

SPECIFICITY OF ELISA FOR ANTIBODY TO β 2-GLYCOPROTEIN I IN PATIENTS WITH ANTIPHOSPHOLIPID SYNDROME

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SUMMARY

The clinical significance of anti- β 2 glycoprotein I (β 2-GPI) antibodies was evaluated in patients with antiphospholipid syndrome (APS), primary and secondary to systemic lupus erythematosus (SLE). Anti- β 2-GPI were tested in 120 patients (39 primary APS, 32 APS with SLE and 49 SLE without APS) by ELISA utilizing irradiated plates in the absence of cardiolipin. Anticardiolipin antibodies (aCL) and antiphosphatidylserine antibodies were also measured in the same patients using standardized assays. Anti- β 2-GPI titres correlated strongly to those of aCL ($r = 0.816$, $P = 0.0001$), and to those of antiphosphatidylserine antibodies ($r = 0.841$, $P = 0.0001$). Anti- β 2-GPI were detected in 53.5% of APS patients (38/71), but only in 4.1% of SLE patients without APS (2/49). In the latter group, 24.5% (12/49) of patients had a positive titre of aCL. The anti- β 2-GPI assay showed higher specificity for APS than the aCL in APS (96 vs 75%, respectively, $\chi^2 = 6.75$, $P = 0.00094$). Our findings suggest that the assay of anti- β 2-GPI may improve the specificity for APS.

KEY WORDS: Anticardiolipin antibody, Systemic lupus erythematosus, Thrombosis, Antiphosphatidylserine antibodies.

SINCE the first study of antiphospholipid antibodies (aPL) by Wassermann, who introduced a serological test for syphilis, the family of aPL has largely expanded to include a heterogeneous group of antibodies, whose specificity is now claimed to be directed not only towards phospholipids, but also towards plasma proteins [1]. The presence of aPL in systemic lupus erythematosus (SLE), in other autoimmune disorders and in apparently healthy people, is associated with arterial/venous thrombosis, recurrent fetal loss, neurological disorders, pulmonary hypertension and thrombocytopenia. The term 'antiphospholipid syndrome' (APS) was coined to link these clinical manifestations with the persistence of aPL, which is now recognized as one of the most frequent causes of acquired thrombophilia [2, 3].

The aCL assay, introduced by our group in 1983 [4], is the most established and standardized method to detect aPL, and most clinical studies rely on aCL ELISA [5]. In 1990, three groups independently reported that aCL associated with APS required a plasma protein as a cofactor to bind cardiolipin on ELISA plates. The plasma protein has been identified as β 2 glycoprotein I (β 2-GPI) [6-8]. Matsuura *et al.* [9] subsequently showed that aCL could recognize β 2-GPI in the absence of cardiolipin if β 2-GPI was coated onto polystyrene plates where oxygen was introduced by radiation. It is now known that the interaction of β 2-GPI with cardiolipin or irradiated ELISA plates allows the exposure of a cryptic epitope which is recognized by aCL [3]. To evaluate the clinical significance of aCL detected as anti- β 2-GPI, we searched for the presence of anti- β 2-GPI using irradiated plates without cardiolipin, in patients with

APS and/or SLE. Although the titre of anti- β 2-GPI correlated highly with that of aCL, anti- β 2-GPI assay proved more specific for APS than the standard aCL assay. None of the sera from our 120 patients bound to β 2-GPI coated on non-irradiated plates. The clinical significance of anti- β 2-GPI assay and the pathogenic roles of these antibodies are discussed.

PATIENTS AND METHODS

Patients

The study population comprised 120 patients (105 female): thirty-nine patients with primary APS (31 female, mean age 40 yr, range 22-60), 32 with APS secondary to SLE (27 female, mean age 40 yr, range 24-62) and 49 with SLE without APS (48 female, mean age 37 yr, range 12-78). The latter group included some patients with clinical features of APS, but who were repeatedly negative for aPL (Table I). All patients fulfilled the proposed criteria for the APS [2, 10] and/or the American College of Rheumatology criteria for the classification of SLE [11].

