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Lupus 1999 8: 121
DOI: 10.1191/096120399678847506

Lupus (1999) 8, 121–126
 0961-2033/99 $12.00
http://www.stockton-press.co.uk/lup

PAPER

[Anti-β_2 Glycoprotein I—β_2 Glycoprotein I] immune complexes in patients with antiphospholipid syndrome and other autoimmune diseases

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Antiphospholipid syndrome (APS) is defined by the presence of aPL antibodies in patients with thromboembolic phenomena. Some antiphospholipid (aPL) antibodies, such as those directed against β_2-glycoprotein I (β_2GPI), are associated with thromboembolism, possess Lupus Anticoagulant (LA) activity and recognize their target antigen only when bound to specific surfaces or to phospholipids (PL). To ascertain whether both free and antibody-bound β_2GPI circulate in APS, we set up an ELISA to detect [IgG anti-β_2GPI–β_2GPI] immune complexes. In this system, rabbit anti-human β_2GPI antibodies were adsorbed onto plastic plates, incubated with patient plasma, and bound complexes were detected by means of alkaline phosphatase-labeled goat anti-human IgG; each assay was stopped when positive controls consisting of *in vitro* generated immune complexes reached an Optical Density (OD) of 0.5 at 405 nm. Plasma from 16 patients with APS showed a mean OD405 of 0.291 (range 0.115–0.558), not statistically different from the mean obtained for 15 age- and sex-matched healthy volunteers (mean OD405 = 0.169, range 0.066–0.264). Surprisingly, levels of immune complexes in 14 patients with other autoimmune diseases and no circulating anti-β_2GPI antibodies were statistically higher (mean OD405 = 0.552, range 0.204–0.991) than those of healthy subjects and patients with APS. These data indicate that while autoantibodies to β_2GPI are mainly unbound in plasma of patients with APS, they are complexed with their antigen in patients with other autoimmune diseases, possibly reflecting a higher binding affinity.

Keywords: anti-β_2-glycoprotein I; immune complexes; antiphospholipid syndrome

Introduction

β_2 glycoprotein I (β_2GPI) is a phospholipid-binding plasma protein discovered more than 35 y ago.[1] This protein circulates at a plasma concentration of 0.2 mg/ml, partly associated with lipoproteins, and is therefore also termed apolipoprotein H. Recent, β_2GPI has gained much clinical interest on the basis of its involvement in the binding of autoimmune anticardiolipin antibodies (aCL) to cardiolipin (CL).[2–4] Furthermore, it has been demonstrated that β_2GPI is in fact the true antigen recognized by aCL when bound onto specific microtiter plates.[5,6]

In 1970, Cohnen[7] was the first to note a slightly increased concentration of serum β_2GPI in patients with Systemic Lupus Erythematosus (SLE); since then, numerous reports on β_2GPI antigen levels in patients with antiphospholipid (aPL) antibodies have produced conflicting results.[8–13]

McNally *et al*[14] recently developed a method to measure free β_2GPI and observed normal levels in patients with aPL antibodies, while total β_2GPI levels were increased in some of them. These elevated β_2GPI levels were due to increased amounts of bound β_2GPI, supporting the view that β_2GPI is complexed with other plasma constituents such as lipoproteins or

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Received 2 June 1998; accepted 6 November 1998

immunoglobulins to form immune complexes. Such circulating Ig-β_2GPI immune complexes might have a pathogenic role in patients with antiphospholipid syndrome (APS).

Recently, we detected complexes formed by IgG-IgM and β_2GPI in a patient with catastrophic APS,[15] and hypothesized a role for these immune complexes, at least in the acute phase of disease. This finding prompted us to develop an ELISA system to detect IgG[aβ_2GPI—β_2GPI] immune complexes.

Materials and methods

Patients

Plasma was obtained from 16 patients with APS, 14 with other miscellaneous autoimmune disorders, and 15 normal individuals. All 16 patients with APS, and none of those with other autoimmune disorders, tested positive in IgG anticardiolipin antibody ELISA.[6] Among the patients with APS, nine had venous thromboembolic disease (five with deep vein thrombosis and four with pulmonary embolism), six had arterial thromboembolic disease (two with acute myocardial infarction, three with stroke, one with renal artery thrombosis) and one patient suffered from multiple foetal losses. One patient with APS was studied during therapeutic plasmapheresis. Among the patients with other autoimmune disorders, three had SLE, two had Sjogren syndrome, four suffered from rheumatoid arthritis, two had systemic sclerosis, and three were affected with undifferentiated connective tissue disease.

