ORIGINAL ARTICLE

Antinucleosome antibodies and primary antiphospholipid syndrome: an observational study

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ABSTRACT

Objective: To study the association of anti-nucleosome (anti-NCS) antibodies in primary antiphospholipid syndrome (APS) and the development of systemic lupus erythematosus (SLE) during follow-up. **Materials and methods:** Thirty-six women with primary APS were evaluated prospectively for clinical features of systemic autoimmune diseases and for the presence of antiphospholipid antibodies, antinuclear antibodies and anti-NCS/chromatin antibodies. **Results:** After a mean follow-up period of 45.7 months, anti-NCS/chromatin antibodies were detected in only one patient (2.8%), who developed features of SLE including polyarthritis, lymphopenia, optic neuritis, multiple sclerosis-like lesions, and an autoantibody profile suggestive of SLE. **Conclusion:** The frequency of anti-NCS/chromatin antibodies in primary APS patients is very low, and they may be associated with the development of SLE manifestations.

Keywords: antiphospholipid syndrome, systemic lupus erythematosus, anticardiolipin antibodies, lupus coagulation inhibitor nucleosomes.

INTRODUCTION

The antiphospholipid syndrome (APS) is characterized by thrombotic manifestations and/or obstetrical morbidity in the presence of antiphospholipid antibodies (APL). The standard APL assays recommended by the latest guidelines on APS are the lupus anticoagulant (LAC) and enzyme immunoassays for antibodies against β_2 glycoprotein I (anti-β_2 GPI) and against cardiolipin (aCL) in the presence of β_2 GPI. APS can occur as a primary disease or associated with some other systemic autoimmune disease, predominantly systemic lupus erythematosus (SLE).[1,2]

SLE is characterized by a wide range of circulating autoantibodies, several of which are directed against nuclear antigens.[3]

In particular, chromatin antigens appear to be a preferential target of autoantibodies in SLE. Nucleosome is the unit of chromatin and consists of 146 base pairs of DNA wrapped around a protein core. The protein core is an octamer consisting of two molecules of each of the histones H2A, H2B, H3, and H4.[4] The frequency of autoantibodies against nucleosome (anti-NCS) and H1 stripped chromatin (anti-chromatin) in SLE varies from 50%–100% and the specificity for SLE diagnosis has been reported from 90%– 99%.[5–15] Anti-NCS/chromatin antibodies have been associated with active glomerulonephritis in SLE patients.[6–10]

Although considered specific for SLE, anti-NCS/chromatin antibodies have been reported in other autoimmune conditions such as systemic sclerosis, Sjögren's syndrome,

mixed connective tissue disease, and type 1 autoimmune hepatitis.[6,8,9,16,17] There is some controversy in the literature and some authors believe that the finding of positive reactivity in non-lupus patients is due to heterogeneity in NCS/chromatin preparations and other reagents used in enzyme-linked immunosorbent assay (ELISA) tests.[6,18–20]

Recently, different groups have reported on anti-NCS/chromatin antibodies in primary APS.[9,21,22] However, the prevalence of anti-NCS antibodies is wide among primary APS patients, ranging from 7%–77%; this may be related either to methodological issues in the detection of anti-NCS/chromatin antibodies or to the inclusion of patients with lupus-like syndrome. Many of the anti-NCS/chromatin positive patients with primary APS were found to subsequently develop SLE.[21,22] Thus, it is not established whether primary APS patients carrying anti-NCS/chromatin antibodies are in fact SLE patients with incipient disease. Therefore, we set to develop an observational prospective study to evaluate the frequency of anti-NCS/chromatin antibodies in patients with primary APS and the development of defined SLE or isolated SLE traits.

MATERIALS AND METHODS

Sampling, recruitment and data collection

Thirty-six women meeting the Sapporo criteria for primary APS[23] underwent clinical evaluation for manifestations of APS and other autoimmune diseases. Peripheral blood was obtained at study entry for determination of LAC and aCL, anti-β_2 GPI and anti-NCS/chromatin antibodies. Patients were prospectively evaluated for a mean of 45.7 ± 9.6 months (range 13–56) with special attention for evidence of systemic autoimmune disease or the establishment of SLE according to the American College of Rheumatology updated criteria for SLE classification.[24] Exclusion criteria at study entry were the presence of systemic autoimmune disease other than APS, chronic infection, malignancy, and age below 18 years. All participants signed an Informed Consent approved by the institutional Ethics Committee.

