Clin Exp Immunol 1994; 95:310–315

IgG2 subclass restriction of anti-β_2 glycoprotein 1 antibodies in autoimmune patients

J. ARVIEUX, B. ROUSSEL, D. PONARD* & M. G. COLOMB† *Laboratoires d'Immunologie Centre de Transfusion Sanguine and *Hôpital Sud, Centre Hospitalier Universitaire, and †Laboratoire d'Immunochimie, INSERM U238, Grenoble, France*

(Accepted for publication 21 October 1993)

SUMMARY

The IgG subclass and light chain distribution of antiphospholipid antibodies (aPL) occurring in autoimmune patients were determined by means of two radioimmunoassays using either cardiolipin- or β_2 glycoprotein 1 (β_2GP1)-coated microtitre plates and mouse MoAbs. Of 50 sera selected for positivity of anticardiolipin antibodies (ACA) of the IgG isotype, 32 (64%) possessed anti-β_2GP1 antibodies and their presence was closely associated with clinical features of the antiphospholipid syndrome. Good correlations were found between ACA and anti-β_2GP1 antibodies when considering antibody level and patterns of light chain and IgG subclass, suggesting that, overall, the same antibodies were being measured. Light chain analysis showed the polyclonal origin of these antibodies and, in most sera, a trend towards use of λ chain. Among sera positive for anti-β_2GP1 antibodies, IgG2 was the major subclass reactive with β_2GP1 and cardiolipin (87% and 74% of the IgG antibody activity, respectively). In contrast, in the group of 18 sera lacking anti-β_2GP1 antibodies, ACA were largely restricted to IgG3, with a lesser contribution by IgG1. A few selected sera from the anti-β_2GP1-positive group were shown to contain mixtures of antibodies that required β_2GP1 (restricted to IgG2 present in large amounts) and did not require this cofactor (restricted to IgG3 and/or IgG1 present in low amounts) for their reactivity with cardiolipin. There was no contribution of glycosylation to the epitopes recognized by anti-β_2GP1 antibodies, even though human anti-carbohydrate antibodies are restricted to the IgG2 subclass. These findings further emphasize the intra- and interindividual heterogeneity of aPL, and should help to discriminate clinically relevant specificities.

Keywords β_2 glycoprotein 1 antiphospholipid antibodies IgG subclass systemic lupus erythematosus

INTRODUCTION

Antiphospholipid antibodies (aPL), usually identified by solid-phase immunoassays using cardiolipin (anticardiolipin antibodies (ACA)), represent a wide group of autoantibodies associated with an increased risk of recurrent thrombosis, pregnancy wastage and thrombocytopenia, a disorder termed 'antiphospholipid syndrome' (APS) [1]. The IgG subclass profile of an antibody response may give insight into the effector mechanisms and T cell regulation of antibody production, as well as the nature of the inducing stimulus. The latter would be crucial for aPL, in view of recent evidence that some of them only bind to anionic phospholipids in the presence of a serum cofactor, β_2 glycoprotein 1 (β_2GP1) [2,3], and of persistent controversy about the true epitope for these β_2GP1-dependent aPL [4–6]. Although repeatedly studied [7–13], the distribution of the IgG ACA subclasses has so far not been clearly established due to conflicting results. These studies have probably been hampered in part by the marked intra- and interindividual heterogeneity of aPL, including the requirement for β_2GP1 for their reactivity, which seems to occur in patients with autoimmune but not infectious disease [1,14].

This led us to re-investigate IgG subclass and light chain patterns of aPL occurring in autoimmune patients by means of two assays using either cardiolipin- or β_2GP1-coated microtitre plates [15]. The specific aims of the current study were to clarify (i) whether ACA and anti-β_2GP1 antibodies have similar IgG subclass and light chain distributions; (ii) whether any particular isotype predominates; and (iii) whether the presence of such a particular IgG type against cardiolipin or β_2GP1 has any relationship to the clinical presentation of these patients.

