Heterogeneity of anti-β2-glycoprotein I antibodies

A factor of variability in test results

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Summary

The aim of this study was to evaluate the heterogeneity of IgG-anti-β_2-glycoprotein I antibodies (IgG-aβ_2GPI) as regarding their reactivity pattern against different sources of human β_2GPI, their avidity and their association with clinical events of antiphospholipid syndrome (APS). Three thousand six hundred and eighty-four consecutive patient sera were routinely tested for IgG-aβ_2GPI over 1 year using an in-house ELISA with 2 different commercial preparations of human purified β2GPI. Of the 340 sera found positive, all those clinically documented were included in this study; 61 were positive with only one preparation (S1) and 59 with both (S2). The results of ELISA were confirmed by Western blot. Heterogeneity was stressed by testing sera with a human recombinant protein and 3 β_2GPI-related peptides. No contribution of glycosylation in the binding to β2GPI was found. The avidity indices for each protein were significantly higher in S1 than in S2 ($p = 0.0021$). S2 were more associated with antiphospholipid antibodies than S1 (75% versus 21% ; $p < 0.0001$). A similar frequency of the main clinical features of APS was found in S1 and S2 sera (69% and 71%, respectively). In conclusion, our data show a heterogeneity in the antigenic reactivity pattern of IgG- aβ_2GPI and a relationship between a binding profile and antibody avidity. This heterogeneity could represent a crucial factor of variability in test results and underlines the difficulty of getting standardisation.

Keywords

β2-glycoprotein I, heterogeneity, avidity, antiphospholipid syndrome

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Introduction

It is increasingly recognized that anti-β_2-glycoprotein I antibodies (aβ_2GPI) are strongly associated with the clinical features of antiphospholipid syndrome (APS) and more specific of this syndrome than antiphospholipid antibodies (aPL) such as anticardiolipin antibodies (aCL) and lupus anticoagulant (LA) (1). Nevertheless, these aPL are considered to be a biological criteria of APS, characterized by clinical disorders including venous or arterial thrombosis and recurrent fetal losses (2). APS can be a primary syndrome or a secondary one when associated with systemic autoimmune diseases, especially systemic lupus erythematosus (3–4). aβ_2GPI though do not yet belong to the biological criteria of APS. However, due to their high correlation with APS clinical events, their investigation appears to be essential for a reliable biological exploration of the latter. Nevertheless, ELISA used for their detection are still not fully standardized although several commercial and "home made" kits are progressively being introduced. A collaborative study concerning an attempt to standardize the aβ_2GPI assays reported discrepancies in results when sera were tested by different laboratories (5). Such conflicting results could be due to the different components of the assays and among them to differences in the β_2GPI preparations.

β_2GPI is a plasma protein belonging to the short consensus protein family with five repeating domains (6–8). Carbohydrates contribute almost 20% of the apparent molecular mass of 50 kDa

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and studies have shown N-linked glycosylation sites located in domains III and IV (7). The fifth domain contains the phospholipid binding site (9). The exact nature of the epitopes recognized by aβ_2GPI has been subject to controversy: either native (10, 11) or cryptic epitopes expressed after the interaction of β_2GPI with anionic phospholipids or irradiated polystyrene surfaces (12). Moreover, the precise location of the epitopes on β_2GPI bound by autoantibodies is still a subject of debate. For some groups, domain I was identified as the major target of aβ_2GPI (13, 14) while for others domain IV could play a critical role in the exposure of cryptic epitopes (15, 16).

Taking into account the possibility of differences in the aβ_2GPI detection attributable to the antigen preparation, sera were tested routinely for IgG-aβ_2GPI over one year using an in-house ELISA with two different preparations of purified human β2GPI. Discrepancies between the results were obtained for some sera according to the β_2GPI preparation used. Thus, the aim of the present study was to analyze the heterogeneity of IgG-aβ_2GPI in terms of antigen reactivity using ELISA and Western blot with one human recombinant β_2GPI and three β_2GPI-related peptides in addition to the two human purified preparations. Moreover, we studied on the one hand the involvement of carbohydrates in the antibody binding and on the other hand the relationship between reactivity pattern and avidity or clinical associations of aβ_2GPI.

Sera and methods

Sera

Three thousand six hundred and eighty-four consecutive serum samples were referred to the laboratory with a request from various departments of medicine for routine aβ_2GPI detection between 2000 and 2001. Sera were collected from people living in the South of France, typical Mediterranean patients (most of them were Caucasians). All sera were run in the same assay with 2 preparations of purified human β_2GPI – one from Stago laboratories (β_2GPI-ST) and the other one from Binding Site laboratories (β_2GPI-BS). Of the 340 sera found positive, all those for which we obtained biological and clinical data, within the year mentioned, were included in this study (n = 120). They were classified into 2 groups according to their reactivity with the two preparations of β_2GPI used as antigen: S1: including 61 sera positive with one of the two preparations of β_2GPI, 20 with β_2GPI-BS (S1-BS) and 41 with β_2GPI-ST (S1-ST); S2: including 59 sera positive with both preparations of β_2GPI.

