Standardized Measurement of Major Immunoglobulin Class (IgG, IgA, and IgM) Antibodies to β_2 Glycoprotein I in Patients With Antiphospholipid Syndrome

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Patients with the antiphospholipid syndrome (APS) have autoantibodies directed against epitopes on β_2 glycoprotein I (β_2 GPI). We describe herein the performance characteristics of standardized enzyme-linked immunosorbent assays (ELISAs) for anti- β_2 GPI of the three major immunoglobulin classes: IgG, IgA, and IgM. All three assays generated highly linear standard curves (5 points, $r \geq 0.993$ for each); precision was excellent both intra-assay and run-to-run, with coefficients of variation (CV) ranging from 2.3% to 6.6%. Values for IgG anti- β_2 GPI correlated strongly with those obtained by an earlier method ($r = 0.80$, $P < 0.0001$). A

study group consisting of 203 healthy subjects was used to generate percentile-based reference intervals for all three classes of anti- β_2 GPI. APS subjects' anti- β_2 GPI were found to differ significantly ($P < 0.0001$ for each) from those of the control population. All three assays correlated well with β_2 GPI-dependent anticardiolipin antibody (aCL) measurements; IgG: $r = 0.94$ ($P < 0.0001$), for IgA: $r = 0.82$ ($P < 0.001$) and for IgM: $r = 0.84$ ($P < 0.0001$). We suggest that these ELISAs may provide valuable standardized measurements of IgG, IgA, and IgM anti- β_2 GPI. *J. Clin. Lab. Anal.* 12:293–297, 1998. © 1998 Wiley-Liss, Inc.

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INTRODUCTION

For much of the time that antiphospholipid syndrome (APS) has been recognized as a distinct clinical entity, antibodies to anionic phospholipids, especially cardiolipin, were thought to be the principle autoantibody specifically associated with this syndrome (1). However, since the discovery that a serum “co-factor”, β_2 glycoprotein I (β_2 GPI) was a requirement for manifestation of anticardiolipin activity in APS patients (2,3), there has been mounting evidence that β_2 GPI, itself, is the marker autoantigen for APS (4–8).

Most studies of the association of anti- β_2 GPI and APS have focused on autoantibodies of the IgG class (4–10). Recently, we proposed the first international standards for IgG anti- β_2 GPI (11) and developed an assay based on these standards (12). The method as described, however, required that the antigen-coated microtiter plates be used within 24 hours of preparation. Because of this antigen instability, the coated plates could not be “batch-prepared” for long-term use, nor were they suitable for commercial development. Furthermore, we observed considerable nonspecific binding and objectionably high backgrounds when we attempted to apply the β_2 GPI-coated plates to the measurement of other classes of anti- β_2 GPI antibodies (i.e., IgA and IgM).

In this article we describe a group of three ELISAs for anti- β_2 GPI autoantibodies of the IgG, IgA, and IgM classes. These assays overcome the shortcomings of the earlier method (12) as described, above. The IgG anti- β_2 GPI assay is based on the standard which was developed earlier (11) and, herein, we compare the data obtained using the two methods. We also describe the first standardized IgA and IgM immunoassays and provide reference intervals for all three immunoglobulin classes of anti- β_2 GPI. Finally, we provide standardized IgG, IgA, and IgM measurements in a group of APS patients, compare these with the reference population and correlate each class of anti- β_2 GPI with the same class of β_2 GPI-dependent anticardiolipin (aCL) antibody.

MATERIALS AND METHODS

Specimens

Healthy individuals ($n = 203$) whose sera were used to develop the reference intervals were recruited from the medical

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center and university communities and were described in detail in an earlier paper (12). Briefly, we included subjects aged 18–65 whose medical history did not indicate chronic illnesses, current infectious diseases or recent hormonal treatment.

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) plasma samples kindly provided by Dr. Thomas Ortel ($n = 8$), The Coagulation Laboratory, Duke University Medical Center and Dr. Ronit Simantov (serum, $n = 22$) 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.

Analytical Methods

Materials used for the antibody-type, enzyme-linked immunosorbent assay (ELISA) have been described previously (13) and were used as reagent kits manufactured by INOVA Diagnostics, Inc. (San Diego, CA). Reference calibrators were used to standardize each of the three (IgG, IgA, and IgM) assays as was done previously for IgG (11,12). Standard units for the assays are SGU for IgG, SAU for IgA, and SMU for IgM.