Anti- β 2-GPI ELISA

Human β 2-GPI, purified from normal human sera by sequential CL-affinity, ion-exchange column and protein A-Sepharose column chromatography [12], was generously provided by Dr Matsuura, Immunology Laboratory, Diagnostics Division, Yamasa Corporation, Japan. Irradiated microtitre plates, Type C (Sumilon Bakelite, Tokyo, Japan), were coated with 4 μ g/ml of purified β 2-GPI in phosphate-buffered saline (PBS) at 4°C and washed twice in PBS. To avoid non-specific binding of proteins, wells were blocked with 150 μ l of 3% gelatin (BDH Chemicals Ltd, Poole). After three washes in PBS containing 0.05% Tween 20 (Sigma Chemical Co., St Louis, MO, USA) (PBS-Tween), 50 μ l of serum diluted with PBS containing 1% bovine serum albumin (BSA) (Sigma) in

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TABLE I
Clinical features of patients

| | APS | | | SLE without APS n = 49 | All patients n = 120 |
|------------------------|-------------------|---------------------|-----------------|---------------------------|-------------------------|
| | Primary n = 39 | Secondary n = 32 | Total n = 71 | | |
| Thrombosis | 34 (87%) | 27 (84%) | 61 (86%) | 6 (12%) | 67 (56%) |
| Venous | 20 (51%) | 15 (47%) | 35 (49%) | 6 (12%) | 41 (34%) |
| Arterial | 23 (59%) | 18 (56%) | 41 (58%) | 2 (4%) | 37 (36%) |
| Recurrent miscarriages | 16/31 (52%) | 10/27 (37%) | 26/58 (45%) | 2/47 (4%) | 28/105 (27%) |
| Thrombocytopenia | 8 (21%) | 12 (38%) | 20 (28%) | 7/47 (15%) | 27 (23%) |

APS, antiphospholipid syndrome; SLE, systemic lupus erythematosus.

1:50 were added in duplicate. Plates were incubated for 1 h at room temperature and washed three times with PBS-Tween. Fifty microlitres per well of the appropriate dilution of alkaline phosphatase-conjugated goat anti-human IgG (Sigma) in PBS containing 1% BSA was added. After 1 h of incubation at room temperature and after four washes in PBS-Tween, 100 μ l/well of 1 mg/ml *p*-nitrophenylphosphate disodium (Sigma) in 1 M diethanolamine buffer (pH 9.8) were added. Following colour development, optical density at 405 nm (OD405) was measured by a Titretek Multiskan MC apparatus (Flow Laboratories, Herts.).

One of the serum samples that had showed high binding to β 2-GPI coated on the irradiated plate was used as a positive control. The titre of IgG aCL of this sample was 89 GPL, according to the international standardization of aCL ELISA [5]. OD405 of doubling dilutions of the control serum (1:50, 100, 200, 400, 800, 1600, 3200 and 6400) was defined as 89, 44.5, 22.3, 11.1, 5.6, 2.8, 1.4 and 0.7 anti- β 2-GPI units (U). The anti- β 2-GPI titre of each sample was derived from the standard curve according to the previous dilutions of the positive control. A normal range was established using 86 healthy controls with a cut-off of 6.0 U being 3 s.d. above the mean.

Anti- β 2-GPI ELISA was repeated using non-irradiated plates, Immulon I (Dynatech Laboratories Inc., Virginia, USA) and Limbro (Nunc A/S, Roskilde, Denmark). The coating efficiency of β 2-GPI to these plates was confirmed by ELISA using rabbit anti-human β 2-GPI antisera (Diagnostica Stago, Asnieres, France) and alkaline phosphatase-conjugated anti-rabbit immunoglobulin.

aCL and antiphosphatidylserine antibodies ELISA

aCL was measured according to the standard aCL ELISA [5]. The cut-off of 5.0 GPL was defined as 3 s.d. above the mean of the same 86 healthy controls in this study.

Antiphosphatidylserine antibody assay was performed by substituting cardiolipin with phosphatidylserine. Antiphosphatidylserine units were defined in the same manner as for anti- β 2-GPI.

Statistical analysis

All statistical analysis was performed by Statview II

(Apple Macintosh software). Comparisons were determined by χ^2 test.

