive cut off, 31 patients had raised levels of anti-β_2-GPI antibodies (total patient number = 280); of these, 19 were identified as having definite or probable APS. In total 22 patients had definite or probable APS, four of which were identified as probable APS but anti-β_2-GPI antibody-negative. The specificity, sensitivity, positive and negative predictive values of the anti-β_2-GPI ELISA for definite or probable APS are 95%, 83%, 0·61 and 0·98, respectively. However, as there was no overlap in the level of β_2-GPI antibodies between patients in any of the control groups and patients with definite APS, an arbitrary cut off of 15 units (the highest level of antibodies in the disease control groups) may be chosen. Using this cut off, 21 patients were identified with raised levels of antibodies to β_2-GPI, of which 19 had definite or probable APS and two had possible APS. Applying this cut-off value the specificity, sensitivity, positive and negative predictive values of the anti-β_2-GPI ELISA for definite or probable APS were 99%, 83%, 0·9 and 0·98, respectively.

Anticardiolipin ELISA. Using the mean + 5 s.d. of the normal control group (15 GPL units/ml), 145 patients had significantly elevated levels of ACA (total patient number = 280), of which 23 were identified as having definite or probable APS. No patient identified with definite or probable APS was ACA-negative. The specificity, sensitivity, positive and negative predictive values of the anticardiolipin ELISA for definite or probable APS are 53%, 100%, 0·16 and 1·0, respectively.

Applying the recommended manufacturer's positive cut-off value (30 GPL units/ml), 64 patients were identified with raised levels of ACA, of which 20 had definite or probable APS. Three patients identified with probable APS were ACA-negative. Using this cut-off value the specificity, sensitivity, positive and negative predictive values of the anticardiolipin ELISA for definite and probable APS are 85%, 87%, 0·31 and 0·99, respectively.

DISCUSSION

Laboratory tests for APS suffer from a lack of specificity, with abnormal results seen in a variety of non-thrombotic disorders, HIV infection [18], AIDS [19], syphilis [20], malignancy [21], pregnancy [22] and following certain drug therapies [23]. Therefore, no single definitive test exists for the laboratory diagnosis of the APS. Establishing an accurate clinical diagnosis of APS is also fraught with difficulty, as the main manifestations of the disorder (recurrent thrombosis, recurrent miscarriage and thrombocytopenia) have many common causes outside of APS. Furthermore, a bewildering array of clinical manifestations has been attributed to the syndrome [24]. To complicate this issue further, APS has been reported to exist both as a primary disorder and also secondary to the multi-system inflammatory disorder SLE [25].

Recent studies have emerged demonstrating that antibodies specific to β_2-GPI are more strongly associated with clinical manifestations of APS than antibodies directed at cardiolipin [26–32]. In this study we established a solid-phase assay to measure antibodies to β_2-GPI in patients with thrombotic and non-thrombotic disorders. Significantly elevated levels of antibodies to β_2-GPI were found only in patients with a putative diagnosis of APS (Fig. 1). Marginally raised levels of antibodies to β_2-GPI were observed in some of the other groups tested, but the median value of β_2-GPI antibodies in these groups was 2–4 AU, in comparison with a median value of 54 AU in the putative APS group.

The hospital charts of patients categorized as having putative APS were retrieved and examined for clinical and laboratory features of APS. These patients were then assigned to one of four groups: definite, probable, possible or not APS. All patients with definite APS ($n = 17$) had significantly elevated levels of antibodies to β_2-GPI (median = 91 AU). Two patients with probable and three patients with possible APS had raised levels of antibodies to β_2-GPI: it will be interesting to follow these patients to see if the presence of antibodies to β_2-GPI marks for the future development of a definite diagnosis of APS. The remaining patients in the putative APS group (probable $n = 3$, possible $n = 3$ and not APS $n = 1$) did not have raised levels of antibodies to β_2-GPI. Moreover, none of the patients with stroke or carotid artery stenosis fulfilled the criteria for definite or probable APS, and none had significantly elevated levels of antibodies to β_2-GPI. Furthermore, there was no overlap between the level of antibody to β_2-GPI found in the definite APS group and any other control group examined. Other studies have also examined the incidence of β_2-GPI antibodies in patients with a history of thrombosis [26–32]. However, to determine the specificity of β_2-GPI for APS it is important to define APS according to established criteria. In this study a group of patients was identified with a working diagnosis of APS and elevated levels of antibodies to cardiolipin. These patients were independently reviewed and characterized according to the rigorous diagnostic criteria of Alarcon-Segovia & Sanchez-Guerrero [17]. The reviewer was unaware of the anti-β_2-GPI antibody results. It is striking that all patients with definite APS had elevated levels of antibodies to β_2-GPI. Furthermore, we have particularly examined patient groups with a high rate of 'false-positive' antibodies to cardiolipin, and none showed significantly raised levels of antibodies to β_2-GPI.