The ELISA used a standard microtiter format. The β_2 GPI-coated wells were incubated for 30 min at RT with 100 μ l of specimens diluted 1:100 in diluent, washed three times and incubated with 100 μ l peroxidase-conjugated antiserum for 30 min at room temperature (22–25°C). The wells were again washed three times 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 acid stop solution. The absorbance at 450 nm was measured in a microtiter plate reader (model ELx800, Bio-Tek Instruments, Inc., Winooski, VT).

Quanta-Lite® assays (INOVA Diagnostics, Inc.) were used to measure aCL. The manufacturer's protocol, as described in the package insert, was followed precisely.

Fig. 1. Five-point standard curves for anti- β_2 GPI antibody assays in standard units (SGU for IgG, circles; SAU for IgA, squares, and SMU for IgM, triangles). Data are means $\pm$ SEM for a series of runs ($n = 13$ for IgG, $n = 9$ for IgA, and $n = 12$ for IgM).

Statistical Methods

All descriptive and inferential statistical analyses were performed with Prism software (GraphPad Software, Inc., San Diego, CA). The distribution from which the reference intervals were developed was tested for normality (goodness-of-fit to a Gaussian distribution) by Dallal and Wilkinson's approximation of Lilliefors' modified Kolmogorov-Smirnov test (14).

RESULTS

Linearity of Reference Standards

The dilutional response of the reference calibrators was used to generate standard curves for the assays. These data, shown in Figure 1, demonstrate excellent linearity (IgG: $r = 0.993$, $P = 0.0006$; IgA: $r = 0.994$, $P = 0.0005$; IgM: $r = 0.999$, $P < 0.0001$).

Precision

Within-run and between-run precision were assessed at three levels of antibody activity. These data are given in Table 1.

TABLE 1. Precision of Anti- β_2 GPI Antibody Assays at Three Levels of Reactivity

	Low			Moderate			High		
	mean	SD	CV%	mean	SD	CV%	mean	SD	CV%
IgG (in SGU)									
Within run ($n=6$)	18.9	0.82	4.3	44.3	2.34	5.3	82.4	2.66	3.2
Between run ($n=6$)	18.9	0.75	4.0	44.3	2.41	5.4	82.4	2.87	3.5
IgA (in SAU)									
Within run ($n=6$)	18.2	1.20	6.6	37.2	1.50	4.0	79.6	4.1	5.2
Between run ($n=6$)	18.2	1.20	6.6	37.2	1.50	4.0	79.6	3.4	4.2
IgM (in SMU)									
Within run ($n=6$)	18.6	0.55	3.0	37.2	1.83	4.9	77.6	1.76	2.3
Between run ($n=6$)	18.7	0.70	3.8	37.2	2.47	6.6	77.7	1.93	2.5

Fig. 2. Distributions of values for IgG, IgA, and IgM anti- β_2 GPI from a reference population of 203 healthy subjects, aged 18–65.

TABLE 2. Descriptive Statistical Summary of Anti- β_2 GPI Data for 203 Healthy Subjects

	IgG (SGU)	IgA (SAU)	IgM (SMU)
Mean	0.3	2.5	2.8
SD	2.1	12.8	6.0
Percentiles:			
50th (median)	0	0	0
75th	0	0	3
90th	0	3	8
95th	4	7	13
97.5th	4	27	17
99th	6	73	31
Maximum	26	134	141

Reference Intervals

The values obtained for the series of healthy subjects are presented as distributions in Figure 2. IgG anti- β_2 GPI ranged from 0 to 26 SGU with 97% of values at 0, IgA anti- β_2 GPI ranged from 0 to 130 SAU with 93% of values at 0, and IgM anti- β_2 GPI ranged from 0 to 41 SMU with 63% of values at 0. As expected, all three distributions tested non-Gaussian ($P < 0.0001$ for IgG, IgA, and IgM). The statistical analyses of these data are given in Table 2.