Correspondence: Dr J. Arvieux, Centre de Transfusion Sanguine de Grenoble, 38701 La Tronche Cedex, France.

PATIENTS AND METHODS

Patients and serum selection

Serum samples, obtained from 50 patients with autoimmune disorders, were selected to cover a wide range of IgG ACA levels, from weakly to strongly positive. Those ACA which required β_2GP1 as a cofactor were identified by a recently developed ELISA using β_2GP1-coated microtitre plates [15]. The cut-off was set at 3 s.d. above the mean of normal controls in both cardiolipin- and β_2GP1-based assays. Thirty-two patients had a diagnosis of systemic lupus erythematosus (SLE) or SLE-like illness (including 12 with features of the APS, defined as secondary APS), 12 had the primary form of antiphospholipid syndrome (PAPS) [1], and the remaining six had unclassified connective tissue disease. There were 40 females and 10 males with mean ages of 40 years and 47 years respectively (range 10–84 years).

β_2GP1-free IgG preparations were obtained from several patient sera as well as control sera by protein G-Sepharose affinity chromatography; their purity was assessed by SDS–PAGE and a sandwich immunoassay using two previously characterized mouse MoAbs to β_2GP1 [16].

Purification and deglycosylation of β_2GP1

β_2GP1 was isolated from human plasma as described previously [15], with the addition of chromatography on a protein G-Sepharose column as the final purification step. N-glycanase (Genzyme) treatment of β_2GP1 was performed according to the manufacturer's instructions, using nine enzyme units for 100 μg β_2GP1 in a final volume of 0·1 ml PBS for 18 h at 37°C.

Radioimmunoassays

The following murine MoAbs were used: clones SG-11, SH-21, SJ-33 and HP6025 (evaluated in the first IUIS/WHO collaborative study [17]) from ICN Immunobiological for IgG1, IgG2, IgG3 and IgG4 determinations, and clones 6E1 and C4 from Immunotech for κ and λ light chain determinations respectively.

For quantification of IgG subclass and light chain distributions of ACA and anti-β_2GP1 antibodies, vinyl microtitre plates were coated overnight at 4°C with 50 μl/well of respectively a 50 μg/ml solution of cardiolipin (Sigma) in methanol and a 10 μg/ml solution of β_2GP1 in PBS. The blocking solution and sample diluent consisted of PBS containing 10% fetal calf serum (FCS) in the ACA assay and 0·1% Tween 20 (PBS-Tween) in the anti-β_2GP1 assay. Duplicate wells were subjected to 1 h successive incubations at room temperature with the serum sample (1 : 50), the anti-IgG subclass or -light chain antibody to be tested (at the appropriate concentration, see below) and finally ^{125}I-labelled goat F(ab')$_2$ anti-mouse immunoglobulin (2×10^5 ct/min per well; specific activity 4×10^7 ct/min per μg). Following final washes performed with PBS in the ACA assay and PBS-Tween in the anti-β_2GP1 assay, individual wells were cut out and counted for γ radioactivity. A pool of normal sera was used in each assay to correct for non-specific binding to the plates, and the ct/min given by this normal pool were substracted from the results for each of the patient's serum sample. The corrected values given by each of the four IgG subclass-specific MoAbs were summed and individual subclasses were expressed as a percentage of this total.

In preliminary experiments, purified human myeloma proteins IgG1 to IgG4, directly coated onto microtitre plates, were used to ensure MoAb reactivity, negate cross-reactivity, and determine appropriate working dilutions of each MoAb in order to obtain (over a wide range of concentrations of immobilized protein) comparable ct/min values in all four subclass assays. Interassay variability, calculated from values obtained for the same serum sample tested on several occasions, ranged from 4% to 21%; intra-assay variability was negligible. The assay for light chain determination was calibrated so that a pool of normal serum immunoglobulins gave similar ct/min values with both κ and γ light chain-specific MoAbs.