β_2GPI preparations

Two preparations of β_2GPI were routinely used in this study – β_2GPI-ST and β_2GPI-BS. Another one, β_2GPI-PH (kindly donated by Pharmacia, Diagnostics GmbH & Co, Freiburg, Germany) was only used to test sera giving conflicting results with the former preparations. The main characteristics of β_2GPI used in this study were given by the manufacturers and are shown in table 1. In our laboratory, the purity of each preparation was checked by SDS PAGE and silver staining showed a single band with a molecular weight similar to that reported in table 1.

Table 1: Characteristics of the different preparations of b2GPI used.

Source	Origin	Purification method	SDS-PAGE analysis
Stago	Normal citrated human plasma	Baryum citrate adsorption, Ion exchange chromatography, Heparin agarose chromatography	One band of about 54 kDa
Binding-Site	Normal human serum	Precipitation with perchloric acid, Heparin-sepharose Chromatography, Affinity purification with a sheep polyclonal antibody anti-β_2-GPI	One band of about 54 kDa
Pharmacia	Human recombinant β_2-GPI produced in Baculovirus-infected Sf9 cells	Ion exchange chromatography (SP sepharose), Hydrophobic interaction chromatography (phenyl sepahrose), Gelfiltration (Superdex 200)	One band of 40 ±5 kDa

IgG-aβ_2GPI ELISA

Irradiated polystyrene microtiter plates (Maxisorp-Nunc, Rockilde, Denmark) were coated with 75 µl / well of β_2GPI (β_2GPI-ST or β_2GPI-BS) diluted at 10 µg/ml in PBS and the plates were incubated overnight at 4°C. After two washes with PBS/Tween 0.1% (PBS/T), the plates were blocked for 1 h at room temperature (RT) with the same buffer. Serum samples diluted 1:100 in PBS/T were added to two β_2GPI coated wells (one with β_2GPI-ST and the other one with β_2GPI-BS) and to one uncoated well to subtract non-specific binding. Plates were then incubated for 1 h at RT. After washing, plates were incubated for 1 h at RT with 100 µl/well of alkaline phosphatase-conjugated affinity-purified goat anti-human IgG antibody, Fcγ fragment specific, (Jackson Immunoresearch, West Grove, USA) diluted at 1:10,000 in PBS/T. The plates were then washed three times and p-nitrophenyl phosphate (1 mg/ml in 1M diethanolamine buffer, 0.5 mM MgCl2, pH = 9.8) was added (100 µl/well). The color reaction was left to develop in the dark at 37°C until the OD at 405 nm of an internal positive control reached 1. Results were expressed in Delta Optical Density. The cutoff values were established from testing of 100 healthy blood donors and were similar with both β_2GPI preparations: 0.16 with β_2GPI-BS and 0.18 with β_2GPI-ST (99 percentiles).

Detection of IgG-aβ_2GPI related peptide antibodies

The ability of aβ_2GPI positive sera to bind β_2GPI related peptides was tested, as previously described (17), using ELISA plates coated with each of the following 3 peptides – A: LKTPRV, B: KDKATF and C: TLRVYK. These hexapeptides were selected from a hexapeptide phage display library; peptide A corresponds to the first and second domains, peptide B to the forth domain and peptide C to the third domain of the β_2GPI molecule.

aCL ELISA

Sera were assayed for IgG-class aCL using an in-house ELISA previously described (18). Values higher than 17 GPLU (97 percentiles) were considered as positive.

Avidity of IgG-aβ_2GPI

IgG-aβ_2GPI avidity was measured with a modification of the aβ_2GPI ELISA as previously described (19). Briefly, after incubation of serum samples with β_2GPI, the wells were soaked with 100 µl of PBS with or without 2M urea for 10 min at RT. The use of 2M urea was determined from preliminary experiments with increasing concentrations of urea (1M, 2M and 4M) showing similar results with 2M and 4M urea. After discarding the urea solution, the wells were extensively washed with PBS /T and the remaining ELISA steps were carried out as in the conventional method. The avidity indices, expressed in percentage, were calculated as follows: (OD value in assay with urea / OD value in assay without urea) X100.