Antibody assays

Anti-cardiolipin antibodies were detected using an in-house standard ELISA technique.[25] Briefly, ELISA plates (NUNC – Thermo Fisher Scientific Inc, Roskilde, Denmark) were coated (50 μL per well) with bovine cardiolipin (50 μg/mL) (Sigma Aldrich Inc, St Louis, USA) overnight at 4 ℃. After blocking with 100 μL of 10% adult bovine serum (ABS) in

phosphate buffered saline pH 7.4 (PBS) for 2 hours at room temperature (RT), 50 μL of patient serum diluted 1:50 in 10% ABS in PBS were added to duplicate wells and incubated overnight at 4 °C. Plates were then washed three times with PBS and wells received alternatively 50 μL anti-IgM or anti-IgG peroxidase-conjugates (Calbiochem, La Jolla, CA, USA) diluted 1:4,000 and 1:5,000 in PBS, respectively, and then were incubated for 90 minutes at RT. After two additional washing steps as before, 50 μL p-nitrophenyl phosphate disodium in diethanolamine buffer (pH 9.8) were added to the wells.

The optic density (OD) was determined by spectrophotometry at 450 nm wave length at 15-minute intervals until the standard positive sample reached an OD between 1,000 and 1,200. The calibration curve was based on international APL standards (Louisville APL Diagnostics Inc, Doraville, GA, USA). All samples were processed in duplicate. Results were expressed in GPL and MPL units for IgG and IgM aCL, respectively, and positive values were considered when above 20 GPL or 20 MPL units. LAC was detected using activated partial thromboplastin time (APTT – Diagnostica Stago, France) and diluted Russell's viper venom time (dRVVT – Trinity Biotech, Wiclow, Ireland, UK) according to international guidelines.[26] Serum IgG and IgM anti-β_2 GPI were detected by ELISA (The Binding Site, Birmingham, UK), according to the manufacturer's instructions with cutoff values of 10 U/mL for IgM and 20 U/mL for IgG.

Anti-NCS/chromatin antibodies were detected by ELISA (INOVA Diagnostics, San Diego, CA, USA), according to the manufacturer's instructions. Antinuclear antibodies (ANA) were determined by indirect immunofluorescence on HEp-2 cells (Bion, Des Plaines, IL, USA) at a screening dilution of 1:80, according to standard protocol.[27,28] Anti-double stranded DNA (dsDNA) tests were performed by indirect immunofluorescence using *Crithidia luciliae* as substrate; anti-extractable nuclear antigens (ENA) were detected by double immunodiffusion technique and included anti-SSA/Ro, anti-SSB/La, anti-Sm, and anti-RNP antibodies. The Western blot technique was used to detect at least one band of three ribosomal P proteins P0 (38 kD), P1 (19 kD), and P2 (17 kD).

Statistical analysis

Statistical analysis was carried out with SPSS 10.0 for Windows, Chicago, USA. Results were expressed as total number and percentage for categorical data and as mean and standard deviation (SD) for continuous variables.

RESULTS

Features of primary APS patients

Thirty-six women with primary APS with a mean age of 38.4 ± 11.8 years participated in this study. Fifteen (42%) were Caucasian-descendants and 21 (58%) were African-descendants. Previous arterial thrombosis episodes occurred in 13 patients (36.1%) being stroke the most frequent manifestation, occurring in 11 (84.6%) of these cases. Previous venous thrombotic episodes occurred in 17 patients (47.2%) mostly in the lower limbs (13 patients, 76.4%). Seventeen patients (47.2%) presented pregnancy morbidity, which was the only APS manifestation in six of them (Table 1). Along the follow-up period, aCL was present in 31 patients (86.1%), IgG aCL was found in 28 (77.8%), and IgM aCL in eight patients (22.2%). Eleven patients (30.6%) had a positive LAC test. Anti-β_2 GPI antibodies were detected in eight patients (22.2%): IgM anti-β_2 GPI in five (13.9%) and IgG anti-β_2 GPI in six patients (16.7%). The concomitant presence of the three APS-associated autoantibodies (aCL, LAC, and anti-β_2 GPI antibodies) occurred in six cases (16.7%) (Table 1). ANA was detected in 12 patients (33.3%) and the most frequent ANA pattern was the nuclear fine speckled pattern, that occurred at 1/320 titer in eight patients. Other ANA patterns, such as nuclear homogeneous (two cases), nuclear envelope (one case), and discrete cytoplasm speckles (one case), were also observed.

