Phospholipid-independent Binding of β₂glycoprotein I by IgA from Patients with Antiphospholipid Syndrome*

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ABSTRACT

β₂glycoprotein I (β₂GPI) is a phospholipid-binding protein of the coagulation system. In patients with the antiphospholipid syndrome (APS), antibodies to β₂GPI contribute to the population of “antiphospholipid antibodies” measured in the anti-cardiolipin antibody (aCL) assay. In fact, both IgG and IgM antibodies from patients with APS bind β₂GPI in the absence of anionic phospholipids if the antigen is bound to a suitable surface, i.e., one which exposes the epitope. The binding of IgA was studied from patients with APS, using an enzyme-linked immunosorbent assay (ELISA), and significantly higher binding of IgA was observed from 39 patients compared to a control group of 50 healthy individuals (p < 0.0001). Moreover, 15 out of 39 APS subjects (38 percent) exhibited binding greater than 5 standard deviations (SD) above the mean of the control group. All 39 APS patients had elevated IgG anti-β₂GPI; however, depletion of IgG from two APS sera diminished, rather than enhanced, binding of IgA. Pre-incubation with purified IgG from a subject with APS led to inhibition of IgA binding at inhibitor levels >125 μg IgG/well. These data demonstrate that patients with APS have IgA anti-β₂GPI autoantibodies and that the epitope(s) which are recognized by these antibodies can be presented in the absence of cardiolipin or other anionic phospholipids.

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Introduction

Antiphospholipid syndrome (APS) is an autoimmune coagulopathy, the pathophysiology of which is largely related to associated thrombotic events.\(^1\) Although APS was originally described as occurring secondary to systemic lupus erythematosus,\(^2\) it is now recognized in some patients who have no other autoimmune disease and in whom it is known as primary APS.\(^3\)

The name APS derives from its well known association with autoantibodies to cardiolipin and other anionic phospholipids.\(^2,4,5\) In 1990, two groups independently determined that serum from patients with APS required a protein cofactor, apolipoprotein H (more commonly referred to as \(\beta_2\)glycoprotein I, \(\beta_2\)GPI), for anticardiolipin reactivity.\(^6,7\) This 50 kDa protein is now generally accepted to be the relevant autoantigen, the role of anionic phospholipids being that of providing a surface which modifies conformation of the native \(\beta_2\)GPI in such a manner as to present a normally encrypted epitope\(^8,9\) or increased antigen density.\(^10\)

Most studies of anti-\(\beta_2\)GPI have focused on IgG and, to a lesser extent, IgM autoantibodies. Although IgA anticardiolipin antibodies have been demonstrated in patients with APS,\(^11,12\) investigation of phospholipid-independent anti-\(\beta_2\)GPI autoantibodies of this immunoglobulin class has not been reported. In this paper our studies describe the binding of \(\beta_2\)GPI by IgA from a group of patients with APS.

Materials and Methods

**Serum and Plasma Specimens**

The APS serum and plasma specimens used in this study were obtained from the following sources: (a) serum samples from patients previously seen on the rheumatology service and stored in our serum bank (n = 8); (b) serum obtained under a prospective protocol approved by the Institutional Review Board of St. Joseph's Hospital and Medical Center (n = 5); and (c) samples kindly provided by Dr. Thomas Ortel (plasma, n = 6), The Coagulation Laboratory, Duke University Medical Center and Dr. Ronit Simantov (serum, n = 20) of Cornell University–New York Hospital. Patient specimens included in this study were from patients diagnosed with APS, based on both clinical and laboratory criteria. Normal sera (n = 50) were drawn from a group of 204 healthy individuals described in an earlier study\(^13\) and maintained in our serum bank.

**Binding Studies**

Binding of IgA to \(\beta_2\)GPI was measured by ELISA using commercially available microtiter plates and reagents.\(*\) The assay was carried out in a manner analogous to that described previously in a study of IgG binding.\(^9\) The \(\beta_2\)GPI-coated wells were incubated for 30 min at RT with 100 \(\mu\)L of specimens diluted as described in the Results, washed thrice with phosphate buffered saline (PBS; 0.138 mol/L NaCl, 0.0027 mol/L KCl and 0.01 mol/L phosphate, pH 7.4)\(\dagger\) and incubated with 100 \(\mu\)L peroxidase-conjugated antiserum for 30 min at RT. The wells were again washed thrice and incubated with chromogen (tetramethyl benzidine with \(H_2O_2\)) for 30 min at RT, after which the reaction was stopped by the addition of 100 \(\mu\)L of \(H_2SO_4\). The absorbance at 450 nm was measured in a microtiter plate reader.\(‡\)

IgG anti-\(\beta_2\)GPI was measured by the method described in detail in a recent paper\(^13\) and by a commercially available assay based on our reference standards.\(^14\)

**Preparation of Purified IgG Fraction of Anti-\(\beta_2\)GPI**

An IgG fraction of a \(\beta_2\)GPI-reactive plasma calibrator (HRM-01), previously described,\(^14\)

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\(*\) INOVA Diagnostics, San Diego, CA. The binding of IgG anti-\(\beta_2\)GPI from 58 subjects was compared using these antigen coated plates and those which were described previously.\(^13\) Strong correlation was found between the two antigen-presentation systems (\(\rho = 0.80; p < 0.0001\)).