SDS-PAGE and Western blot

The human purified or recombinant β_2GPI were electrophoresed in 10% SDS-PAGE performed in gels under non-reducing conditions and were then transferred to a nitrocellulose membrane (100 V for 1 h) in 25 mM Tris, 192 mM glycine, pH 8.3 in 20% methanol (V/V). Membranes were blocked with 1% BSA, 0.1% Tween 20 in PBS overnight at 4°C, then incubated for 1 h with patient sera and positive or negative controls diluted at 1:200 in PBS. After washes in PBS-0.1%Tween 20, membranes were incubated for 1 h in peroxydase-conjugated anti-human IgG (1:10,000, goat anti-human IgG, Fcγ fragment specific; Jackson ImmunoResearch). Peroxydase activity was revealed by chemoluminescent reaction (ECL, Amersham Biosciences, Buckinghamshire, UK).

Enzymatic deglycosylation

Human purified β_2GPI from Binding Site or Stago laboratories were treated by N-glycanase (1 enzyme unit for 1 µg of β_2GPI) according to the manufacturer's method (Roche Diagnostics GmbH, Mannheim, Germany) with an overnight incubation at 37°C.

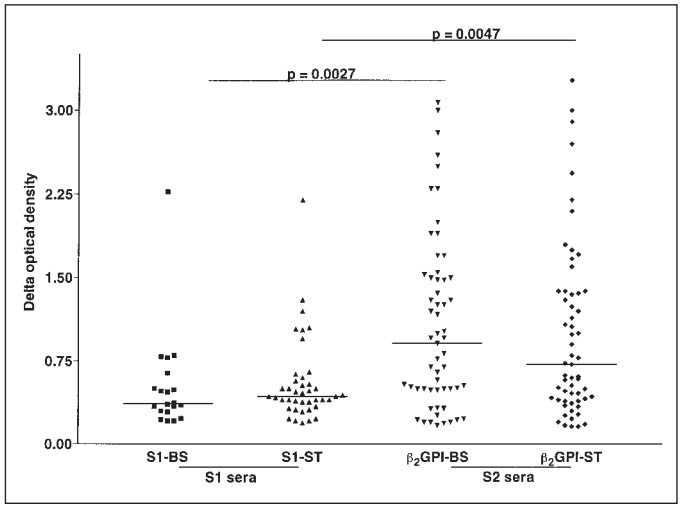

Figure 1: Distribution of IgG-aβ_2GPI levels in both subgroups of S1 sera (S1-BS and S1-ST) and in S2 sera tested with β_2GPI-ST or β_2GPI-BS. Lines represent the median of delta OD. Cutoff values were 0.16 with β_2GPI-BS and 0.18 with β_2GPI-ST, respectively.

Statistics

The Chi-square, the Fisher test, the Mann-Whitney test and the Spearman's rank correlation test for nonparametric data were used when appropriate. *p* values <0.05 were assumed to be statistically significant.

Results

aβ_2GPI ELISA

Of the sixty one S1 sera, 67% only reacted with β_2GPI-ST (S1-ST) and 33% only with β_2GPI-BS (S1-BS). The comparison of aβ_2GPI levels between S1-BS and S1-ST sera showed no significant difference (p = 0.34) (Fig. 1). With regard to S2 sera, reactivity was similar with each preparation of β_2GPI (p = 0.07), and a positive correlation was found between the aβ_2GPI levels (r = 0.92 ; *p* <0.0001).

The levels of aβ_2GPI were then compared between both subgroups of S1 and S2 tested with β_2GPI-ST or β_2GPI-BS. The results showed that the median of aβ_2GPI levels was significantly higher in S2 than in S1 irrespective of the β_2GPI preparation used: 0.72 and 0.91 in S2 sera versus 0.47 and 0.36 in S1 sera with β_2GPI-ST (*p* = 0.0047) and with β_2GPI-BS (*p* = 0.0027), respectively (Fig. 1).

In order to further analyze the variability of the reactivity of sera according to the source of β_2GPI, 40 representative sera, 22 S1 (including 9 S1-BS and 13 S1-ST) and 18 S2, were tested by ELISA using a recombinant human β_2GPI (B2-PH). The reactivity against the recombinant protein was compared to that against β_2GPI-BS and β_2GPI-ST in the same assay. All 18 S2 sera gave similar qualitative (positive/negative) and quantitative results with β_2GPI–PH and β_2GPI-BS (p = 0.0003) or β_2GPI–ST (p = 0.0021) (Fig. 2). On the contrary, in S1 sera, S1-BS but not S1-ST gave similar qualitative results when tested with β_2GPI-PH. Nevertheless, for each subgroup of S1 sera, there was no significant correlation between the aβ_2GPI levels obtained with β_2GPI-PH and those with β_2GPI-BS (p = 0.13) or β_2GPI-ST (p = 0.78) (Fig. 2).