RESULTS

A positive titre of anti- β 2-GPI was found in 40/120 of all patients (33.3%) and in 38/71 APS patients (53.5%) [21/39 primary APS patients (53.8%) and 17/32 secondary APS patients (53.1%)], whereas aCL were detected in 62/120 of all patients (51.7%) and in 50/71 of APS patients (70.4%) [28/39 primary APS patients (71.8%) and 22/32 secondary APS patients (68.8%)]. Patients were divided into four subgroups: (1) aCL-positive APS; (2) lupus anticoagulant-positive but aCL-negative APS; (3) aCL-positive SLE without APS; (4) aCL-negative SLE without APS. Most anti- β 2-GPI-positive patients belonged to group 1, and none of the patients in groups 2 and 4 had anti- β 2-GPI (Fig. 1). The titre of anti- β 2-GPI correlated highly to that of aCL (correlation coefficient: $r = 0.816$, $P = 0.0001$), and to that of antiphosphatidylserine ($r = 0.841$, $P = 0.0001$) (Fig. 2A and B).

In 71 patients with APS, possible correlations between clinical manifestations and anti- β 2-GPI were examined. The presence of anti- β 2-GPI did not correlate significantly with any independent clinical features (arterial or venous thrombosis, recurrent miscarriages or thrombocytopenia) (data not shown).

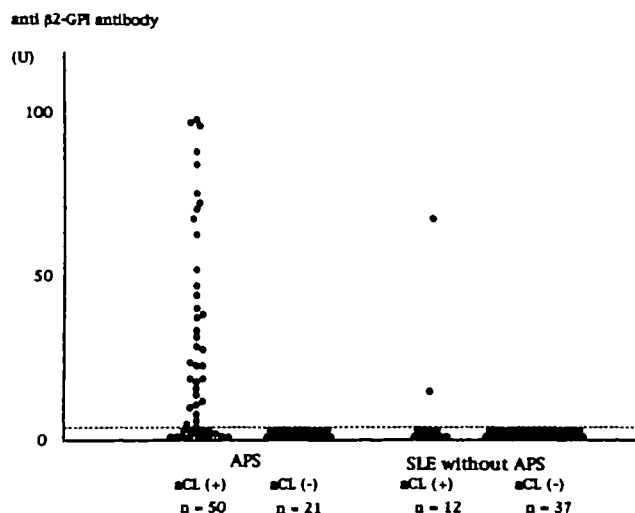


FIG. 1.—Distribution of IgG anti- β 2-GPI in patients with/without APS. None of the 58 patients without aCL had anti- β 2-GPI.

In 49 SLE cases without APS, only two had a positive titre of anti- β 2-GPI (4.1%), but 12 patients were aCL-positive (24.5%). The specificity of anti- β 2-GPI for APS in this study group was 95.9%, a value significantly higher than that of aCL (75.5%) ($\chi^2 = 6.75$, $P = 0.0094$).

Anti- β 2-GPI were not detected in 12 aCL-positive patients with APS (16.9%). Seven (58.3%) had primary APS and five (41.6%) had secondary APS. The frequencies of arterial or venous thrombosis, recurrent miscarriages and thrombocytopenia in these patients were not significantly different from those of other APS patients. Lupus anticoagulant was measured in 10/12 anti- β 2-GPI-negative patients, and was positive in six (60%).

β 2-GPI was coated on two different non-irradiated ELISA plates, and ELISA was performed in the same way. Coating efficiency was examined using polyclonal rabbit anti-human β 2-GPI serum (Fig. 3). None of the 120 patients showed significant binding to β 2-GPI coated on any of the two plates (data not shown).

