Monoclonal Gammopathy, Nicola Bizzaro, Paola Pasini, and Bruno Finco (Laboratorio di Patologia Clinica, Ospedale Civile, 30027 San Donà di Piave, Venice, Italy; *author for correspondence: fax 00-39-0421-227571, e-mail nbizzaro@dacos.it)

We report here that measurements of IgA anti-cardiolipin (aCL) and anti-β2-glycoprotein I (β2GPI) antibodies are unreliable in patients with IgA monoclonal gammopathies (MGs). MGs are characterized by monoclonal immunoglobulins produced by a proliferating B-lymphocyte clone. Monoclonal gammopathy of undetermined significance (MGUS) is the most common type of MG (1); the monoclonal component (MC) is IgA in 15–25% of MGs (2).

aCL antibodies, originally described in patients with systemic lupus erythematosus (3), have been detected in patients with other autoimmune diseases (4, 5) and in the antiphospholipid syndrome (6, 7) as well as in nonrheumatologic disorders (8) and in healthy individuals (9–11). In patients with connective tissue diseases and antiphospholipid syndrome, aCL antibodies are most frequently IgG, followed by IgM. Kalunian et al. (12), however, reported a high incidence of IgA, rather than IgG-aCL antibodies associated with thrombosis and fetal loss, and other reports have suggested that IgA-aCL antibodies may have a clinical meaning even in the absence of IgG- or IgM-aCL (13–21). Their prevalence in healthy subjects seems negligible; in a series of 389 healthy pregnant women, Lynch et al. (22) found only one positive case (0.3%). Therefore, to properly assess the risk of thrombosis, thrombocytopenia, and recurrent abortions in systemic lupus erythematosus patients, the determination of IgA-aCL antibodies has been recommended (18, 21). Very recently, the same recommendation was also made for anti-β2GPI antibody determination because anti-β2GPI restricted to the IgA isotype can be found in the sera of systemic lupus erythematosus patients with thrombosis (23).

IgG- or IgM-aCL activity has been described occasionally in subjects with MG (24–27), but to our knowledge the association of IgA-MG with IgA-aCL and anti-β2GPI antibodies is not known. The objective of this study was to evaluate whether and to what extent subjects with IgA-MG show IgA-aCL and anti-β2GPI activity.

We studied 135 subjects (mean age, 70 years; range, 44–89 years; 70 men, 65 women), of whom 20 had IgG-MGUS (10k, 10a), 20 had IgM-MGUS (10k, 10a), 45 had IgA-MGUS (24k, 21a), and 50 were age- and sex-matched healthy controls. Of the 45 patients with IgA-MGUS, 20 belonged to the initial protocol; the other 25 were subsequently entered on the basis of preliminary findings that showed a high positivity of IgA-MGUS sera. Patients with known autoimmune diseases, in whom the MC was detected as part of the evaluation of their primary disease, were excluded from this study, as were patients with biclonal gammopathies. None of the patients had a clinical history of thromboembolic episodes, thrombocytopenia, or recurrent fetal loss. A TPHA test was performed in every case to rule out cross-reacting antibodies to Treponema pallidium.

The presence of a MG was first detected by serum protein electrophoresis and then identified by immunofixation electrophoresis with the Paragon System (Beckman Instruments). MCs were then quantified by densitometric analysis. The concentration of each immunoglobulin isotype was also quantified with monospecific antisera and calibrators, using the Behring Nephelometer BNA (Behring Diagnostics). IgA subclasses were assayed by nephelometry and immunofixation electrophoresis, using commercial reagents (The Binding Site); 43 patients had an IgA1 and 2 had an IgA2 MC. Most of the sera samples were stored at −85°C up to 1 year before testing, whereas a small portion were tested within 48 h of collection.

aCL antibodies (IgG, IgM, and IgA) were measured in serum samples diluted 1:100 in phosphate-buffered saline (0.1 mol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4) supplemented with 100 g/L fetal calf serum by ELISA using commercial aCL antibody kits (CLS for IgG and IgM; Reaads Medical Products and Elias for IgA), according to the manufacturers’ instructions. All samples were analyzed in duplicate for all immunoglobulin isotypes and with the two different kits for IgA. IgA-anti-β2GPI antibodies were measured in the same sera by an ELISA method (Elias). In every assay, nonspecific background absorbance was measured in wells filled with sample diluent alone, and this value was routinely subtracted from the test absorbance values. Moreover, to exclude the possibility of nonspecific binding between the antibodies and the plates (28), all IgA-MGUS sera were also examined on antigen-free plates.