Binding to β_2GPI by Western blot analysis

In order to confirm the heterogeneity of aβ_2GPI reactivity observed in ELISA, 30 out of the 40 sera were tested by Western blot performed with the different preparations of β_2GPI (5 S1-BS, 12 S1-ST and 13 S2). Representative profiles of the binding of several sera to different preparations of β_2GPI are exemplified in figure 3A. The immunoreactive band obtained with β_2GPI-PH was lower than that with β_2GPI-ST or β_2GPI-BS because of the low degree of glycosylation of the recombinant protein. As shown in table 2, perfect concordance was obtained for the 32 sera between ELISA and Western blot.

Purification of β_2GPI using perchloric precipitation could induce a loss of carbohydrates, and β_2GPI-BS was purified with such a method in contrast to β_2GPI-ST. The β_2GPI-reactivity of sera was similar with β_2GPI-BS and β_2GPI–PH and different between both these preparations and β_2GPI-ST. These results prompted us to study an involvement of carbohydrates in the binding sites of β_2GPI. Thus, we tested 10 out of the 30 sera, 6 S1 (3 S1-BS and 3 S1-ST) and 4 S2, with the native and deglycosylated forms of β_2GPI-BS and β_2GPI-ST. All the sera tested

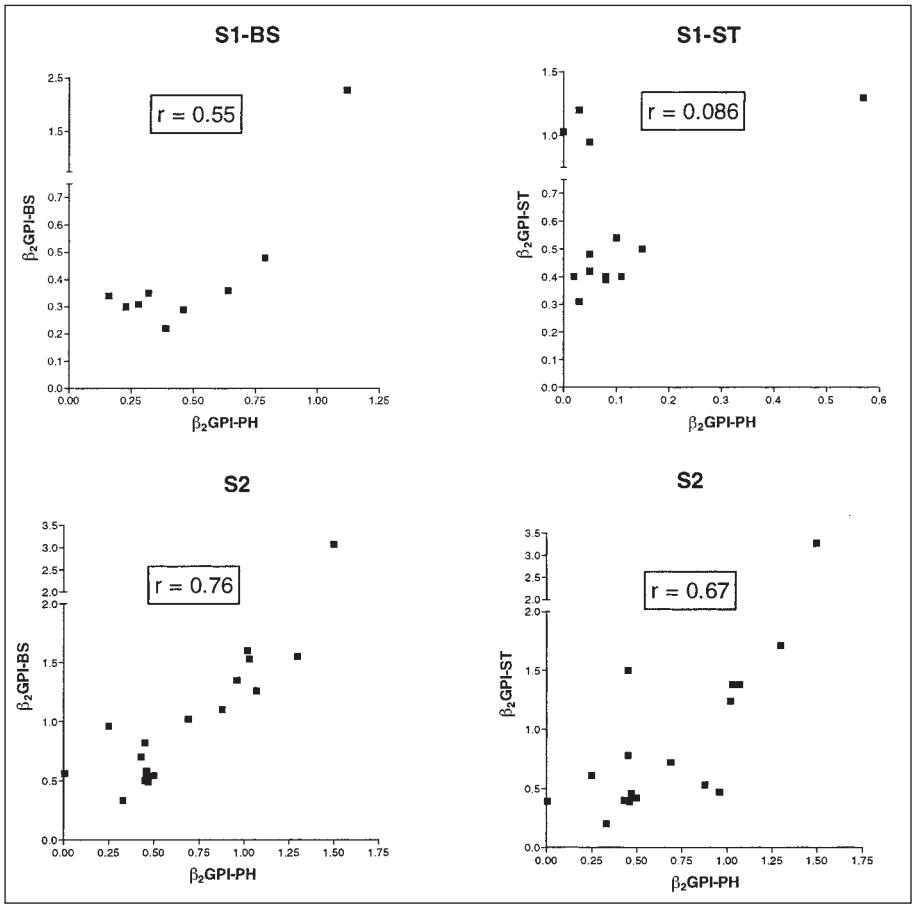

Figure 2: Correlation between IgG-ab2GPI levels measured with b2GPI-PH and those with b2GPI-BS or b2GPI-ST in S1-BS, S1-ST and S2.

