Antiphosphatidylserine Antibodies in Patients With Autoimmune Diseases and HIV-Infected Patients: Effects of Tween 20 and Relationship With Antibodies to β 2-Glycoprotein I

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Antiphospholipid antibodies (aPL) react with negatively charged phospholipids, which may often be complexed with a protein cofactor such as β 2 glycoprotein (β 2GPI) and prothrombin. Cofactor requirements may be assessed by measuring antibodies to β 2GPI or by adding Tween 20 to some reagents in the assays for aPL (anticardiolipin and antiphosphatidylserine). We have measured anticardiolipin antibodies (aCL), antiphosphatidylserine antibodies (aPS), and anti β 2 glycoprotein antibodies (a β 2GPI) in the serum of 10 normal subjects, 20 patients with systemic autoimmune diseases (SAD) diagnosed as having systemic lupus erythematosus (SLE) or antiphospholipid syndrome (APS), and 12 patients with HIV infection. Adding Tween 20 to aPS, the assay couldn't differentiate protein cofactor dependent from independent antibodies, but this can be done by measuring a β 2GPI

($P = 0.0008$). There was a significant correlation between aCL and a β 2GPI in the control group and in the patients with SAD, but not in the HIV-positive (HIV+) patients. After excluding the HIV+ patients, the best Spearman correlation was obtained between a β 2GPI and aCL (0.64, $P < 0.0005$). In 3 out of 7 patients with positive a β 2GPI and in 5 out of 6 patients with moderate or high positive aCL of the group of SAD, there was a history of venous thrombosis. The presence of moderate or high values of aCL either alone or together with a β 2GPI was significantly associated with a history of venous thrombosis ($P < 0.05$). Moderate or high aCL concentrations and their association with a β 2GPI seems to be useful for the assessment of the risk of venous thrombosis in unselected patients with SLE or APS. *J. Clin. Lab. Anal.* 13:59–64, 1999. © 1999 Wiley-Liss, Inc.

Key words: antiphosphatidylserine antibodies; anticardiolipin antibodies; anti β 2 glycoprotein antibodies; autoimmune diseases; HIV infection

INTRODUCTION

Antiphospholipid antibodies (aPL) are a family of closely related immunoglobulins that react with negatively charged phospholipids, which may often be complexed with a protein cofactor such as β 2-glycoprotein (β 2GPI) and prothrombin. The negatively charged phospholipids against antibodies that have been measured are: cardiolipin (aCL), phosphatidic acid, phosphatidylserine (aPS), and phosphatidylinositol (1). The presence of aPL is associated with a disorder termed antiphospholipid syndrome (APS) characterized by thrombocytopenia, recurrent thromboembolic phenomena, recurrent fetal loss, and other diverse manifestations (2–5). The APS is often present in subjects with systemic autoimmune diseases (SAD) such as systemic lupus erythematosus (SLE), and in

subjects without an underlying autoimmune disease. Also, aPL may be present in subjects with infectious diseases such as syphilis and HIV infection, although these infectious diseases are not usually associated with APS (6,7).

The phospholipid-binding plasma protein, β 2GPI is the antigenic target of autoantibodies in patients with SAD whereas in patients with syphilis and others infectious diseases, aCL antibodies are detected in absence of seric proteins, and its binding is inhibited very little by added bovine

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β 2GPI (2). The dependence of aPL on a protein cofactor such as β 2GPI or prothrombin, has been proposed as a means to differentiate between aPL present in APS and aPL present in infectious diseases (8–11). Also, it has been suggested that adding Tween 20 to some ELISA reagents can distinguish between GPI-dependent (those found in APS) and GPI-independent (those found in infectious diseases) anticardiolipin antibodies (12).

The aim of the present study was to assess: (1) the effect of Tween 20 on aPS measured by ELISA; (2) if the relationship between aCL and aPS is modified by Tween 20 addition on aPS assay; and (3) the relationship between aPL and β 2GPI antibodies (a β 2GPI).

Subjects, Materials, and Methods

Subjects

The subjects were seen at the Departments of Internal Medicine and Hematology of the University Hospital of Salamanca where their diagnoses were made. They were divided in three groups: healthy blood donors ($n = 10$), all men (ranged 8–25 years); HIV-positive (HIV+) patients with aCL > 80 GPL/ml ($n = 12$), 5 women and 7 men (ranged 19–37 years); and patients with SAD ($n = 20$), 14 women and 6 men (ranged 24–64 years). SLE was diagnosed in 17 patients, according to the American College of Rheumatology criteria (13). Seven patients with SAD had a past history of venous thrombosis and fulfilled Harris criteria for APS (14). Four patients had APS arising from their SLE and three had primary APS. In ten patients with SLE, the existence of thromboembolism was clinically excluded. In three patients the clinical history doesn't include antecedents of venous thrombosis but it couldn't be totally excluded. In three HIV+ patients an acquired immunodeficiency syndrome was diagnosed, and in two other HIV+ patients thromboembolic phenomena were detected, one associated with a factor V Leyden deficiency and the other associated with serum aPL.