Blood sampling

Venous blood from patients and controls was collected in one-tenth volume of 3.8% trisodium citrate. Plasma, obtained by centrifugation at $2000 \times g$ for 15 min at 4°C, was filtered through a 0.22 µm BioDisc filter unit (CAM Bio srl, Milan, Italy) and stored at −20°C until use.

Purification of β_2 glycoprotein I

Human β_2GPI was purified from normal plasma by means of perchloric acid precipitation and affinity chromatography on heparin Sepharose (Hi-trap Heparin, Pharmacia Biotech Europe, Brussels, Belgium), followed by cation exchange chromatography (Resource S, Pharmacia Biotech Europe, Brussels, Belgium).[6] The purified protein migrated as a single 50 kDa band in SDS-PAGE. Protein concentration was assessed by the Bradford method (Roti-Quant Carl Roth GmbH Co., Karlsruhe, Germany).

Immunization protocol

Rabbits. Two 3-month-old New Zealand white rabbits were injected intramuscularly with 1 ml of purified human β_2GPI (250 µg/ml) in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). After two weeks, a second booster injection prepared under the same conditions was administered. Two weeks later, the rabbits received a third immunization with 250 µg/ml of β_2GPI in incomplete Freund's adjuvant injected subcutaneously at multiple sites. Blood samples were drawn weekly and the titers of anti-β_2GPI antibodies were determined by ELISA.

Mice. Four BALB/c mice were immunized subcutaneously with 200 µl of a 10 µg/ml solution of β_2GPI in incomplete Freund's adjuvant, followed by two booster injections of antigen in incomplete Freund's adjuvant, administered one week apart. Two weeks later, production of ascites fluid was induced by injecting the mice intraperitoneally with 500 µl of β_2GPI (20 µl/ml) in incomplete Freund's adjuvant. The titers of anti-β_2GPI antibodies in the resulting polyclonal mouse antisera and ascites were determined by ELISA.

Isolation of IgG

Whole IgG was isolated by affinity chromatography on Protein A Sepharose CL 4B (Sigma Chemical Co., St. Louis, MO), eluted with 0.1 M glycine/HCl, pH 3.0, neutralized with 1 M Tris, pH 8.4, and dialyzed against Tris buffer, pH 7.4. IgG from plasma of a β_2GPI-immunized rabbit was used as a coating agent in ELISA to capture immune complexes, while IgG isolated from normal plasma was used as a negative control.

Total plasma β_2GPI determination

To measure the plasma concentration of β_2GPI, a single radial immunodiffusion was performed. Twenty-four microliter samples (diluted 1 : 2 in Tris-buffered saline) were applied to 4.0 mm wells of a 1% agarose gel containing 6% v/v polyclonal anti-β_2GPI

rabbit antiserum. Purified β_2GPI was serially diluted with PBS, pH 7.4, to give a calibration curve ranging from 0.050–0.294 mg/ml.

Detection of IgG anti-β_2GPI antibodies

Antibodies to human β_2GPI were measured in ELISA as previously described.[6] Plasma samples were diluted 1 : 100 in 10% FBS (Celbio, Milan Italy)/PBS. The colour reaction was stopped when an in-house positive control reached 1.5 OD units at 405 nm, after an average time of 20 min. The titer of anti-β_2GPI antibodies in polyclonal rabbit antisera was determined by diluting antisera at 1 : 100 and developing the reaction using a 1 : 41 000 dilution of phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO); under these conditions rabbit antisera showed ~0.6 OD units at 405 nm. Polyclonal mouse aβ_2GPI ascites were also tested in ELISA at a 1 : 64 dilution, and bound IgGs were detected using a 1 : 6500 dilution of phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). The mouse ascites was found to be highly reactive in anti-β_2GPI ELISA (OD405 ~ 0.8).

'In vitro' generation of [anti-β_2—β_2GPI] immune complexes

The optimal concentration of antigen and antibody was determined by the quantitative precipitin test developed by Heidelberg and Kendall.[16] To a series of tubes containing a fixed amount of polyclonal mouse anti-human β_2GPI (100 µl), increasing amounts of human β_2GPI (from 0 µg/ml to a maximum of 33 µg/ml final concentration) were added. The mixtures were incubated for 1 h at 37°C and then at 4°C overnight. The tubes were then centrifuged at 3000 *g* for 5 min and the immune precipitation was observed. Maximal precipitation (equivalent zone) was obtained at a β_2GPI concentration of 5.2 µg/ml. This concentration was routinely employed for *in vitro* generation of immune complexes and used in ELISA as a positive control.