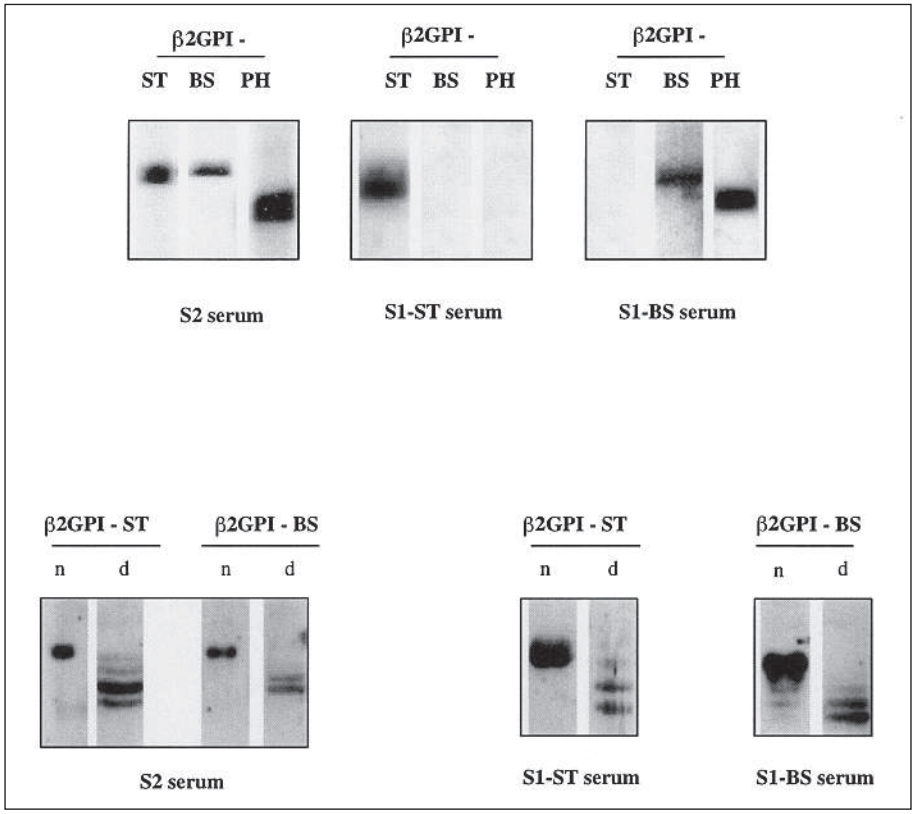

Figure 3: Western blot analysis of aβ_2GPI reactivity. A: representative experiments of the patterns of reactivity against β_2GPI-ST, β_2GPI-BS and β_2GPI-PH with one S2 serum positive with the three preparations and two S1 sera, one S1-ST and one S1-BS.
B: reactivity against β_2GPI-ST or β_2GPI-BS untreated (n) and treated with N-glycanase (d) of one S2 serum positive with both preparations, one S1-ST and one S1-BS.

reacted by Western blot with the native as well as the digested protein whatever the preparation of β_2GPI (Fig. 3B). Due to the cleavage of N-linked oligosaccharide residues of β_2GPI by N-glycanase, three or more bands were obtained with the digested protein.

Reactivity against three β_2GPI related peptides

Fourteen sera (3 S1-BS, 3 S1-ST and 8 S2) were tested with peptides A, B and C by ELISA. Only 2 of them were negative (1 S1-BS and 1 S2). The reactivity was mainly directed against peptide B (Fig. 4) in S1 as well as in S2 sera. In both groups of sera, peptide A was not recognized in the absence of the other peptides, while 4 out of 10 sera positive with peptide B bound peptide B alone. Peptide C was recognized by 1 S1-BS, 1 S1-ST and 4 S2 sera. The number of sera tested with these three peptides was too low to permit a statistical analysis of the peptide reactivity in S1 and S2 sera. Nevertheless, no reactivity profile was found to be representative of each group of sera.

Avidity of IgG-aβ_2GPI from S1 and S2 sera

To further analyze the heterogeneity of aβ_2GPI, antibody avidity indices were compared between S1 and S2 sera. Thirty representative sera (11 S1 and 19 S2) were tested using aβ_2GPI ELISA in the presence of urea 2M in order to determine the antibody avidity. Among the S1 sera, 5/11 were S1-BS and 6/11 were S1-ST. The aβ_2GPI ELISA avidity indices for S1 sera ranged from 29 to 98% (mean and median indices were 64.55% and 62%, respectively). S2 sera were tested with both preparations of aβ_2GPI and the measurement of the avidity indices with each β_2GPI preparation was very similar; indices ranged from 14 to 79% with β_2-GPI-BS (mean = 36% and median = 37%) and from 18 to 79% with β_2GPI-ST (mean = 34% and median = 33%). Thus, the mean of avidity indices obtained with both preparations of β_2GPI was calculated for each S2 serum in order to compare avidity indices between S1 and S2 sera. Since no significant difference of the avidity indices was found between both subgroups of S1 (data not shown), all S1 sera were compared to S2 sera. As shown in figure 5, the avidity indices were significantly higher in S1 than in S2 sera ($p = 0.0021$).