APS Patient Data

Anti- β_2 GPI antibodies were measured in a group of APS patients. These data are presented in Figure 3. For IgG, 36/43 (87.7%) were above the 99th percentile of the normal group and for IgM 21/43 (48.8%) were above this percentile. For IgA, 6/43 (14%) were above the 99th percentile, however, all but 11 specimens (32/43, 74.4%) were above the 95th per-

Fig. 3. Histograms for IgG, IgA, and IgM anti- β_2 GPI antibodies in a group of APS patients compared with the 90–99th percentile of the reference population shown in Fig. 2. The percentiles are indicated by dotted lines with the

99th percentile on top followed by the 97.5th, 95th, and 90th percentiles on bottom (note that for IgG, both the 90th and 95th percentiles are at 0 SGU).

Fig. 4. Scattergram showing comparison of IgG anti- β_2 GPI by this method and by the original standardized IgG anti- β_2 GPI method (12). Fifty eight data points were correlated by Spearman's rho ($\rho = 0.80$, $P < 0.0001$).

centile of the normal serum value. The values for all three immunoglobulin classes were significantly different than those from the reference group ($P < 0.0001$ for IgG, IgA, and IgM).

Correlation with Other Methods

We examined the correlation of IgG anti- β_2 GPI with the methods described earlier (12) and the correlation of IgG, IgA, and IgM anti- β_2 GPI with anti-cardiolipin antibodies of these immunoglobulin classes. A scattergram of the comparison of the IgG anti- β_2 GPI method described herein and that which we published earlier is presented in Figure 4. Because the data are bimodally distributed, we applied a nonparametric statistic (Spearman's rho, ρ) to examine the strength of the correlation. This analysis yielded $\rho = 0.80$ ($P < 0.0001$) suggesting a strong concordance between the two methods.

Figure 5 demonstrates the correlation of 21 APS patient aCL and anti- β_2 GPI antibodies of the IgG (Fig. 5 top), IgA (Fig. 5 middle), and IgM (Fig. 5 bottom) classes. All three correlated strongly with Pearson coefficients (ρ) of 0.94, 0.82, and 0.84 for IgG, IgA, and IgM, respectively ($P < 0.0001$ for each comparison).

DISCUSSION

It is becoming clear that the name applied to the hypercoagulable state described as antiphospholipid syndrome is a misnomer. A mounting body of evidence suggests that marker antibodies in this autoimmune coagulopathy are directed toward an epitope on phospholipid-bound proteins such as β_2 GPI (15–17) and prothombin (18) rather than to the phospholipids themselves. In fact, measurements of antibodies to β_2 GPI in phospholipid-independent assay systems appear to predict APS better than the more commonly applied aCL assays which have been shown to require β_2 GPI as a “co-factor” for optimal reactivity (19,20).

Fig. 5. Correlation of anti- β_2 GPI and aCL for IgG: $\rho = 0.96$; for IgA: $\rho = 0.82$; and for IgM: $\rho = 0.84$ ($n = 21$, $P < 0.0001$ for each).

In 1995, our two laboratories independently began the process of standardizing ELISAs for anti- β_2 GPI. We recognized the need to develop international standards for calibrators and methodology to provide a basis for a set of common measurements upon which the clinical specialties studying APS could rely. This led to the establishment of calibration standards for IgG anti- β_2 GPI (11) and the development of a reference interval for healthy subjects based on the use of these calibrators in an ELISA (12). That method suffered from the lack of stability of the antigen-presentation system, requiring that the β_2 GPI-coated polymer be freshly prepared.

Herein we describe the performance characteristics of a new series of ELISAs for anti- β_2 GPI antibodies of all three

major immunoglobulin classes. The analytical parameters of linearity and precision are excellent for ELISAs of this type. Moreover, the distribution of values from an acceptably-sized reference population yields intervals which readily allow the discrimination of pathological values. In fact, even the broadest reference ranges, those for IgA, provided minimal overlap with the data from the APS group.

The data also suggest that anti- β_2 GPI measurements correlate with aCL of the same immunoglobulin class. These methods, interestingly, yield values of measurement units which are comparable; SGU, SAU, and SMU (standard G, A, and M units) are numerically similar to GPL, APL, and MPL, the units developed by the phospholipid standardization laboratory in Louisville, Kentucky (USA), for aCL. This should allow clinicians, familiar with the aCL units to associate them with the anti- β_2 GPI units.

In conclusion, we believe that these data support the contention that the measurement of antibodies to β_2 GPI represents an important contribution to the study of APS. Standardization of these assays provides the clinician with a valuable new tool that will allow comparison between assays from different health care facilities and laboratories.

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