\(\dagger\) Sigma Biosciences, St. Louis, MO.

\(‡\) Model ELx800; Bio-Tek Instruments, Inc., Winoski, VT.
was purified by ligand affinity chromatography on recombinant protein G bound to beaded sepharose§ in a Genex System 100 chromatograph, as described in an earlier paper.\textsuperscript{15} Briefly, one mL of HRM-01 was prefiltered through a 0.45 $\mu$m (pore size) particulate filter\textsuperscript{a} then passed through a column containing 1.6 mL (packed volume; column dimensions, 10 mm height by 20 mm width) GammaBind G-Superose\textsuperscript{™}. The unbound components were washed off the column with washing/binding buffer (0.010 mol/L Na$_3$PO$_4$, 0.15 mol/L NaCl, 0.010 mol/L EDTA, pH 7.0) and the bound IgG was eluted with acetic acid (0.5 mol/L, adjusted to pH 3 with ammonium hydroxide). This procedure was repeated with four additional aliquots and the five eluates pooled. The column was cleaned with 1.0 mol/L acetic acid and re-equilibrated with PBS between aliquots. The pool was then concentrated and buffer was exchanged (with PBS) using a Centriprep-30\textsuperscript{™} ultrafiltration apparatus.\textsuperscript{§} The concentration of the IgG fraction thus obtained was 7.0 g/L, which was diluted with PBS to a final concentration of 5.0 g/L.

PREPARATION OF IgG-DEPLETED SERA

GammaBind G-Superose\textsuperscript{™} was washed thrice with PBS and used to deplete the IgG from three sera from the APS group. Individually, 0.060 mL sera were added to 0.415 mL PBS and 0.250 mL of a 50% slurry of GammaBind G-Superose\textsuperscript{™} in PBS. Thus, these suspensions contained serum at a dilution of one part in 10 parts PBS and 0.125 mL of GammaBind G-Superose\textsuperscript{™} as solids. These mixtures were incubated 5 hr at 37°C in a tube rotator to provide mild agitation. The suspensions were then centrifuged at 1500 x g for 15 min and the supernatants collected. The binding procedure was repeated with another 0.025 mL aliquot of the 50 percent slurry. The supernatants were adjusted with PBS to a final serum dilution of 1:48. Control specimens were subjected to the same procedure, except that 0.125 mL PBS was substituted for 0.250 mL of the affinity adsorbant slurry.

IMMUNOGLOBULIN MEASUREMENTS

Immunoglobulin in the IgG fraction of HRM-01 was measured by radial immunodiffusion (RID) in polyacrylamide gels; the ultralow levels of IgG in the IgG-depleted sera and of the IgA in the purified IgG fraction were measured by particle-enhanced RID.* End point measurements were made according to Mancini et al.\textsuperscript{16}

STATISTICAL ANALYSIS

Distributed data were tested for Gaussian fit by Dallal and Wilkinson’s approximation of Lilliefors’ modified Kolmogorov-Smirnov test.\textsuperscript{17} All non-normally distributed data were evaluated by non-parametric statistical methods; Spearman’s rho ($\rho$) was used for correlation and the Mann-Whitney test (U) was used to compare IgA binding by the control with that of the APS group.

Parametric methods were used for regression analyses to test goodness-of-fit of linear relationships and to compare the differences between lines by analysis of variance (ANOVA). ANOVA was also used to evaluate multi-group comparisons. All statistical analyses were performed with Prism\textsuperscript{™} software.\textsuperscript{†}

RESULTS

SCREENING OF APS SERA FOR IgA BINDING

To identify specimens which might contain $\beta_2$GPI-binding IgA, 39 APS specimens were screened, all of whom had IgG anti-$\beta_2$GPI, and the absorbances resulting from 1:100 dilutions were compared to these sera with those from 50 healthy control serum specimens (fig-

\textsuperscript{§} GammaBind G-Superose\textsuperscript{™}; Pharmacia Biotechnology, Inc., Piscataway.
\textsuperscript{a} Autovial\textsuperscript{™}; Whatman, Inc., Clifton, NJ.
\textsuperscript{†} Amicon Div., W. R. Grace & Co., Danvers, MA.