In order to check that urea treatment did not affect the binding or the molecular structure of β_2GPI, plates were treated with urea after the antigen coating step and before incubation of the sera. ELISA was then performed as described above. No difference in reactivity was observed with urea treated and non-treated plates (data not shown).

Table 2: Reactivity according to the source of β_2GPI and the method.

Sera	Western-blot			ELISA		
	ß2GPI-ST	ß2GPI-BS	ß2GPI-PH	ß2GPI-ST (CO*= 0.18)	ß2GPI-BS (CO*= 0.16)	ß2GPI-PH (CO*= 0.15)
S1						
1	+	-	-	0.4	0.03	0.02
2	+	-	-	1.03	0.05	0
3	+	-	-	0.4	0.04	0.11
4	+	-	-	0.54	0.06	0.1
5	+	-	-	0.39	0.06	0.08
6	+	-	-	0.42	0.05	0.05
7	+	-	-	0.95	0.01	0.05
8	+	-	-	0.7	0.045	0.04
9	+	-	-	0.5	0.03	0.13
10	+	-	-	0.48	0.01	0.05
11	+	-	-	0.38	0.023	ND
12	+	-	-	1.2	0.11	0.03
13	-	+	+	0.07	0.29	0.46
14	-	+	+	0.036	0.62	0.71
15	-	+	+	0.08	0.21	0.17
16	-	+	+	0.07	0.5	0.28
17	-	+	+	0.07	0.22	0.39
S2						
18	+	+	+	0.2	0.6	0.6
19	+	+	+	0.53	1.1	0.88
20	+	+	+	0.47	1.35	0.96
21	+	+	+	1.71	1.55	1.3
22	+	+	+	1.38	1.53	1.03
23	+	+	+	1.5	0.5	0.45
24	+	+	+	0.35	0.52	0.45
25	+	+	+	0.42	0.54	0.5
26	+	+	+	1.24	1.6	1.02
27	+	+	+	1.38	1.26	1.07
28	+	+	+	0.78	0.82	0.45
29	+	+	+	0.72	1.02	0.69
30	+	+	+	0.61	0.96	0.25

*CO : cutoff value

Finally, no correlation was found between avidity indices and aβ_2GPI levels in S1 as well as in S2 (data not shown).

Biological and clinical associations

The biological and clinical associations of S1 and S2 were studied and compared (Table 3). aβ_2GPI were found associated with antiphospholipid antibodies in 75% of S2 and in only 21% of S1 ($p < 0.0001$). In S1, aPL were mainly found in S1-ST (9/13 aPL positive sera). In S2, LA and aCL were simultaneously positive in 80% of the aPL positive sera. Sex ratio and mean age were similar between both groups of patients. Associations with the main clinical facts of APS, thrombosis and/or pregnancy loss, were also analyzed. Thrombotic events were diagnosed clinically and with conventional tests (Doppler ultrasound, venography, ventilation/perfusion lung scanning and/or angiography, and magnetic resonance imaging studies). Forty-two out of the 120 patients in this study were diagnosed APS according to current criteria (2), 6 from S1 group (1 S1-BS and 5 S1-ST, all were APS I) and 36 from S2 group (25 APS I and 11 APS II with SLE). Interestingly, the number of patients having clinical features of APS was similar between S1-BS (60%), S1-ST (73%) and S2 (71%) groups. Nevertheless, among these patients, thromboses were more frequent in S2 than in S1 group (95% versus 76%, $p = 0.029$) without any significant difference between the site of thrombosis, venous or arterial. As regards pregnancy losses, they were significantly more frequent in patients from S1 than in those from S2 group (31% versus 12%, $p = 0.03$). Most of them occurred mainly before the 10th week of gestation in S1 as well in S2 groups. Finally, in patients with thrombotic or obstetrical complications, no significant difference in the number of events was observed between both groups.

The clinical status of patients without APS features was also compared between S1 and S2 group. Interestingly, the prevalence of SLE was found to be much higher in S2 than in S1 group (82% versus 37%, respectively ; $p = 0.001$).

Discussion

This study is the first to demonstrate, from a large panel of consecutive sera, that human IgG-aβ_2GPI represent a heterogeneous group of antibodies as regards their binding to human β_2GPI from different preparations and their avidity. This conclusion is supported by 1). the ability of sera to recognize either only one or both purified human preparations in ELISA, 2). the confirmation by Western blot of this difference in reactivity, 3). a diverse reactivity against three β_2GPI-related peptides, 4). a significant difference of the antibody avidity between S1 and S2 sera. Moreover, analysis of the clinical and biological data of the patients showed association with the main clinical features of APS whatever the β_2GPI reactivity or the association with the presence of aPL.