For aCL activity, the results are expressed as phospholipid units (GPL for IgG, MPL for IgM, and APL for IgA), based on a calibration curve prepared with international reference samples (29). A plasma with known aCL (IgG, IgM, and IgA) concentrations was included in each assay. Samples were scored as positive when values exceeded the normal cutoff concentrations (11.3 GPL, 10.1 MPL, and 12.7 APL) obtained by calculating the mean value plus 2 SD in 83 healthy individuals studied previously in our institution. For anti-β2GPI, results are expressed in kilounits/L, based on a calibration curve. According to the cutoff indicated by the manufacturer, values >15 kilounits/L were considered positive. Statistical analysis was performed using the SPSS/PC+ package. The Mann–Whitney rank-sum U-test was used to compare most of the variables; a P value <0.05 was considered significant. The Spearman rank correlation (r) test was used to detect associations of aCL or anti-β2GPI with IgA concentration.

Monoclonal IgA concentrations measured densitometrically in the 45 IgA-MGUS sera ranged from 6.56 to 46.5 g/L (reference range of serum IgA, 1.5–4.8 g/L). IgA-aCL antibodies were detected in all 45 patients (100%) with IgA-MGUS. 4 were weakly positive (13–40 APL), 31 moderately positive (40–80 APL), and 10 strongly positive (>80 APL), with perfect agreement between the results of the two tests used (Reaads and Elias). No differences in reactivity were observed between
monoclonal IgA bearing κ or λ light chains or belonging to the IgA1 or IgA2 subclass. No sample with an IgG MC showed aCL activity, whereas three samples with an IgM MC (15%) were positive for IgM-aCL antibodies (two with low and one with high titer). The IgA-aCL titer was correlated with densitometric concentrations of IgA MC (r_s = 0.926; P < 0.0001; Fig. 1A). None of the 50 control sera passed the threshold of positivity, and no positive result was obtained on antigen-free plates.

Of the 45 IgA-MGUS sera tested for IgA anti-β2GPI, 43 (96%) were positive: 28 with low titers (15–40 kilounits/L), 5 with moderate titers (41–80 kilounits/L), and 10 with high titers (>80 kilounits/L). None of the 50 control sera showed positive results (>15 kilounits/L), and the anti-β2GPI titer was correlated with serum IgA concentrations (r_s = 0.928; P < 0.0001; Fig. 1B).

To verify whether the positive results were indeed attributable to IgA MC, immunosubtraction of IgA was performed by incubating patient sera with equal amounts of agarose-sheep immunoglobulin anti-human IgA complexes (Beckman Analytical) for 3 h at room temperature with constant mixing. After centrifugation at 10,000 g for 30 min, the supernatants were collected, reconstituted, and retested by ELISA. We observed a decrease in IgA concentrations (mean, 39.1%; range, 16.3–62.5%; P < 0.00001) determined nephelometrically, as well as IgA-aCL (mean, 32%; range 11.4–86.0%) and anti-β2GPI antibody concentrations (mean, 28%; range, 8.4–72.6%), the entity of which was also directly related to the MC concentration in whole serum (P < 0.0001 for aCL and P < 0.0002 for anti-β2GPI antibodies).

Purified monoclonal IgA was prepared from the serum of 10 patients (5 with IgA κ light chain and 5 with λ light chain) who were positive for aCL and anti-β2GPI activity by means of affinity chromatography using agarsose-bound jacalin (Pierce) according to the method described by Gregory et al. (30). Jacalin is an α-d-galactose lectin extracted from jack-fruit seeds that selectively binds IgA (31). Purified IgA was then eluted with 0.1 mol/L melibiose monohydrate in phosphate-buffered saline, and the fractions with the highest absorbance at 280 nm were collected. Melibiose was removed from the eluates by a buffer-exchange gel filtration/desalting column (Pierce). The mean recovery of IgA was 52.7%. The samples were then reconstituted to the initial volume and tested for aCL and anti-β2GPI activity. Finally, serial dilutions of purified polyclonal IgA (product no. I-0633; Sigma BioSciences) were tested for aCL and anti-β2GPI binding activity. IgA concentration in the diluted samples ranged from 2.25 to 25 g/L. Both jacalin-purified monoclonal IgA obtained from patient sera and commercial polyclonal IgA (Sigma) showed positive reactions for aCL and anti-β2GPI activity, with absorbance values correlated with IgA concentration. Interference occurred at IgA concentrations >5 g/L.