ELISA for [anti-GPI—β_2GPI] immune complexes

One hundred microliters of anti-human β_2GPI IgG (1.5 mg/ml in PBS, pH 7.4) isolated from rabbit plasma were applied to a polyvinylchloride plate (Costar, Cambridge, MA), and adsorbed overnight at 4°C; all subsequent incubations were carried out at room temperature. The plate was then washed two times with PBS and blocked with a 0.3% gelatin/PBS solution for 2 h; this buffer was also used for diluting samples and conjugated antibodies. One hundred microliters of a 1 : 50 dilution of patient and control plasmas were then added and the plates were incubated for 1 h; 100 µg of purified β_2GPI and a 1 : 4 dilution of control IgG (0.2 mg/ml) served as a negative control, while 100 µl of indiluted *in vitro* generated immune complexes were used as positive reference control. Following six washes, bound IgG was detected using 100 µl of a 1 : 6500 dilution of phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) for the positive control and 100 µl of a 1 : 1000 dilution of alkaline phosphatase γ chain-specific goat anti-human IgG for negative controls and sample plasmas. After 1 h of incubation followed by six washes in PBS, 100 µl of freshly prepared *P*-nitrophenylphosphate solution (1 mg/ml in 10% diethanolamine, pH 9.8) were added to each well. The colour reaction was stopped by addition of 50 µl of 3 M NaOH when the positive control reached an absorbance of 0.5 at 405 nm. Results were expressed as the mean value of three replicates. To exclude non-specific charge-dependent binding of anti-β_2GPI to β_2GPI in autoimmune patients, plasma from a patient who tested strongly positive for complexes was extensively dialyzed against increasing concentrations of NaCl (0.15 M, 0.5 M, 1.0 M) and then tested for the presence of immune complexes. Some samples that tested positive were re-assayed at increasing dilutions to obtain binding curves.

Statistics

To compare the results obtained for controls, APS and other autoimmune patients, the Bonferroni multiple comparison test was applied and considered significant at a *P* value below 0.05.

Results

All 16 APS patients tested positive for IgG anticardiolipin antibodies (range 40–147 GPL units; normal values < 15) and five were also positive for IgM anticardiolipin (range 32–93 MPL units; normal values < 18). All 16 patients also tested positive for IgG anti-β_2GPI antibodies, with their OD values at 405 nm ranging from 0.427–1.894 (cut-off value, 0.401) (Figure 1). One patient in the autoimmune group and one in the control group had IgG aβ_2GPI

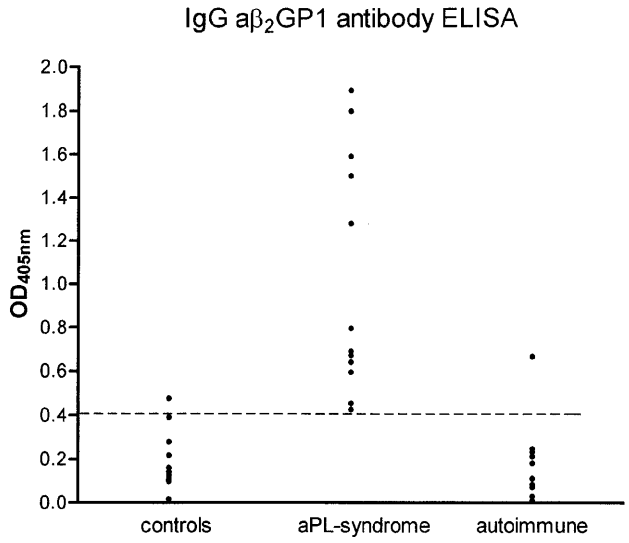

Figure 1 IgG aβ_2GPI antibody ELISA in controls ($n=15$), patients with APS ($n=16$), and miscellaneous autoimmune disorders ($n=14$).