On the basis of the results from ELISA, using two preparations of purified human β_2GPI, two types of sera have been defined in this study – S1 reacting with only one β_2GPI preparation and S2 reacting with both. We attempted to understand the significance of such findings. First, we investigated the part of ELISA in this variable reactivity by testing S1 and S2 sera by Western blot, and found similar results which excludes the possibility of artifacts. Moreover, the ability of these sera to react with β_2GPI when using Western blot suggests a recognition of native epitopes on the protein cofactor by S1 as well as by S2 sera as reported by others (20). Secondly, we analyzed the influence of the nature of the antigen on the detection of aβ_2GPI and assessed the sera reactivity with a recombinant protein, β_2GPI-PH. Interestingly, S1 sera gave the same results with this protein as with β_2GPI-BS. Thus, the variable reactivity of sera towards β_2GPI could be considered as the reflection of antibody heterogeneity as regards the recognition of various epitopes expressed on the protein. In line with this view, some sera of S1 and S2 groups were tested with three β_2GPI related peptides. The results confirmed the heterogeneity of aβ_2GPI, showing different profiles of peptide reactivity. Recently, Shoenfeld et al. (21) studied, in 295 APS patients, the presence of antibodies against six β_2GPI related peptides including peptides A, B and C. They found a heterogeneous activity of antiphospholipid antibodies directed against various epitopes of the β_2GPI molecule. Interestingly, in

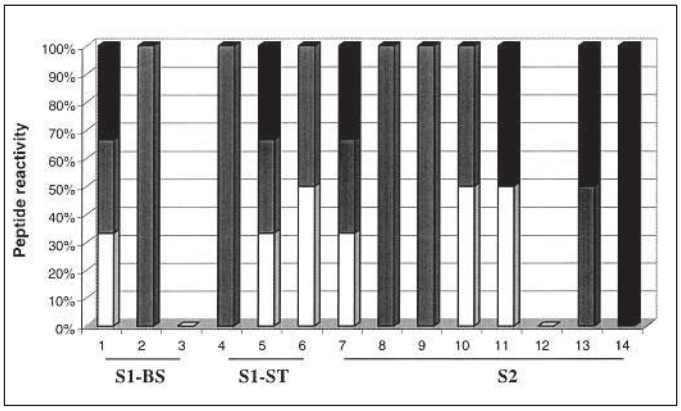

Figure 4: Reactivity of 14 sera, 6 S1 and 8 S2, against three β_2GPI related peptides:
peptide A;
peptide B;
peptide C.

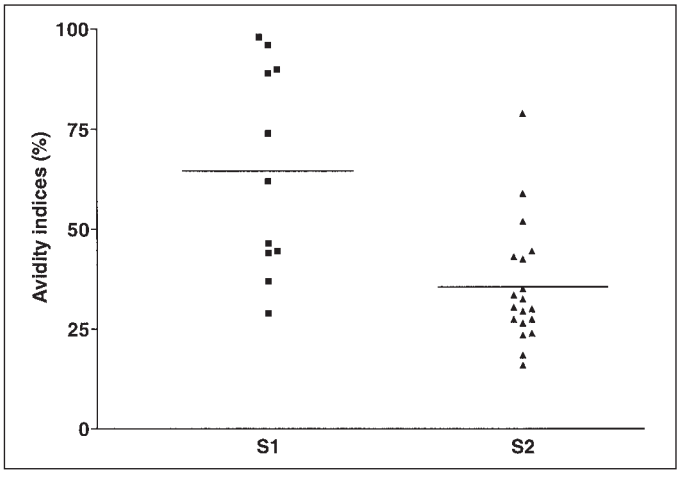

Figure 5: Avidity indices in S1 and S2 sera. Lines represent the median of avidity indices.

our study, among the three peptides tested, peptide B was mainly recognized by sera as reported by Shoenfeld et al.

Finally, in a further attempt to gain a better understanding of the difference in reactivity to β_2GPI-ST as compared to that to β_2GPI-BS and β_2GPI-PH, we studied the role of carbohydrates with regard to antibody recognition. Indeed, variation in the carbohydrate content was involved in the microheterogeneity of the molecule (22–24). As mentioned before, a perchloric acid-precipitation was used for purification of β_2GPI-BS but not for that of β_2GPI-ST. This method has been reported to generate damage to the protein and induce a loss of carbohydrates (24). In this current study, the results of Western blot with native and deglycosylated proteins showed no involvement of N-linked carbohydrates in the epitopes recognized by the antibodies. Similar results have previously been reported by others (25). Moreover, the reactivity of some S1 and of all S2 sera with β_2GPI-PH, a recombinant protein poorly glycosylated, reinforces these results. The similarity of the reactivity between β_2GPI-BS and β_2GPI-PH could be explained by the fact that perchloric treatment of β_2GPI-BS induced a molecular conformation close to that of the recombinant protein.