To further verify whether nonspecific binding was present, 10 IgA-MGUS sera were also tested by immunoenzymatic assay for the presence of IgA anti-gliadin antibody (ImmunoCAP System; Pharmacia-Upjohn) and anti-transglutaminase IgA antibody (Medipan); all 10 sera were positive in both tests.

In this study we observed that all sera from subjects with an IgA MC were positive for IgA-aCL activity. Although IgG and/or IgM MCs with functional autoantibody activity to many specific proteins, including phospholipids, have been described occasionally in MGUS patients (24, 25, 32), this unexpected finding suggested that we might have been dealing with false-positive results attributable to the high concentration of IgA MCs. Indeed, interferences from IgG or IgM MCs on aCL ELISAs have been reported in patients with hypergammaglobulinemia (33–35). Hughes et al. (33) also observed that serum concentrations of IgG >16 g/L and of IgM >3 g/L were responsible for nonspecific binding on polyvinyl plates. Other laboratory methods, such as spectrophotometry, nephelometry, or radioimmunoassays, are also subject to interference by MCs (36–40), and the phenomenon does not depend on the antibody specificity of the monoclonal immunoglobulins but on their physicochemical properties. The positive results obtained with other
laboratory tests to detect the presence of IgA autoantibodies (anti-gliadin and anti-transglutaminase) confirmed the absence of specific binding to cardiolipin. Moreover, the negative results observed after immunosubtraction and the close correlation between the antibody concentrations of both mono- and polyclonal purified IgA and aCL activity as well as anti-β₂GPI concentrations clearly demonstrated that the reactivity was related to IgA antibodies.

The precise mechanism responsible for this very high rate of IgA immunoreactivity is not clear. Several possible clues were considered. A high background signal, as demonstrated by Cowchock et al. (35) for IgM-aCL antibodies, was excluded because tests conducted in the absence of serum as well as in the absence of antigen gave only a weak reactivity. In addition, aCL assays in our 50 controls consistently showed absorbance values below the cutoff point.

We also considered the possibility that the positive findings might be related to prolonged storage because most serum samples were stored at −85 °C for up to 1 year before testing; however, this was excluded because the samples that were tested immediately after collection also gave positive results. Moreover, it was demonstrated that long-term storage of frozen samples does not influence the results of aCL measurements (41, 42). In addition, samples of sera with IgG and IgM MCs were consistently negative before and after long-term storage. The most likely explanation is that IgA antibody molecules might have a greater capacity than other immunoglobulin isotypes to form nonspecific bonds with various substrates.

This study demonstrates that a serum IgA concentration >5 mg/L may cause interferences; it also shows that measurement of IgA-aCL and anti-β₂GPI activity in patients presenting with an IgA-MG is not reliable and that the serum factor identified by ELISA should not be interpreted as a specific aCL or anti-β₂GPI antibody. Laboratories using IgA assays for aCL and anti-β₂GPI determinations should be aware of these potential interferences and should question the credibility of their results.

References

Lectin ELISA for Analysis of α2-Acid Glycoprotein Fucosylation in the Acute Phase Response, Ingar Rydén,1 Arne Lundblad,2 and Peter Pählsson2 (1 Department of Clinical Chemistry, Kalmar County Hospital, S-39185 Kalmar, Sweden; 2 Department of Biomedicine and Surgery, Division of Clinical Chemistry, Linköping University Hospital, S-58185 Linköping, Sweden; * author for correspondence: fax 46-480-81025, e-mail ingvar.ryden@swipnet.se)

In recent years, increasing attention has been directed to the specific changes in glycosylation of glycoproteins that occurs in response to certain physiologic and pathologic conditions (1–6). α2-Acid glycoprotein (AGP; orosomucoid) is a heavily glycosylated acute phase protein with five glycosylation sites carrying N-linked, complex-type oligosaccharides (N-glycans). The function of AGP is not well understood, but immunomodulating properties have been suggested that may be dependent on the expression of the sialyl LewisX (SLeX) epitope, in which fucose is a necessary component (7). However, methods for analysis of the glycosylation of acute phase proteins have been time-consuming and not suitable for routine analysis in a clinical laboratory (3, 6, 8).