To compare within individual patients the patterns of ACA IgG subclasses involved in β_2GP1-dependent and -independent binding, the ACA assay described above was modified by substituting bovine serum albumin (BSA) for FCS to block wells and dilute samples. Also, purified patient IgG was used in place of serum, and purified β_2GP1 was then added to the sample diluent as required.

Western blot analysis

β_2GP1 (2 μg per lane) was resolved by SDS–PAGE in 10% acrylamide gels under non-reducing conditions, followed by electrotransfer to PVDF membranes (ProBlott, Applied Biosystems) for 16 h at 0·05 A in 10 mM 3-cyclohexyl amino-1-propane sulphonic acid, 2·5% methanol (pH 11). Membranes were blocked for 2 h with 2% BSA in PBS-Tween and subjected to 1 h successive incubations with serum samples (1 : 50 in PBS-Tween) and peroxidase-conjugated rabbit anti-human IgG (1 : 1000, Dako) separated by washes of 5 min each with six changes of PBS-Tween. Peroxidase activity was then revealed using H_2O_2 and 4-chloro-1-naphthol.

Statistical analysis

The Spearman's rank correlation test (correlation coefficient, RSp) for non-parametric data and Student's t-test for two independent samples were used as appropriate. The association between clinical features of the APS and the presence of anti-β_2GP1 antibodies was assessed by the χ^2 test. $P < 0.05$ was considered to indicate statistical significance.

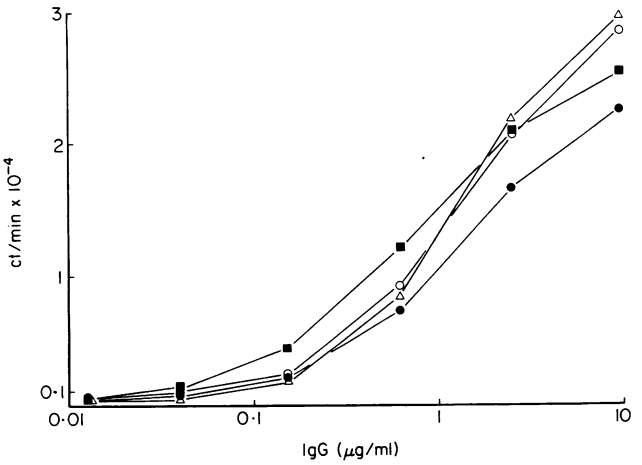

Fig. 1. Binding of MoAbs specific for IgG1 (●), IgG2 (○), IgG3 (■) and IgG4 (△) to purified myeloma proteins IgG1 to IgG4 (homologous combinations), directly applied to microtitre plates in decreasing concentrations. MoAbs were used diluted from ascites 1 : 100 (anti-IgG1 and -IgG3) and 1 : 10 000 (anti-IgG2 and -IgG4).

Table 1. Spearman rank correlations between levels of IgG antibodies to cardiolipin (ACA) and β_2-glycoprotein 1 (β_2GP1) and their IgG subclass distribution in 50 serum samples from autoimmune patients

	Per cent total IgG			
	IgG1	IgG2	IgG3	IgG4
IgG ACA level and IgG ACA subclass	−0·07	0·65*	−0·65*	0·13
IgG ACA level and IgG anti-β_2GP1 subclass	0·44**	0·58*	0·42**	0·36***
IgG anti-β_2GP1 level and IgG ACA subclass	−0·29***	0·79*	−0·67*	−0·02
IgG2 anti-β_2GP1 (%) and IgG ACA subclass	−0·36***	0·73*	−0·63*	0·07

*$P<0·001$; **$P<0·01$; ***$P<0·05$.