DISCUSSION

This study showed that anti- β 2-GPI could be detected using irradiated ELISA plates in the absence of cardiolipin in aCL-positive APS patients, and that the titre of anti- β 2-GPI correlated highly not only with

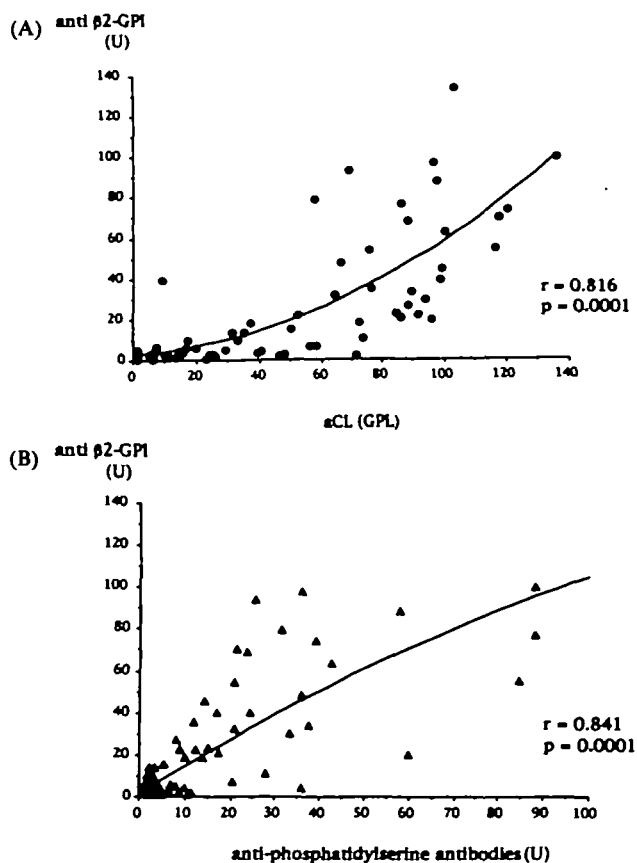


Fig. 2.—Correlation of the titres of anti- β 2-GPI to those of (A) aCL and (B) antiphosphatidylserine antibodies in sera from patients with APS and/or SLE ($n = 120$).

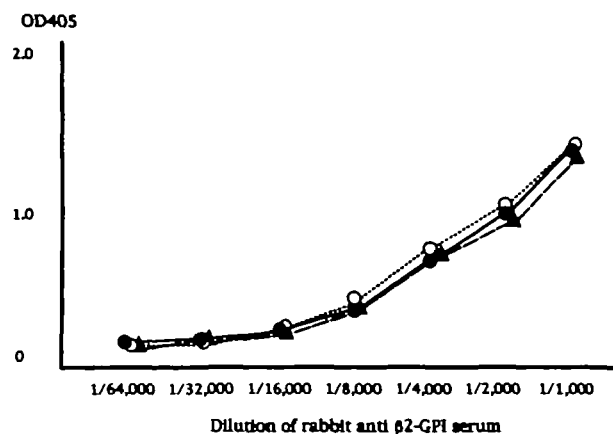


Fig. 3.—Binding of sera from human β 2-GPI-immunized rabbit to β 2-GPI, coated on irradiated plate, Type C (●—●), non-irradiated plate, Immulon 1 (○—○) and another non-irradiated plate, Limbro (▲—▲). OD values obtained from each plate were similar.

that of aCL, but also with that of antiphosphatidylserine, another anionic phospholipid. Furthermore, in APS and SLE patients, the anti- β 2-GPI assay was more specific for APS than the standard aCL assay.

Since 1990, β 2-GPI has been thought to play an important role in APS. It has recently been demonstrated that the fifth C terminal domain of β 2-GPI contains the major phospholipid binding site (Cys281–Cys288), a region critical for aCL binding [13], confirming the importance of β 2-GPI in the pathogenesis of APS. Experimental studies utilizing monoclonal aCL derived from lupus-prone mice [9], and human monoclonal aCL derived from patients with APS [14], showed that either monoclonal could bind to β 2-GPI coated onto irradiated plates also in the absence of cardiolipin. In particular, human monoclonal aCL bound other anionic phospholipids, such as phosphatidylserine, only in the presence of β 2-GPI, suggesting that aCL can recognize the epitope on β 2-GPI in the presence of other anionic phospholipids.