In this study, we have also shown that heterogeneity of aβ_2GPI was not restricted to antigenic recognition on β_2GPI molecule but was also linked to differences in antibody avidity. Thus, the highest avidity indices were found in S1 sera, most of them being negative for aPL conversely to S2 sera mainly associated with aPL. Moreover, the difference in antibody avidity between S1 and S2 did not appear to be related to the levels of aβ_2GPI, higher in S2 than in S1. In view of our findings, it can be speculated that aβ_2GPI, restricted to the recognition of some epitopes only expressed on a type of β_2GPI preparation, exhibits a higher avidity than those able to react with various epitopes on the two β_2GPI preparations and even on the complex β_2GPI-anionic phospholipids. This study is the first to show a relationship between binding profile and avidity of aβ_2GPI. Furthermore, the fact that S1 sera were mainly collected from non-APS patients suggests that aβ_2GPI avidity is higher in these patients than in APS ones conversely to the results from a previous study showing a relationship between APS and a high avidity of aβ_2GPI (19). Differences in the β_2GPI preparations used could be responsible for discrepancies between the results.

Finally, comparison of clinical features associated with the presence of aβ_2GPI revealed a similar frequency of APS clinical events between S1 and S2 groups, thrombosis and/or pregnancy loss. However, it should be noted that the recruitment criteria of the sera, positivity for aβ_2GPI and availability of clinical data, did not permit evaluation of their clinical relevance. Nevertheless, the comparison between both groups of sera showed significant differences when thrombotic and obstetrical complications were taken into account separately. Thrombotic events were significantly more frequent and more associated with the presence of aPL in patients from S2 than in those from S1 group. Conversely, pregnancy losses were more frequent in patients from S1 group, frequently negative for aPL, than in those from S2 group. It is worth mentioning that no significant difference was found between each subgroup of S1 sera regarding clinical associations as well as aβ_2GPI levels and avidity.

In the literature, there are inconsistencies in the association between aβ_2GPI and aPL in patients with thrombosis or pregnancy loss in APS. These antibodies have been described in thrombotic patients as always associated with aPL (26) or isolated (20, 27, 28) or as an independent risk factor for venous thrombosis (29, 30) or not (31). A recent review of the literature underlines the difficulty of establishing whether aβ_2GPI can be considered independent risk factors (32). In the same way, the relationship between aβ_2GPI and pregnancy loss has been described in women who are negative for aPL, either weak (33, 34) or strong (35).

Sera (n[1])	Mean age Sex ratio (M/F)	aPL[2] +	aPL –	With features of APS	Without features of APS
S1 (n=61)	46 ± 16 (16/45)	n = 13 LA +aCL+ : 4 LA + : 5 aCL + : 4	n = 48	n = 42 ***Thromboses = 32*** *. venous = 22* (recurrent = 7) *. arterial = 10* (recurrent = 0) ***Miscarriages = 12*** . with thromboses = 3 . recurrent = 7 ***SLE = 0***	n =19 ***SLE = 7*** ***Others = 12*** . CTD[3]= 3 . Infections = 3 . Cancers = 3 . MS[4] = 1 . VI[5] = 1 . HELLP = 1
S2 (n=59)	42 ± 15 (12/47)	n = 44 LA +aCL+ : 35 LA + : 2 aCL + : 7	n = 15	n = 42 ***Thromboses = 40*** *. venous = 32* (recurrent = 14) *. arterial = 15* (recurrent = 2) ***Miscarriages = 5*** . with thromboses = 3 . recurrent = 4 ***SLE = 11***	n = 17 ***SLE = 14*** ***Others = 3*** . Cancers = 2 . Healthy but with a familial history of thrombosis = 1

[1]n : number ; [2]aPL : antiphospholipid antibodies ; [3]CTD: Connective Tissue Diseases
[4]MS = Multiple Sclerosis ; [5]VI = Venous Insufficiency.

Table 3: Main biological and clinical features associated with S1 and S2 sera.

Our data show that despite a similar association with APS clinical features, whatever the antigen specificity of aβ_2GPI, most of those found in well diagnosed APS patients recognized various preparations of β_2GPI. Nevertheless, the clinical significance of aβ_2GPI, binding only one preparation of β_2GPI and not associated with aPL, remains to be defined. Moreover, this study underlines the crucial role of β_2GPI preparation in the discrepancies between results from different aβ_2GPI assays and shows the difficulty of obtaining standardisation.

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