RESULTS

Relationship of anti-β_2GP1 antibodies to clinical complications of APS

Of 50 sera from patients with autoimmune disorders (mostly SLE and PAPS) selected for raised IgG ACA, 32 (64%) had anti-β_2GP1 antibodies detectable. Among the latter group of 'double-positive' patients, 23 presented clinical manifestations of APS (including 11 secondary APS and all 12 PAPS), compared with one of 18 patients in the group lacking anti-β_2GP1 antibodies ($P<0·001$). This patient had in fact a variant of APS with haemolytic anaemia due to ACA of the IgG3 and IgM isotypes [18]. The positive or negative status of a given patient for anti-β_2GP1 antibodies tended to remain stable with time, and their levels correlated well with those of ACA (RSp = 0·82, $P<0·001$). IgG anti-β_2GP1 levels averaged 11 099 ct/min *versus* 17 470 ct/min for IgG ACA, with marked differences between the two groups of patients with and without anti-β_2GP1 antibodies (25 380 and 3407 ct/min respectively, $P<0·001$).

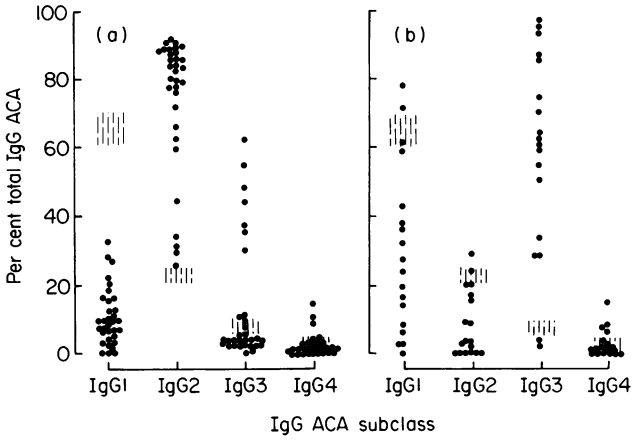

Fig. 2. Percentage distribution of IgG anticardiolipin (ACA) subclasses in the sera of 32 patients with anti-β_2 glycoprotein 1 antibodies (a) and 18 patients without anti-β_2 glycoprotein 1 antibodies (b). The hatched areas represent normal ranges for each IgG subclass in serum.

IgG subclass distribution of ACA and anti-β_2GP1 antibodies

To circumvent well known difficulties in the quantification of IgG subclasses [17,19], a set of subclass-specific murine MoAbs was used at predetermined dilutions that were found to give comparable ct/min values in calibration experiments with immobilized myeloma proteins (Fig. 1). In both ACA and anti-β_2GP1 antibody assays, results for total IgG binding (as measured using radiolabelled goat anti-human IgG) correlated well with those obtained by summation of independently measured IgG1 to IgG4 antibodies.

Correlations were performed between the level of IgG binding to cardiolipin or β_2GP1 and the IgG subclass distribution of these antibodies, expressed as a percentage of the total specific IgG activity contributed by each subclass (Table 1). There were strong correlations between binding to both cardiolipin and β_2GP1 and IgG ACA of subclasses IgG2 (positive correlation) and IgG3 (negative correlation) ($P<0·001$ in all cases). Similar positive and negative correlations existed between ACA of IgG2 and IgG3 subclasses respectively and IgG2 anti-β_2GP1 ($P<0·001$ in both cases). Weaker correlations were seen between reactivity of IgG (or IgG2) anti-β_2GP1 and ACA of the IgG1 subclass ($P<0·05$). The only weak correlation concerning the binding activity of IgG4 was between IgG4 binding to β_2GP1 and IgG ACA levels ($P<0·05$).