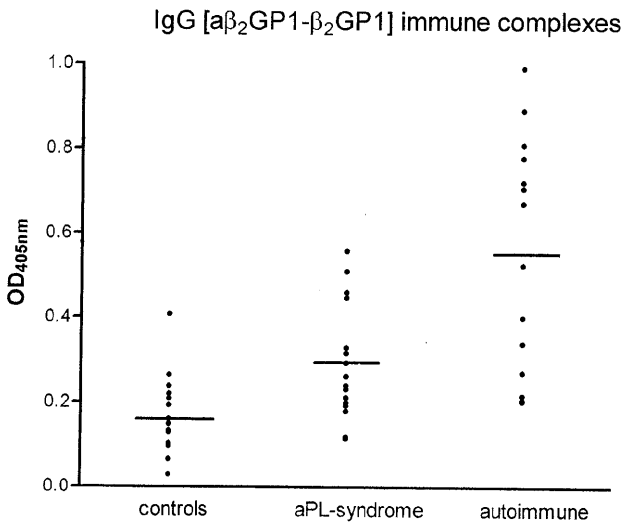

Figure 2 [IgG aβ_2GPI—β_2GPI] immune complexes in controls ($n=15$), patients with APS ($n=16$), and miscellaneous autoimmune disorders ($n=14$). (— = mean values).

OD values above the cut-off value (namely 0.668 and 0.476, respectively).

In the ELISA for [IgG aβ_2GPI—β_2GPI] immune complexes, indiluted *in vitro* generated immune complexes were included as a positive control, and assay plates were stopped when this control reached an OD405 of 0.5 (range 0.527–0.571), which occurred after a mean of 40 min. Human purified β_2GPI and normal IgG were used as negative controls, their OD405 values ranging from 0–0.031 and from 0–0.093, respectively. Each sample was also tested for binding to a parallel uncoated ELISA plate; as results of this control assay never exceeded 0.1 OD405, these values were not subtracted from those obtained with coated plates. To test for the possible charge-dependent non-specific binding of immune complexes, plasma from an autoimmune patient that tested strongly positive for the presence of immune complexes was dialyzed against increasing NaCl concentrations and then retested. Results showed that this pre-treatment step to disrupt charge-charge interactions did not decrease the positivity of the sample (OD405 0.841–0.15 M; OD405 0.796—0.5 M; OD405 1.243–1.0 M). Furthermore, serial dilution of two positive samples (one APS and one autoimmune) yielded binding data that paralleled each other (OD405 of 0.402 and 0.441 at standard 1 : 50 dilution, 0.226 and 0.370 at 1 : 100 dilution; 0.165 and 0.182 at 1 : 200, 0.056 and 0.088 at 1 : 400. We also tested the effect of pre-treatment (1 h at room temperature) of coated plates with pure β_2GPI (0.5 mg/ml) on the binding of [IgG aβ_2GPI—β_2GPI] immune complexes of two positive autoimmune patients and two positive APS patients. This pre-treatment step markedly reduced the binding values obtained for samples from both autoimmune patients (decrease in OD405 from 0.481–0.143 for one patient and from 0.700–0.073 for the second patient) as well as the APS patients (decrease in OD405 from 0.270–0.007 for one patient and from 0.276–0.029 for the second patient).

As shown in Figure 2, results of ELISA to detect IgG[aβ_2GPI—β_2GPI] immune complexes did not reveal significant differences between normal controls (mean value OD405 = 0.169, range 0.066–0.264) and patients with APS (mean OD405 = 0.291, range 0.115–0.558). On the contrary, the OD405 values for immune complexes were significantly higher for patients with autoimmune disorders (mean OD405 = 0.552, range 0.204–0.991) compared to control and aCL patients ($P<0.001$).

Similar observations were made when comparing mean values of β_2GPI plasma levels (Figure 3). However, in this case β_2GPI levels in APS patients (mean 0.369 mg/ml) were significantly higher than those of controls (mean 0.210 mg/ml, $P<0.05$), but did not differ significantly from those of autoimmune patients (mean 0.470 mg/ml).

The APS patient with the highest titer of IgG[aβ_2GPI—β_2GPI] immunocomplexes (OD405 = 0.558) underwent therapeutic plasmapheresis daily for 3 d. As shown in Figure 4, the first plasmapheresis resulted in a substantial decrease in the titers of both IgG anti-β_2GPI antibodies and IgG[aβ_2GPI—β_2GPI] immune complexes, the second and third treatments were less effective.