Blood samples were obtained after an overnight fast and allowed to coagulate at room temperature. Serum was obtained by centrifugation and an aliquot was frozen at -20°C until assayed.

ELISA for IgG antiphosphatidylserine antibodies

aPS antibodies of IgG isotype were measured by a commercial ELISA technique (Reads Medical Products, CO, USA) that uses bovine phosphatidylserine as antigen and bovine serum as the source of β 2GPI in the sample diluent. Samples, calibrators, and controls were 50-fold diluted in sample diluent and applied to the wells. Bound antibodies were detected using horseradish peroxidase-labeled antibodies against human IgG. Interassay coefficients of variation (CV) were 8.3% and 6.9% for low (< 10 GPS/mL) and high (40 GPS/mL) aPS concentrations, respectively. To assess the effect of

Tween 20 on APS measurement, we replaced washing buffer by 0.05% Tween 20/washing buffer and sample diluent by 0.05% Tween 20/sample diluent. Samples were read at 450 nm and concentrations expressed as GPS units/mL. These units are traceable to the reference preparations of Louisville Antiphospholipid Laboratory. Reference values were obtained in our laboratory and did not differ from those recommended by the manufacturer (mean + 3 SD), 16 GPS/mL.

ELISA for IgG anticardiolipin antibodies

aCL antibodies of IgG isotype were measured by using a commercial ELISA technique (Cheshire Diagnostic Limited, Chester, England). Plates were coated by cardiolipin and blocked with a β 2GPI solution. This cofactor was also added in the sample diluent (20 $\mu\text{g/mL}$). Standards are expressed as GPL/mL according to aCL International Standard. Alkaline phosphatase conjugated IgG antibody was the secondary antibody and p-nitrophenyl phosphate the substrate. The absorbance was measured by an optical reader at 405 nm. Interassay CV were 10.5% and 7.8% for 9.5 GPL/mL and 32.0 GPL/mL concentrations, respectively. The reference cut-off (13 GPL/mL) was calculated as the mean GPL antibody concentration of 130 healthy blood donors plus three standard deviations.

ELISA for IgG anti β 2GPI antibodies

a β 2GPI of IgG isotype were measured by Quanta Lite β 2GPI assay (Inova Diagnostic, Inc., San Diego, CA). Irradiated plates were coated with purified β 2GPI and stabilized. 100 μL of prediluted calibrator and control and 100 μL of 1/101 diluted samples were added to each well. Standards were referenced to the reference standards available from the Rheumatology Laboratory, Seton Hall University, St. Joseph's Hospital and Medical Centre (15). Antihuman IgG horseradish peroxidase labelled conjugate was used. Interassay CV were 4.3% and 3.5% for negative (18.9 U/mL) and high reactive (82.4 U/mL) samples, respectively. Manufacturer recommended negative range values 0–20 U/mL.

All the ELISAs were automated in a Guest System (Chemila, Menarini, Italy).

Statistical methods

The results were analyzed by a commercial statistic program (SPSS) on a personal computer. Nonparametric descriptive statistics, median and interquartile range, were used. Kruskal–Wallis and Wilcoxon tests allowed comparison of antibody concentrations obtained in each of the groups. Kappa Statistic was applied to evaluate the agreement (positive/negative) between tests. The Binomial Exact Test was used to evaluate the association between the presence of antibodies and clinical history of the patient. The Spearman's Rank Correlation Test was used to assess the relationship between the values of the antibodies measured. Finally, the relationship

between aPS and aPSTween 20 concentrations were examined using linear regression analysis.

RESULTS

aPS concentrations were elevated in patients with both SAD and HIV infection with respect to the control subjects ($P < 0.00005$). aPSTween 20 values were higher than aPS values in the three groups studied ($P < 0.001$) (Table 1). When both the difference (aPSTween20-aPS) and the ratio aPSTween20/aPS were analyzed, greater values were obtained in HIV+ patients and patients with APS than in both patients with SLE and control subjects ($P < 0.00005$ for the difference and $P = 0.0064$ for the ratio) (Fig. 1). However, neither the difference nor the ratio changed between patients with APS and HIV+ patients (NS). The regression analysis showed a positive linear relationship between aPSTween20 and aPS concentrations (Fig. 2). Thus, 94% aPSTween20 values may be explained as a function of the initial aPS concentration of the specimen, and an increase of 1.56 GPS/mL aPSTween20 for each GPS/mL aPS is expected when adding detergent.