Of the 20 SLE patients reviewed, one patient was classified as probable APS and the remaining 19 had no clinical evidence of APS. When considering patients with SLE, it is apparent that the criteria used to identify APS may be less specific. For example, patients with SLE may have thrombocytopenia with haemolytic anaemia and thus fulfil the criteria for definite APS [17]. Furthermore, patients with SLE frequently have multiple predisposing factors for thrombosis unrelated to APS. This is an important issue which needs to be addressed in evaluating patients with SLE for evidence of APS.

The relative frequency of primary *versus* secondary APS is unclear. Of the 17 patients categorized as having definite APS, three could be classified as having SLE. Therefore, applying rigorous criteria, 83% of patients identified with definite APS had it as a primary disorder.

The specificity of antibodies for β_2-GPI was demonstrated by absorption studies with fluid- and solid-phase β_2-GPI. Antibody reactivity was absorbed specifically with solid-phase β_2-GPI. Absorption with fluid-phase β_2-GPI at the highest concentration used, resulted in no decrease in antibody level in any sera tested, suggesting that the antibodies do not recognize fluid-phase β_2-GPI. It is possible that the antibodies are recognizing an exquisite epitope exposed on β_2-GPI subsequent to binding to a high-affinity plastic plate [26,33]. This binding may mirror β_2-GPI binding to a platelet, endothelial cell or phospholipid surface *in vivo*. However,

others have shown that anti-β_2-GPI antibodies may bind to fluid phase with lower affinity ($K_d \approx 10^{-5}$ M) [30].

Patients with definite APS had the full spectrum of disease manifestations, namely recurrent thrombosis, recurrent miscarriage and thrombocytopenia (Table 2). Although the numbers of patients with these manifestations are too small for meaningful analysis, there was no difference in the frequency or level of antibodies to β_2-GPI in patients whose main or sole clinical problem was either thrombosis, miscarriage or thrombocytopenia. Nonetheless, it is tempting to speculate that such patients may have identical underlying pathologies, and the incidence of antibodies to β_2-GPI in the different manifestations of APS may help clarify this issue.

In contrast to the levels of antibodies to β_2-GPI, significantly elevated levels of ACA were observed in patients with SLE, RF, syphilis, VDRL false-positive serology for syphilis, stroke, infectious mononucleosis and HIV. This is in agreement with previous experience with the anticardiolipin assay, where ACA are found in many non-thrombotic disorders [25,34,35]. For the anticardiolipin assay, a positive cut-off value was established from the mean +5 s.d. of normal healthy controls. This was the reference interval used for the anti-β_2-GPI ELISA and gives a true indication of the relative incidence of ACA. The manufacturers of this assay propose a positive cut off which is much higher (30 GPL units/ml). Applying this cut-off value improves the specificity of ACA for APS, but its specificity for APS is still significantly less than that of the anti-β_2-GPI ELISA.

In this study it is difficult to estimate accurately the true incidence (sensitivity) of antibodies to β_2-GPI in patients with APS. It is possible that there may be additional patients with APS, representing a different spectrum of the disorder, who are ACA-negative and have not been included in the study due to selection bias. However, such patients are likely to represent a minority of patients with APS [36].

In conclusion, we report a simple, rapid and highly specific assay for the diagnosis of APS. This assay represents a significant advance in the laboratory diagnosis of APS, and probably identifies APS patients with identical underlying pathologies.

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