Patient sera were subdivided into two groups based on the presence or absence of anti-β_2GP1 antibodies, and the results of their respective ACA IgG subclass distribution are shown in Fig. 2a, b. It may be seen that the ACA response was markedly restricted to the IgG2 subclass in sera positive for anti-β_2GP1 antibodies and to the IgG3 subclass in sera negative for these antibodies. Within these respective groups, an average of $74\pm19\%$ (mean $\pm$ s.d.) was IgG2 and $58\pm29\%$ IgG3, which is far above the normal range for these subclasses in whole serum IgG. Anti-β_2GP1 antibodies distributed as $6\pm7\%$ IgG1, $87\pm12\%$ IgG2, $3\pm4\%$ IgG3 and IgG4. These fractional amounts were significantly higher for IgG2 and lower for IgG3 ($P<0·01$ in both cases) and IgG1 ($P=0·035$) than those of ACA in the same sera. The isotype restrictions observed for ACA and anti-β_2GP1 antibodies were not reflected in total serum IgG, which exhibited a normal subclass distribution in the few sera examined (not shown).

We would therefore predict from the above data that some sera from the anti-β_2GP1-positive group contain mixtures of antibodies that require β_2GP1 (restricted to IgG2 present in large amounts) and do not require this cofactor (restricted to

Table 2. IgG anticardiolipin antibody (ACA) level and subclass distribution using purified patient IgGs in the modified ACA assay in the presence and absence of β_2-glycoprotein 1 (β_2GP1)

Added β_2GP1 (μg/ml)	IgG ACA level*	Per cent total IgG			
		IgG1	IgG2	IgG3	IgG4
Patient 1					
0	2·39	14·1	0	85·9	0
20	15·65	3·5	84·1	11·0	1·4
Patient 2					
0	3·63	22·2	20·2	57·0	0·6
20	63·49	12·1	82·1	3·4	2·4

*Ct/min $\times 10^{-3}$; values obtained with normal human IgG have been subtracted.

IgG3 and/or IgG1 present in low amounts) for their reactivity with cardiolipin. To analyse further this issue, we used IgG purified from selected patient sera in a modified ACA assay and examined the role of purified β_2GP1 in binding activity of various IgG subclasses. Typical results are shown in Table 2. Differences appeared, as expected, in both level of IgG binding to cardiolipin and IgG subclass distribution between β_2GP1-dependent and -independent antibodies.

Light chain distribution of ACA and anti-β_2GP1 antibodies

We then determined the light chain distribution of these same specificities in order to confirm partial overlap between assays for ACA and anti-β_2GP1 antibodies in autoimmune patients, and to search for an oligoclonal or monoclonal origin of these antibodies. κ/λ light chain ratios within the group of anti-β_2GP1-positive sera assayed for ACA and anti-β_2GP1 antibodies correlated significantly (Fig. 3). These antibodies were polyclonal, since no serum was found with a single light chain type determining antibody activity. With the exception of two sera showing a marked predominance of κ light chain, κ/λ ratios for ACA and anti-β_2GP1 antibodies were somewhat lower than the expected values in serum IgG, although the differences were not statistically significant.

Independence of anti-β_2GP1 antibody binding on carbohydrates

Reports that antibodies to carbohydrate antigens are restricted primarily to the IgG2 subclass in human or its analogue in other species [20] prompted us to search for an involvement of carbohydrates in the binding sites of anti-β_2GP1 antibodies. β_2GP1 was thus digested with protease-free N-glycanase to remove the four N-linked oligosaccharides attached to the molecule, and the effect on its recognition by 15 patient sera was assessed by immunoblotting. Positive staining of five bands (representing the native protein and the serial removal of the oligosaccharides) was obtained with nine among the strongest anti-β_2GP1 sera, as exemplified in Fig. 4, whereas the other six were unreactive with either form of the molecule. Using anti-IgG2 MoAb, followed by enzyme-conjugated anti-mouse immunoglobulin as a detection system, led essentially to the same results.