A lectin ELISA has been used previously for analysis of a tumor marker (9). The Aleuria aurantia lectin (AAL) was used in an ELISA for analysis of fucosylation of serum cholinesterase in liver disease (10). We developed a lectin ELISA that uses biotinylated AAL and a capture antibody specific for AGP to study the fucosylation on AGP in the acute phase response. As a model for acute inflammation, we monitored the daily changes in AGP fucosylation in patients with severe burns.

The plasma concentration of AGP and C-reactive protein (CRP) was analyzed on a Cobas Integra 700 (Roche). AAL was purified from locally picked mushrooms as described previously (7). AAL fractions were pooled, lyophilized, and stored at −20 °C until biotinylation was performed according to the manufacturer’s instructions (ImmunoprobeTM Biotinylation Kit; Sigma). Biotinylated AAL was stored at 4 °C. Microtiter plates (Nunc-ImmulonTM Maxisorp) were coated with polyclonal antibodies directed against human AGP (anti-human orosomucoid, cat. no. A0011; Dako), diluted 1:100 in coating buffer (15 mmol/L Na2CO3, 35 mmol/L NaHCO3, and 0.2 g/L NaN3 pH 9.6), for 12 h at 4 °C. The following procedures were then performed at room temperature.

Two hundred microliters of blocking agent [phosphate-buffered saline (PBS), pH 7.4 containing 50 g/L bovine serum albumin (BSA)] was added to the wells, and the plates were incubated on a shaker for 60 min. The wells were then washed four times with a washing solution (9 g/L NaCl, 7.5 g/L Tween). Patient samples (serum or plasma) were diluted 1:200 in PBS containing 10 g/L BSA. The diluted samples (100 µL) were individually added to wells and incubated on a shaker for 60 min. Samples were added in duplicates for each patient. The plates were washed six times with washing solution and incubated with 100 µL of biotinylated AAL diluted 1:100 in PBS containing 10 g/L BSA, incubated for 60 min on a shaker, washed six times, and incubated with ExtrAvidin (Sigma) diluted 1:1000 in PBS containing 10 g/L BSA. After incubation for 60 min on a shaker, the plates were washed six times and incubated with 100 µL of the substrate (1 tablet of ABTS dissolved in 5 mL ABTS buffer; Boehringer Mannheim) for 30 min on a shaker. Absorbances were read at 405 nm. The Statistica 5.1 software package (StatSoft) was used for statistical analysis.

Plasma was collected from healthy donors, pooled, and stored at −70 °C to be used as the control. A fucosylation ratio (AGP-FR) was calculated as a ratio of the mean absorbance of patient samples to the mean absorbance of the control pool, after subtraction of blank values:

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\text{AGP-FR} = \frac{\text{mean } A_{405} \text{ patient sample} - \text{blank}}{\text{mean } A_{405} \text{ control pool} - \text{blank}}
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There was no difference between the lectin ELISA results for serum, EDTA plasma, or heparin plasma. The specificity of lectin binding was tested by addition of free l-fucose to the AAL solution before addition to the microtiter plate. Fucose inhibited lectin binding in a dose-dependent manner, with complete inhibition at a concentration of 0.1 g/L. Samples from 30 patients with increased AGP (range, 1.2–3.5 g/L) were analyzed both before and after adjustment of AGP concentration to 0.7 ± 0.2 g/L. A very minor effect of the AGP concentration on lectin binding was noted. However, to avoid any interference in a clinical study, the AGP concentration was adjusted to 0.7 ± 0.2 g/L in samples having an AGP concentration >1.1 g/L.

To test for linearity, a pool of sera with highly fucosylated AGP (AGP-FR = 5.5) was diluted with the control pool (AGP-FR = 1.0). Both pools had the same concentration of AGP. A linear correlation \(r^2 = 0.98\) was found in this interval, as shown in Fig. 1A. The within-run coefficient of variation (CV) was 5% for the control pool and 3% for a sample with highly fucosylated AGP. The total CV for the control pool was 10%.

We compared the lectin ELISA results to high-pH anion-exchange chromatography with pulsed ampero-