The number of specimens classified as positive for aPSTween20 with respect to aPS did not change in the patients with SAD (7/20), while a positive result was obtained in the control group (1/10), and all results were positive for aPSTween20 in the HIV+ patients (12/12) (Table 1). The normal crossing point for aPS is 16 GPS/mL and the corrected crossing point according to the regression line for aPSTween20 was 25 GPS/mL. The agreement between aPS and aPSTween20 measurements was 0.73 (IC kappa, 95%: 0.53 to 0.92). aPS and aPSTween20 were present in the serum in most of the samples with aCL and there was a good correlation for both aCL vs. aPS ($r^2=0.91$) and aCL vs. aPSTween20 values ($r^2=0.97$).

We did not find any a β 2GPI in the serum of the control group (0/10) or in the serum of the HIV+ patients (0/10). Two HIV+ patients with thromboembolic events were excluded. In one patient there was a factor V Leyden deficiency and in the other there was no other alteration of coagulation than the presence of aPL. In 7/20 of the patients with SAD, a β 2GPI was found, 5/9 with aCL(+), and 2/11 with aCL(-) (Table 2). After excluding HIV+ patients, the following Spearman correlation coefficients were obtained: a β 2GPI vs. aCL (0.64, $P < 0.0005$), a β 2GPI vs. aPS (0.45, $P < 0.0005$), and a β 2GPI vs. aPSTween20 (0.58, $P < 0.001$).

Although in patients with APS there was an increased a β 2GPI prevalence (3/7) with regard to the control group (0/10) ($P = 0.05$, Fisher exact test), a β 2GPI also was detected in 4/10 patients with SLE without thrombosis (SLE/T-) (Table 3). Of all the antibodies studied, a significant association was only obtained between moderate or strong aCL(+) and APS, as can be seen in Table 3. The combination of either moderate or strong aCL(+) and a β 2GPI increases specificity but decreases sensitivity (Table 3).

DISCUSSION

Antiphospholipid antibodies comprise a wide spectrum of antibodies that react in the analysis systems that use phospholipids. In patients affected with autoimmune diseases such as SLE and APS, aCL and aPS appear to crossreact (16,17). This fact could justify the good correlation obtained in this study. Either aPS or antiphosphatidylinositol antibodies are more prevalent than aCL in a few patients (18). All patients with elevated aPS also had high aCL in the present study.

Currently, it is accepted that the aCL test detects a β 2GPI, aCL, and antibodies against other serum proteins (which bind to cardiolipin to a lesser extent) and, in general, phospholip-

Fig. 1. Box-plot for differences in aPS serum concentrations when Tween20 is added. $\chi^2 = 28.53$, $P < 0.00005$, Kruskal-Wallis 1 way ANOVA.

Fig. 2. Relation between aPS concentrations obtained in the presence and absence of Tween20: $b = 1.56$, $IC_{95\%} = 1.44-1.68$; $a = 0.32$; $n = 42$; $r^2 = 0.94$.

ids. In the patients with SAD, antibodies are mainly directed against $\beta 2GPI$, which binds with a high protein density to anionic surfaces of phospholipids. While in patients with syphilis and other infectious diseases, aCL is detected in the absence of serum proteins, and its binding is less inhibited by bovine $\beta 2GPI$ added in most of tests (19). Adding detergent Tween20 in the ELISA reagents has been proposed as a method to distinguish $\beta 2GPI$ -dependent and $\beta 2GPI$ -independent aCL (12). In the present study, we added Tween20 to the sample diluent and the washing solution in the ELISA for aPS, and we found that aPS concentration increased in all the groups studied. However, we didn't find that detergent addition allowed us to distinguish $\beta 2GPI$ -dependent (SAD) and β -independent (HIV infection) aPS. The detergent effect seems to

be a function of initial concentration of aPS. It has been demonstrated by other authors that Tween20 addition to buffer washing solution potentiates a $\beta 2GPI$ binding to $\beta 2GPI$ in the patients with SLE (5).

In HIV+ patients we have not observed the presence of a $\beta 2GPI$ in serum, in spite of high concentrations of aCL. These results are in agreement with the fact that antiphospholipid antibodies react with phospholipids in a protein cofactor independent way. Similar results were obtained in patients with syphilis (9). When patients whose aCL can be considered as cofactor-independent were excluded, a significant correlation ($r = 0.64$) between aCL and a $\beta 2GPI$ was obtained; although it was lower than that obtained by other authors, in patients with either APS (0.90) or SLE (0.79) (8,9). The correlation coefficient decreased when aPS were considered, especially when Tween20 was not added. This fact could indicate that $\beta 2GPI$ is not so important as an aPS cofactor as it is for aCL, perhaps because there were a greater binding of other proteins to phosphatidylserine.