Several studies have shown that over one-third of SLE patients had a positive aCL titre [15, 16] and confirmed the strong association between aCL and thrombotic events across a large spectrum of autoimmune diseases [16, 17]. However aCL are often present in SLE patients without thrombotic events and/or thrombocytopenia, and they do not correlate with disease activity in SLE [4]. Some investigators reported that IgG aCL were highly specific for the prediction of clinical features of APS, but to accomplish this, they set the cut-off of their IgG aCL assay at a large number of standard deviations above the mean, in order to reduce the number of false positives [18, 19]. This implies that high titres of aCL indeed become very specific for the diagnosis of APS, but at the expense of a large number of 'potential' true positives in patients with SLE.

In our study, all 40 patients with anti- β 2-GPI had aCL, suggesting that anti- β 2-GPI could have been detected by standard aCL assay. Most of these patients

belonged to the primary/secondary APS group. In addition, anti- β 2-GPI strongly correlated with conventional aCL and with antiphosphatidylserine antibodies. This is in keeping with experimental data from studies utilizing monoclonal antibodies, where it was shown that anti- β 2-GPI could be detected not only by aCL but also by other anionic antibody assays [14]. Furthermore, in our SLE patients without APS, the rate of false positives for anti- β 2-GPI was only 4.1%, whereas that for aCL was 24.5%. Therefore, anti- β 2-GPI markedly increased the specificity for a diagnosis of APS in patients with SLE, who might produce 'non-specific aCL', probably low-affinity antibodies deriving from polyclonal B cell activation, which do not recognize β 2-GPI and are not associated with thrombosis. High titres of aCL may give similar information as anti- β 2-GPI. However, some patients in our study with moderate titres of aCL did not have anti- β 2-GPI, implying that anti- β 2-GPI may be more valuable than setting up a higher cut-off in aCL assay. None of our patients' sera showed binding to β 2-GPI coated onto non-irradiated plates. Since polyclonal anti-human β 2-GPI obtained from β 2-GPI immunized rabbits showed binding to β 2-GPI coated on both irradiated and non-irradiated plates, the coating efficiency of β 2-GPI to those plates was similar. A logical conclusion is that aCL may not be able to recognize β 2-GPI without a conformational change induced by irradiated polystyrene surface, which subsequently allows the exposure of the cryptic epitope on β 2-GPI.

Recently, antibodies against β 2-GPI in the absence of cardiolipin were shown in patients with SLE or primary APS, and they were correlated with aCL [20, 21]. Cabiedes *et al.* [22] showed, however, that in SLE patients anti- β 2-GPI were found in 89.7% (35/39) of patients with clinical manifestations of APS, and especially in aPL-negative APS patients [88.9% (16/18)]. They suggested that some anti- β 2-GPI might be independent of aCL. None of our patients with aCL-negative APS bound to β 2-GPI coated onto irradiated ELISA plates. This discrepancy between our data and those of Cabiedes may be due to the difference in the method used to prepare human β 2-GPI as an antigen. Our study confirms the clinical significance of anti- β 2-GPI in a large number of APS patients, and also reasonably supports the hypothesis that 'aCL' associated with APS recognize the cryptic epitope expressed on β 2-GPI.

Owing to its interaction with negatively charged proteins involved in the coagulation process, β 2-GPI may play more than one role in the pathogenesis of thrombosis in the APS. It inhibits ADP-induced platelet aggregation [23], Factor XII [24], contact activation of the intrinsic clotting pathway [25] and Factor Xa-generating activity [26]. Anti- β 2-GPI may abolish these anticoagulant properties of β 2-GPI when its cryptic epitope is exposed in particular conditions, and favour a prothrombotic state.

Finally, having shown that anti- β 2-GPI ELISA may be a valuable test for APS, 16.9% of our APS patients

with a positive aCL titre did not have anti- β 2-GPI. In 40% of these patients, a lupus anticoagulant was not detected, suggesting that testing for aCL is still a useful tool for the diagnosis of APS in spite of its low specificity. Considering that aCL is a well-established and standardized assay worldwide, and that none of our aCL-negative patients had anti- β 2-GPI, aCL should remain the basic screening ELISA for APS, followed by the anti- β 2-GPI which could identify those patients at risk of developing APS.

In conclusion, because of its higher specificity to detect 'aCL-related APS', the anti- β 2-GPI assay using irradiated ELISA plates could be performed in conjunction with the standard aCL ELISA to improve the recognition of the APS.

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