Prevalence of Antiphospholipid Antibodies in Chilean Patients With Rheumatoid Arthritis

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Antiphospholipid (aPL) antibodies found in patients with autoimmune diseases are also detected in those with inflammatory diseases. The purpose of this study was to examine the prevalence of these antibodies in patients with rheumatoid arthritis (RA), and to evaluate the association of these antibodies with thrombosis and/or other clinical characteristics of this inflammatory disorder. Eighty-four patients with RA and 82 normal controls were studied. Anticardiolipin (aCL), anti- β_2 glycoprotein I (anti- β_2 GPI), and antiprothrombin (aPT) antibodies and the lupus anticoagulant (LA) activity were determined. Seven out of 84 (8.3%) patients were positive for aCL, six out of 84 (7.2%) for anti- β_2 GPI, and six out of 84 (7.2%) for aPT, while in controls the

overall prevalence of aPL antibodies was 3.6% (3 out of 82). All patients and controls were LA negative. There was no correlation between the presence of aPL with thrombosis and/or other clinical features of the antiphospholipid syndrome. We found aPL antibodies in 19.1% (16 out of 84) of the patients with rheumatoid arthritis and this prevalence was statistically higher than in normal controls ($P < 0.003$). In this study, the presence of aPL antibodies was not associated with the development of thrombosis and/or thrombocytopenia. Whether the presence of aPL antibodies implies an increased risk for thrombosis and atherosclerosis in these patients should be studied further. J. Clin. Lab. Anal. 20:190–194, 2006.

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INTRODUCTION

Antiphospholipid (aPL) antibodies are a heterogeneous group of antibodies that have been associated with thrombosis and recurrent fetal loss (1,2) in patients with antiphospholipid syndrome (APS), systemic lupus erythematosus (SLE) (3,4), and rheumatoid arthritis (RA) (5). Similarly, the presence of these antibodies has been associated with the use of some drugs and some infections (6–8).

Most aPL antibodies are auto antibodies directed against phospholipid-binding proteins such as β_2 Glycoprotein I (β_2 GPI) (9) and prothrombin (PT) (10), among others. The evaluation of anti- β_2 GPI antibodies was recently added as a new laboratory criterion of the APS (11). Rheumatoid arthritis is an autoimmune disease. The frequency of thrombosis in individuals

with RA is greater than in normal individuals (12). The presence of anticardiolipin (aCL) antibodies has been related to activation and prognosis of RA (13). In patients with RA the prevalence of aCL antibodies has been reported from 4–50% (14,15) and 1% in lupus anticoagulant (LA) (16). Their presence is associated with thrombotic (17) and vascular manifestations including livedo reticularis, thrombophlebitis, and leukocytoclastic vasculitis (15).

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The purpose of this study was to determine the prevalence of different aPL antibodies in Chilean patients with RA and to study an eventual correlation with thrombosis and/or other clinical characteristics.

PATIENTS AND METHODS

Patients and Controls

Eighty-four patients with RA attending the Regional Hospital of Talca, Chile, were studied and all fulfilled the diagnostic approach of the American Rheumatism Association (18). The mean age was 53 ± 14 years, 79 females (94.1%) and five males (5.9%). Thirty-four out of 84 patients (40.1%) presented classic thrombosis risk factors: hypertension (15.5%), diabetes (9.5%), dyslipidemias (3.6%), obesity (2.4%), smoking (1.2%), and being over 60 years of age (35.7%) (Table 1).

The normal control group included 82 healthy blood donors (50 ± 9.3 years, females 34% and males 66%), attending the primary health care clinics of Talca, Chile.

Methods

An amount of 10 mL of blood were collected by venipuncture. From this amount, 3.5 mL were anticoagulated with sodium citrate 3.2% in a ratio of 9:1 (blood:anticoagulant) for detection of LA activity, and 6.5 mL were used to obtain serum for all the enzyme-linked immunosorbent assay (ELISA) determinations. Serum samples were separated and stored at -70°C until testing.