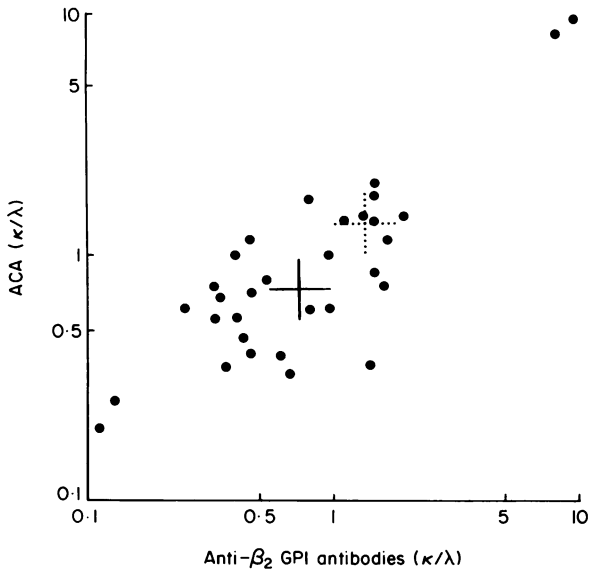

Fig. 3. Correlation between κ and λ light chain ratios of anticardiolipin antibodies (ACA) and anti-β_2 glycoprotein 1 (β_2GP1) antibodies in the sera of 32 patients. Spearman's correlation coefficient was 0·70 ($P < 0·001$). Horizontal and vertical dotted lines represent mean values (1·33 for both ratios) and continuous lines, median values (0·73 for both ratios).

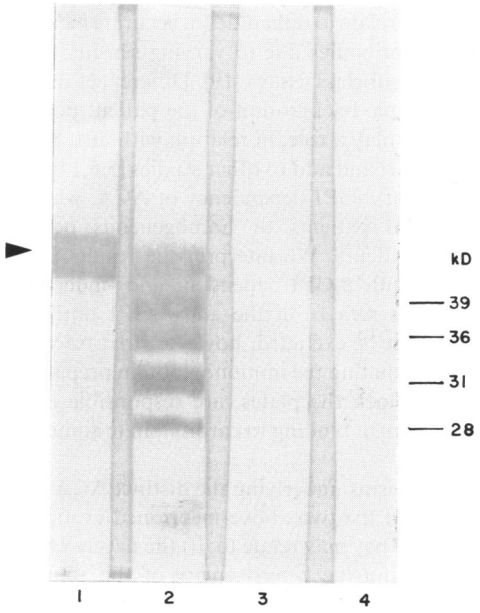

Fig. 4. Western blot analysis of β_2 glycoprotein I (β_2GP1) untreated (lanes 1 and 3) and treated with N-glycanase (lanes 2 and 4). Lanes 1 and 2 were reacted with a patient's serum positive for anti-β_2GP1 antibodies, and lanes 3 and 4 with a serum lacking these antibodies. The location of native β_2GP1 is indicated by the arrow.

DISCUSSION

We have recently developed an ELISA that uses β_2GP1 on the solid phase with the aim of improving the detection of clinically relevant aPL [15]. In this respect, the close association of anti-β_2GP1 antibodies with features of the APS, noted in the present study, is in keeping with a previous report of SLE patients [21]. Although the IgG ACA isotype appears to be most strongly associated with clinical complications [1], we observed occasional patients with one or more features of the APS, that possessed ACA and anti-β_2GP1 antibodies of the IgM isotype exclusively (unpublished observation). The population of 50 autoimmune patients studied here segregated into two groups on the basis of both the presence of anti-β_2GP1 IgG antibodies and pattern of ACA IgG subclass. A different distribution of IgG ACA subclass was found between sera with and without anti-β_2GP1 antibodies, in that they were mainly restricted to IgG2 and IgG3, respectively. In line with our results is the report by Gharavi *et al.* [8] that the clinical complications, thrombosis, fetal loss or thrombocytopenia, defining the APS were found more frequently in patients with IgG2 and IgG4 ACA than in patients with IgG1 and IgG3 ACA. Also in agreement with us, Loizou *et al.* [10] noted predominantly IgG1 and IgG3 ACA in autoimmune sera with low ACA levels, in contrast to a strong predominance of the IgG2 subclass in those with high ACA levels.