* The Binding Site, San Diego, CA.
† GraphPad Software, Inc., San Diego, CA.
The APS group exhibited significantly greater binding of IgA (0.382 ± 0.428) than controls (0.018 ± 0.043; U = 42.00, p < 0.0001). Fifteen of 39 (38 percent) APS patients had IgA binding greater than 5 SD above the control group mean.

IGA Binding in IgG-depleted Sera

Specimens 5084 and 5700 were subjected to IgG depletion in order to determine whether or not this maneuver would alter the binding of β₂GPI by IgA. Specifically, it was sought to determine if there was adequate antigen present to detect IgA in the presence of high concentrations of IgG anti-β₂GPI. In figure 2, the results of IgG depleted samples 5084 (figure 2A) and 5700 (figure 2B) were compared with their respective whole serum analogues. There was no change in quantity of IgA in the samples following IgG depletion. It can be seen that in both cases significantly greater (p < 0.0001 for 5084 and p = 0.003 for 5700) binding was observed in the presence of IgG, i.e., the IgA from IgG depleted sera gave a significantly smaller signal throughout the range of serum dilutions studies.

Inhibition of IGA Binding by IgG

Based on observation of the results presented in figure 2, the effect of pre-incubation with a purified IgG anti-β₂GPI on IgA binding (figure 3) was examined. In these experiments, the antigen coated wells were pre-incubated with 100 µl of dilutions of the purified, reactive IgG (initial concentration, 5.0 g/L) in PBS for 30 min at RT. As a control study (shown in inset to figure 3), binding of anti-IgG to the purified IgG was measured as well as the signal from our anti-IgA antiserum. A typical saturation curve was obtained for anti-IgG binding; negligible binding from anti-IgA was observed. It should be noted that, using particle enhanced RID, <10 mg/L residual IgA was measured in this specimen.

The binding of IgA to the antigen-coated wells after incubation with the reactive IgG preparation is shown in figure 3 (main figure). These data represent the means ± 1 SEM for six experiments (1:100 dilution of six different sera). Although inhibition is observed at IgG levels greater than 125 µg/well, a slight enhancement of IgA binding seems to occur at lower concentrations of inhibitor IgG. Although in several of the individual experiments represented (not shown), there was a substantial increase in binding at these lower inhibitor concentrations. This effect was not statistically significant (by ANOVA) for the group as a whole.

Discussion

Since the initial report of the presence of IgA antiphospholipid antibodies in patients with APS, their measurements have become an integral part of APS diagnostic panels. In fact, measurement of IgA anticardiolipin antibodies (aCL) may substantially enhance the diagnostic sensitivity of aCL.
Moreover, IgA aCL appear to have increased stability to freeze-thaw degradation of immunoreactivity compared with IgG and IgM aCL and, thus, may be less sensitive to poor specimen handling practices.19

There is by now a substantial body of evidence that aCL and other antiphospholipid antibody assays in patients with APS depend upon the ability of anionic phospholipids to bind β2GPI8,9,10,20 and other proteins of the coagulation system.21 It is believed that once bound to the phospholipid surface, β2GPI presents an epitope which is encrypted when the protein is free in solution.8,9 Several studies on patients with APS (both primary and secondary to SLE) have also demonstrated that anti-β2GPI antibodies of the IgG class are associated with APS22-24 and that this autoantibody associated more closely with APS than either aCL or lupus anticoagulant.25

Although IgM anti-β2GPI antibodies have not been widely investigated, several studies
have reported the isolation of monoclonal anti-
β₂GPI from patients with APS. In fact, the
earliest demonstration of an antibody with in-
vitro anticoagulant activity suggesting a lupus anticoagulant was made with a monoclonal population of antibodies of the μλ isotype. In this study, the specific issue of the binding of β₂GPI by IgA from patients with APS has been examined.

Among our 39 specimens obtained from
APS patients, 15 exhibited IgA binding to
β₂GPI greater than 5 SD above a group of 50 healthy control subjects. Because these measurements were made in whole serum or plasma containing IgG anti-β₂GPI, studies were pursued to learn whether or not there was adequate antigen present in our assay system to overcome the possible quenching of β₂GPI epitopes by the IgG anti-β₂GPI. To accomplish this, a highly specific ligand-based adsorbent, recombinant protein G-sepharose, was used to deplete the sera of IgG. It was found that this maneuver did not increase the amount of IgA bound; in fact, it significantly decreased binding of β₂GPI by IgA. An alternate experimental system, wherein purified IgG was pre-incubated with β₂GPI, did demonstrate inhibition.

In conclusion, it has been demonstrated by us that some patients with APS exhibit IgA anti-β₂GPI with APS exhibit IgA anti-β₂GPI activity, independent of cardiolipin or other anionic phospholipids. As has been done for IgG, both other major immunoglobulin class (IgA and IgM) anti-β₂GPI assays require complete standardization so that larger cohorts may be studied prospectively. This should be a goal of those laboratories investigating the role of anti-β₂GPI in APS.

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References