TABLE 1. Characteristics of the 84 patients with rheumatoid arthritis

Risk factors of thrombosis	
Hypertension	13 (15.5%)
Obesity	2 (2.4%)
Smoking	1 (1.2%)
Dyslipidemias	3 (3.6%)
Diabetes	8 (9.5%)
More than 60 years	30 (35.7%)
Clinical antecedents	
Associated with APS	
Recurrent fetal loss	2 (2.4%)
Thrombosis	1 (1.2%)
Inflamed articulations (n = 83)	32 (38.1%)
Morning rigidity (n = 82)	25 (30.5%)
Sjogren syndrome (n = 78)	21 (26.9%)
Painful articulations (n = 81)	12 (14.8%)
Cutaneous nodule (n = 79)	2 (2.5%)
Bone erosions (n = 79)	1 (1.3%)
Anemia (n = 75)	27 (36.0%)
Thrombocytopenia	1 (1.2%)
Leukopenia (n = 75)	0 (0%)

n, number of patients with available information.

aCL ELISA

To determine aCL (IgG, IgM, and IgA) an ELISA assay was used as described (19,20). Briefly, non-irradiated microtiter plates (Maxisorp 469914-A; Nunc, Denmark) were coated with cardiolipin at a concentration of $50 \mu\text{g/mL}$ in 70% ethanol. The plates were blocked with 10% fetal bovine serum (FBS) (Gibco BRL, Gibco, Grand Island, NY) in phosphate buffer saline (PBS) and serum samples (1:50 dilution in FBS-PBS). Negative and positive controls were added in duplicate. To perform aCL ELISA, international standards were included to express the results in GPL, MPL, and APL units. They were provided by Dr. Silvia Pierangeli, Antiphospholipid Standardization Laboratory, Morehouse School of Medicine, Atlanta, GA. The optical densities (OD) were read at 405 nm by a StatFax-2600 microplate reader (Awareness Technology Inc., Palm City, FL). The activity of aCL antibodies was expressed in international units (GPL, MPL, and APL). The cut-off value for the three immunoglobulin isotypes was 15 units. The positive samples were confirmed by testing twice.

Lupus Anticoagulant

For the detection of the lupus anticoagulant activity, two clotting tests were used: 1) Dilute tissue thromboplastin inhibition (dTTI) was performed as described in other studies (8,21). Briefly, $100 \mu\text{L}$ of thromboplastin (Pacific Hemostasis, Fisher Diagnostics, Middletown, VA) diluted 1:500 in saline was added to $100 \mu\text{L}$ of platelet-poor plasma (PPP) and incubated for 5 min at 37°C . The reaction was started by adding 0.025 M CaCl_2 and the clotting time was determined using a Clot-1-Coagulometer (RAL; Tecnica para el laboratorio S.A., Barcelona, Spain). Determinations were carried out in duplicate. When the patient time/control time ratio was > 1.30 , the test was repeated using a mixture of 1:1 with normal plasma. If the clotting time was not corrected, the platelet neutralization procedure (PNP), using a platelet lysate as a source of phospholipids, was used as a confirmatory test. 2) The second test was Kaolin clotting time (KCT) (8,22). Briefly, $100 \mu\text{L}$ of 0.1% kaolin in PBS was incubated with $200 \mu\text{L}$ of PPP at 37°C for 3 min. The reaction was initiated by adding $200 \mu\text{L}$ 0.025 M CaCl_2 , and the clotting time was registered using a Clot-1-Coagulometer. Criterion for considering a sample positive was similar to that used for dTTI.

Anti- $\beta_2\text{GPI}$ ELISA

IgG, IgM, and IgA anti- $\beta_2\text{GPI}$ antibodies were determined by ELISA as described (20,23,24). In brief, gamma irradiated microtiter plates (Maxisorp 269787; Nunc) were coated overnight with a solution containing

20 µg/mL β_2 GPI, purified in the laboratory as described (25), and plates were blocked with 3% bovine serum albumin (BSA). Patient samples and controls (diluted 1:50 in 3% BSA solution) were added to the plates in duplicate. A sample was considered positive when its OD was greater than 3 standard deviations (SD) from the average of normal controls (cut-off). The activity of these antibodies was expressed as the ratio OD_{405} patient/ OD_{405} cut-off. Positive controls were provided by Dr. A. Cabral (Department of Immunology and Rheumatology, Instituto Nacional de la Nutrición Salvador Zubiran, Mexico City, Mexico).