As a whole, however, conflicting data have appeared in the literature regarding the IgG subclass distribution of aPL as well as the absolute requirement of β_2GP1 in their binding to anionic phospholipids [5,6,22]. Amongst patients with SLE or related autoimmune disorders, IgG ACA activity has been reported to involve all four IgG subclasses [8,12], selectively IgG1 and IgG2 [7,11], selectively IgG2 and IgG4 [9], and IgG1 and IgG3 together with predominant elevation of IgG2 in a subgroup of 25–28% of patients [10,13]. A potential explanation for these differences may relate to difficulties in accurate measurements of IgG subclass antibodies due to varying sensitivity in the four individual IgG subclass assays [19]. Differences in the selection criteria and ethnic background of the patient populations for study may also play a role, in relation with aPL heterogeneity. Our data support and add to other studies [5,6,14,22] regarding the variability in β_2GP1 dependency of ACA, whether derived from individual patients or homogeneous populations of autoimmune patients. We interpret the results in Table 2 to indicate that both β_2GP1-dependent and -independent ACA coexist in some sera from the anti-β_2GP1 antibody-positive group. It cannot be excluded, however, that trace amounts of β_2GP1, contaminating the immunoglobulin preparations or the BSA used to block the plates, are responsible for seemingly β_2GP1-independent binding to cardiolipin in some of the above studies.

The mechanisms underlying the distinct ACA IgG subclass responses within the two above-mentioned groups of patients are of interest. They may relate to: (i) the nature of the inducing stimulus, stressing the heterogeneity of the actual targets of ACA; and (ii) the T cell control of antibody response, especially the nature and amounts of the cytokines produced. Evidence from the murine system suggests that differential cytokine production by helper T cells is centrally important in determining the isotype profile of an antibody response [23]. In this respect, it is of interest that an imbalance of the cytokine profile (with decreased production rate of IL-2, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF)) has been reported in a murine model of PAPS [24]. Our results demonstrating similar restriction of anti-β_2GP1 antibodies to the IgG2 subclass in patients with PAPS and APS secondary to SLE may indicate identity in the nature of the inducing antigen and, presumably, the form in which it is presented to the immune system in both diseases. With regard to the generation of cofactor-dependent aPL, Bevers *et al.* [25] have proposed an attractive hypothesis in which expression of procoagulant surfaces upon platelet activation and/or endothelium damage would result in binding and cryptic epitope exposure of β_2GP1 and prothrombin, thereby inducing an immune response to these modified lipid-bound plasma proteins. Recent demonstrations, using MoAbs to β_2GP1 as probes, that phospholipid binding could lead to changes in β_2GP1 antigenicity are in accordance with such a model [26]. Despite the fact that anti-carbohydrate antibodies are restricted to human IgG2 or its analogue in other species [20], the epitopes recognized by anti-β_2GP1 antibodies do not appear to involve any of the four oligosaccharide chains, as demonstrated by immunoblotting after N-glycanase digestion of β_2GP1.

The IgG2 subclass restriction of anti-β_2GP1 antibodies is likely to be of biological significance, since each IgG subclass exhibits a distinct range of potential effector functions. In view of the relatively poor ability of IgG2 to activate complement through the classical pathway and Fcγ receptor-bearing cells [19], it seems unlikely that such effector mechanisms are intimately involved in the pathogenicity, if any, of anti-β_2GP1 antibodies. In particular, this would make our recent observation that murine IgG1 MoAbs against β_2GP1 activate human platelets through β_2GP1-mediated binding and Fcγ receptor cross-linking irrelevant to the thrombotic pathophysiology of APS [27]. However, matters are complicated by the self-associating properties of the analogue of human IgG2, mouse IgG3, accounting for enhanced binding to multivalent antigens (intermolecular cooperativity) [28]; these interactions between Fc regions of adjacent bound molecules would in turn help to activate effector mechanisms, such as the complement cascade and Fcγ receptor-dependent processes.

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