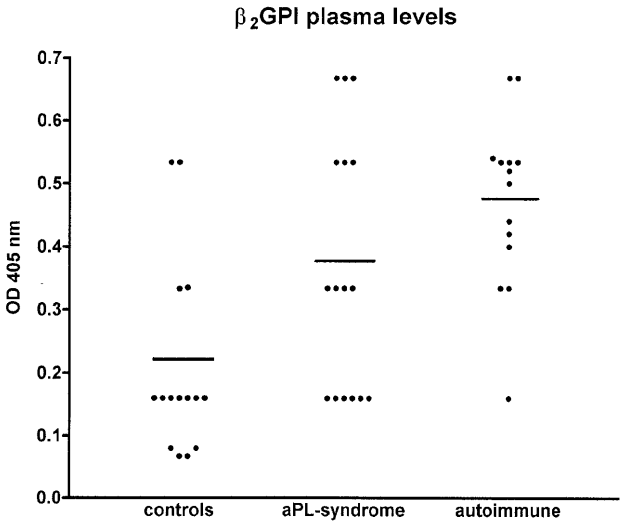

Figure 3 β_2GPI plasma levels in controls ($n=15$), patients with APS ($n=16$), and miscellaneous autoimmune disorders ($n=14$). (— = mean values).

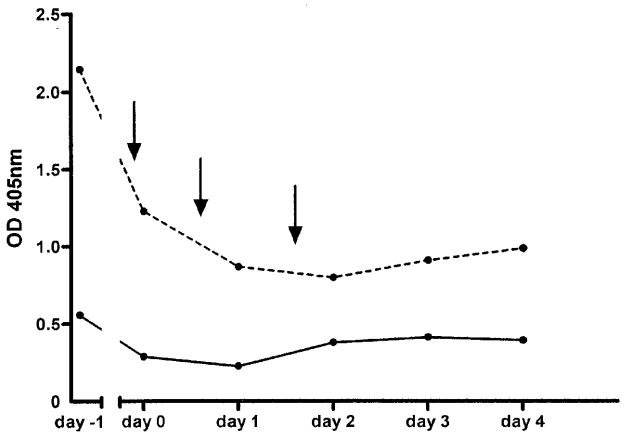

Figure 4 IgG aβ_2GPI antibodies (dashed line) and IgG[aβ_2GPI—β_2GPI]immune complexes (solid line) measured during therapeutic plasmapheresis in a patient with APS. Arrows indicate the administration of plasmapheresis daily for 3 d.

Discussion

β_2GPI is a phospholipid-binding plasma protein whose physiological role remains uncertain.[17–21] *In vitro*, β_2GPI inhibits thrombin formation both at the level of contact activation and prothrombinase activity.[22,23] Although antibodies binding to an 'anticoagulant' protein should theoretically predispose to thromboembolic phenomena, congenital β_2GPI deficiency is not associated with thrombosis.[24] This leads to the proposal that the interaction of β_2GPI with specific autoantibodies to form immune complexes may be more important in the pathogenesis of thrombosis than the levels of β_2GPI *per se*. McNally *et al*[14] found circulating material linked to β_2GPI in patients with APS, and our laboratory identified Ig bound to β_2GPI in a patient with catastrophic syndrome.[15] Nevertheless, the present analysis showed that IgG[aβ_2GPI—β_2GPI] immune complex levels in plasma of patients with APS did not differ from those of control subjects (Figure 2). However, we must emphasize that these patients were tested for immune complexes at least six months after their index thromboembolic event. Moreover, we know that anti-β_2GPI autoantibodies exhibit low binding affinity,[25] as they do not recognize their antigen in plasma[6] or when bound to a physiological surface such as heparin.[26]

In APS, immune complexes might be formed *in vivo* when anionic PL becomes exposed on the surface of cells, and thus might be detectable only in such instances. Acute exposure of anionic PL might be associated with immune complex formation and acute thromboembolic events.[27] Moreover, complex formation can be expected to result in a parallel decrease in circulating β_2GPI, as was the case of the patient with catastrophic syndrome.[15]

Conclusions

The detection of high levels of circulating IgG[aβ_2GPI—β_2GPI] immune complexes in the absence of circulating anti-β_2GPI antibodies in patients with autoimmune disease was unexpected (Figure 2). Anti-β_2GPI antibodies in these patients should differ from those found in APS by their ability to recognize circulating β_2GPI in the absence of exposed PL. Formation of immune complexes in these patients might lead to chronic consumption of β_2GPI, which in turn could promote an over-compensating production by the liver, therefore explaining the increased β_2GPI levels measured in these patients.[14]

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