Antiprothrombin ELISA

Antiprothrombin antibodies were determined by ELISA (20,26). Briefly, non-irradiated microtiter wells (Maxisorp 469914-A; Nunc) were coated with a solution containing 5 µg/mL human prothrombin (Sigma-Aldrich Corp., St. Louis, MO), and wells were blocked with PBS-Tween-BSA 1%. Patient samples, positive and normal controls diluted 1:100 in PBS-Tween-BSA were added to the plates. The positivity of samples and the activity of antiprothrombin (aPT) antibodies were determined as mentioned in the anti- β_2 GPI assay. A positive control (Department of Hematology, Universidad Favaloro, Buenos Aires, Argentina) was included.

Statistical Analysis

The prevalence of each different aPL antibody in patient vs. control groups were compared using the chi's square test or Fisher Exact test. A five percent level of significance was used.

RESULTS

Prevalence and Isotype Distribution of aCL Antibodies and LA

Seven out of 84 (8.3%) patients and three out of 82 controls (3.6%) were positive for aCL antibodies (for at least one isotype). The difference between the two groups was not statistically significant ($P = 0.1$) (Table 2). Seven samples were positive for IgG and two for IgM aCL antibodies (Table 3). Isolated isotypes were observed more frequently (IgG = 71.4%) than combinations of isotypes (28.6%). The mean and standard deviations of activity of the different isotypes of aCL antibodies are shown in Table 3. All aCL positive cases were <40 GPL, MPL units. All positive cases among the control group were low positive (IgG = 16.1 ± 0.9 , IgM = 17.1 ± 0.9 , and IgA = 16 ± 0.9). The LA test was negative in all cases (Table 2).

TABLE 2. Antiphospholipid antibodies in patients with rheumatoid arthritis

Antibodies	Patients (n = 84)		Controls (n = 82)		P
	n	%	n	%	
aCL	7	8.3	3	3.6	0.1
Anti- β_2 GPI	6	7.2	3	3.6	0.2
aPT	6	7.2	3	3.6	0.2
LA	0	0	0	0	

aCL, anticardiolipin antibodies; aPT, antiprothrombin antibodies; anti- β_2 GPI, anti- β_2 glycoprotein I antibodies; LA, lupus anticoagulant.

TABLE 3. Isotype distribution and activity of anticardiolipin, anti- β_2 glycoprotein I, and antiprothrombin antibodies in 84 patients with rheumatoid arthritis*

Isotypes	aCL n (%)	anti- β_2 GPI n (%)	aPT n (%)
Any isotype ^a	7 (8.3)	6 (7.2)	6 (7.2)
IgG	7 (8.3) (19.9 ± 5.8)	6 (7.2) (1.7 ± 0.3)	6 (7.2) (1.2 ± 0.1)
IgM	2 (2.4) (16.9 ± 1.4)	4 (4.8) (1.5 ± 0.6)	0 (0)
IgA	0 (0)	0 (0)	0 (0)

*The activity of antibodies is shown in parentheses (aCL: GPL, MPL, APL international units for anticardiolipin antibodies; anti- β_2 GPI and aPT antibodies: OD_{405} patient/ OD_{405} cutoff).

^aAt least one of the three isotypes positive.

aCL, anticardiolipin antibodies; anti- β_2 GPI, anti- β_2 glycoprotein I antibodies; aPT, antiprothrombin antibodies.

Prevalence and Isotype Distribution of Anti- β_2 GPI Antibodies

Six out of 84 (7.2%) patients and three out of 82 (3.6%) controls were positive for anti- β_2 GPI antibodies (for at least one isotype). The difference between the two groups was not statistically significant ($P = 0.2$) (Table 2). Six were IgG and four were IgM anti- β_2 GPI antibodies (Table 3). Isolated isotypes were observed less frequently (IgG = 33.3%) than combinations of isotypes (66.7%). The levels of the different isotypes of anti- β_2 GPI antibodies are shown in Table 3 and two anti- β_2 GPI antibodies had high activity. All the positive values among the control group were low positive (IgG = 1.4 ± 0.2 , IgM = 1.4 ± 0.3 , and IgA = 1.4 ± 0.3).