In patients with SAD, the presence of antiphospholipid antibodies detected as lupus anticoagulant or with aCL tests

TABLE 1. aPS, aPSTween20, and aCL Distributions^a

	Total n = 44	Control n = 10	SAD n = 20	HIV+ n = 12
aPS GPS/mL	12.85 (34.60) 19	7.35* (4.72) 0	12.85* (10.30) 7	48.08* (28.38) 11
aPSTween20 GPS/mL	21.90 (64.07) 21	9.10 ^ (6.90) 1	18.40 (25.50) 7	78.28 ! (45.55) 12
aCL GPL/mL	13.95 (115.6) 21	5.3 (3.30) 0	12.3 (31.43) 9	196.8 (135.1) 12

^aResults are expressed as median (interquantic range), and positive results. * $\chi^2 = 18.58$, $P < 0.00005$, Kruskal-Wallis Test. aPSTween20 vs. aPS in control ^ ($z = 2.67$, $P < 0.00005$), SAD · ($z = 3.3$, $P = 0.0007$), HIV+ ! ($z = 5.17$, $P < 0.00005$), Wilcoxon Test. The SAD group includes patients with systemic lupus erythematosus and patients with antiphospholipid syndrome.

TABLE 2. Association Between a $\beta 2GPI$ and aCL^a

	Control (aCL-)	SAD (aCL-)	SAD (aCL+)	HIV+ (aCL+)
a $\beta 2GPI$ -	10	9	4	10
a $\beta 2GPI$ +	0	2	5	0
Total	10	11	9	40

^aSAD group includes patients with SLE and patients with APS. $\chi^2 = 12.71$, $P = 0.0053$, Kruskal-Wallis Test. SAD(aCL+) vs. SAD(aCL-), $P = NS$ (Binomial Test). Two HIV+ patients and thromboembolic events were excluded.

TABLE 3. Prevalence of aPL and α 2GPI in Patients With SLE Without Clinical History of Thrombosis and Patients With Thrombosis and APS

	SLE (T-) n = 10	APS n = 7	P Fisher
α 2GPI > 20 U/mL	4	3	NS
aCL > 13 GPL/mL	4	5	NS
aCL > 20 GPL/mL	1	5	0.02
aPS > 16 GPS/mL	3	4	NS
aPSTween20 > 25 GPS/mL	5	6	NS
aCL > 20 GPL/mL	0	3	0.05
Y α 2GPI > 20 U/mL			

is associated with venous thromboembolic phenomena. In this work aCL were detected in 9/20 of the patients with SAD, and in 6/20 (five of them with aCL greater than 40 GPL/mL) when only > 20 GPL/mL values were considered. There were clinical antecedents of thrombosis with a significant association in 5/6 of these patients, which also has been demonstrated by others (2,3). Prevalence of α 2GPI was increased (5/9) in patients with SAD aCL(+) and was greater than in patients with SAD aCL(-) (2/11), although without statistical significance. Forastiero et al. have described greater prevalences in SLE patients and venous thromboembolic phenomena (9). The degree of prevalence seems to depend on aCL concentration and the number of patients with thrombosis, because when antecedents of aCL(+) and thrombosis were taken into account, 4/5 patients have positive α 2GPI. Roubey et al. think that α 2GPI are more associated with venous thrombosis than aCL, and that they have greater positive predictive value (14% vs. 35%) (20). In our study, however, when results obtained for aCL were not taken into account, patients with APS (3/7) didn't have α 2GPI more prevalent than patients without thrombosis, since it was detected in 4/10 SLE patients without thrombosis, 2 of them with aCL (+) and the other 2 with aCL (-). It is possible that these patients could develop a thromboembolic phenomenon in the future (21), or perhaps the cut-off should be elevated, with respect to that recommended by the manufacturer, to increase specificity as was done with aCL. However, some authors, such as Emlen, suggest that only approximately one third of the patients with SLE and α 2GPI will have a clinical history of thrombosis (22). However, in order to detect venous thromboembolic phenomena in patients with SAD, in our study the best sensitivity was obtained with aCL at moderate or high levels, and the best specificity when aCL is associated with α 2GPI measurement.

In summary, in the ELISAs for measurement of aPS that include β 2GPI as a cofactor, adding of Tween20 potentiates the binding of aPL to phosphatidylserine. However, this addition doesn't allow to differentiate β 2GPI-dependent from β 2GPI-independent aPS. Results obtained for aPS and aCL were correlated and they agreed in a high number of assayed samples of both the patients with SAD and controls. More-

over, in these groups there was a significant correlation between aPL analyzed and α 2GPI. In 3/7 patients with SAD (α 2GPI+) there were clinical thrombotic events, in spite of it not being possible to rule out a late development of thrombotic events in the rest of the patients. In our study and for autoimmune diseases (SLE and APS), the best sensitivity to identify the patients affected by venous thrombosis has been obtained with aPL(aCL) at moderate to high concentrations, whereas the best specificity has been obtained when both positive aPL(aCL) and α 2GPI were obtained.

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