Prevalence and Isotype Distribution of aPT Antibodies

Six out of 84 (7.2%) patients and three out of 82 (3.6%) controls were positive for aPT antibodies (for at least one isotype). The difference between the two groups was not statistically significant ($P = 0.2$).

(Table 2). All positive aPT antibodies were of the IgG isotype (Table 3). The activity of different isotypes of aPT antibodies is shown in Table 3 and all had a low activity. The positive values of the control group were in the low positive range (IgG = 1.3 ± 0.2 , IgM = 1.2 ± 0.3 , and IgA = 1.4 ± 0.1).

Prevalence of aPL Antibodies

Sixteen out of 84 (19.1%) patients with RA had some type of aPL antibodies. It was statistically significantly higher than the frequency in the normal control group (three out of 82, 3.6%, $P < 0.003$).

Six out of 84 (7.2%) patients presented only aPT, four (4.8%) only aCL, and three (3.6%) only anti- β_2 GPI antibodies. In three patients (3.6%) more than one aPL was detected (aCL + anti- β_2 GPI antibodies). The prevalence of aCL, aPT, and anti- β_2 GPI antibodies was not different among patients younger or older than 60 years of age.

Thirteen out of 84 (15.6%) patients with rheumatoid arthritis presented aPL as the only identified thrombosis risk factor.

Ten out of 16 (62.5%) patients with aPL presented the classic risk factors of thrombosis (hypertension: three; diabetes: two; obesity and dyslipidemias: one; over 50 years of age: seven) and eleven out of 16 (68.8%) had clinical antecedents of rheumatoid arthritis (Sjorgren syndrome: eight; inflamed articulations and morning rigidity: seven; anemia: six; painful articulations and cutaneous nodules: one).

DISCUSSION

The prevalence of RA in Chile is 0.5%, and it is more frequently found in women than men (3–4:1) (27). Evidence exists that patients with RA present an increased risk of cardiovascular disease and death, but these risk factors are not fully understood (28). Several possible factors have been described: hyperhomocysteinemia, antiphospholipid antibodies, altered serum levels of lipoproteins, and inflammatory processes (29). The purpose of this study was to determine the prevalence of different aPL antibodies in Chilean patients with RA and to study a possible correlation with thrombosis and/or clinical characteristics of the RA disorder.

Our results show that 16 out of 84 (19.1%) patients with RA had some type of aPL. This is in agreement with previous studies showing a wider range (4–50%) (30–32).

The prevalence of aCL antibodies was 8.3% in our patients but all presented low levels of activity. Sherer et al. (30) found 22% of prevalence of aCL antibodies but 60% of the patients had low levels. Unikiene et al. (31) found 8.2% of prevalence, Avcin et al. (16) 46.4%

in children with juvenile idiopathic arthritis (JIA) but during the follow-up the frequency of aCL decreased to 21.4%, and Assous et al. (33) 19% of prevalence in patients with RA. The LA test was negative in our patient group, in accordance with data published by Avcin et al. (16) about patients with JIA.

Our prevalence of anti- β_2 GPI antibodies was 7.2%, and 3.6% of these had high levels of activity. Ambrozic et al. (5) found a high prevalence of 21%, which greatly differs with other reports (30) that show a low prevalence of around 5% in patients with RA.

Our prevalence of aPT antibodies was 7.2% and in all cases the aPT activity was low. No other reports have been published about aPT antibodies in RA.

In summary, we found that 19.1% of patients with RA had aPL antibodies that do not have the main clinical manifestations of APS. Similar results were found in other studies. There were no correlations between aPL antibodies and thrombosis and/or other clinical features of RA. These discrepancies might indicate that the development of aPL antibodies, for example, is associated with an infectious trigger or that the low positive antibody activity could explain the limited prothrombotic potential of antiphospholipid antibodies in patients with RA. Whether the presence of aPL antibodies indicates an increased risk for thrombosis and atherosclerosis in these patients should be studied further.

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