Anti-NCS/chromatin antibodies and disease follow-up in primary APS patients

Anti-NCS/chromatin antibodies were positive in only one patient (2.8%) with primary APS (Table 1). Considering that the recommended cut-off for the test is 20 IU/mL and strongly positive reactivity is set above 60 IU/mL, it is relevant to mention that this patient presented 147 IU/mL (Figure 1). Along the follow-up period (45.7 ± 9.6 months) this patient with reactivity for the anti-NCS/chromatin assay developed manifestations of SLE such as lymphopenia, polyarthritis, and neurological involvement characterized by optic neuritis and white matter demyelinating lesions resembling multiple sclerosis on brain MRI. She also developed an autoantibody profile compatible with SLE, including a 1:1280 antinuclear test (Hep-2 ANA) with homogeneous pattern, anti-dsDNA 1/10, anti-SS-A/Ro and anti-ribosomal P antibodies. This patient had a non-reactive test for anti-aquaporin 4 antibody. Among patients with no reactivity for anti-NCS/chromatin antibodies, two developed rheumatoid-like polyarthritis and had non-reactive

Table 1
Clinical features and autoantibody profile in primary APS patients

| Variables	Patients
Thrombosis	30 (83.3%)
Venous manifestations	17 (47.2%)
Lower limbs	13
Pulmonary embolism	3
Intracranial venous thrombosis	2
Upper limbs	1
Arterial manifestations	13 (36.1%)
Stroke	11
Peripheral occlusion	3
IMyocardial infarction	1
Pregnancy morbidity	17 (47.2%)
APS-related autoantibodies (frequency)	
LAC	11 (30.6%)
IgG aCL	28 (77.8%)
IgM aCL	8 (22.2%)
Anti-β2 GPI	8 (22.2%)
All three antiphospholipid antibodies	6 (16.7%)
Anti-nucleosome/chromatin antibodies (frequency)	
Anti-chromatin	1 (2.8%)
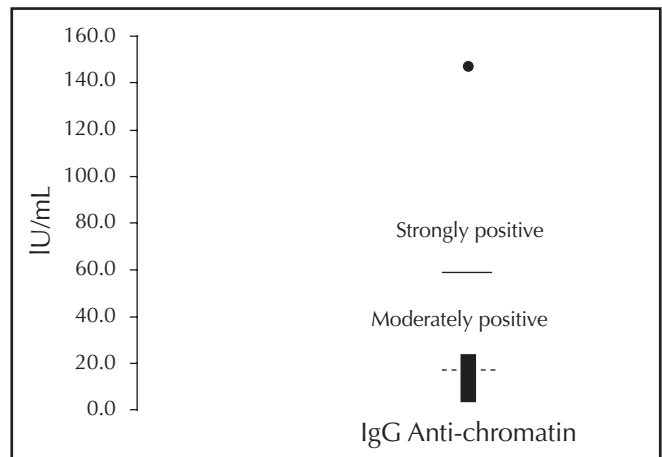
Figure 1
Magnitude of IgG anti-NCS/chromatin antibody reactivity in primary APS patients. Levels between 20.0 IU/mL and 60.0 IU/mL are considered moderately positive and levels above 60 IU/mL, strongly positive.

tests for rheumatoid factor and cyclic citrullinated peptide antibody. One of them was treated with corticosteroids and methotrexate and another one with methotrexate and leflunomide. One death occurred due to myocardial infarction in one patient after a mean follow-up time of 13 months. No other patient developed symptoms and signs suggestive of systemic involvement. No other patient was reactive for anti-dsDNA and ENA.

DISCUSSION

The present study has demonstrated a low frequency of antibodies against chromatin components in primary APS. In fact, only one patient (2.8%) had a positive anti-NCS/chromatin test and developed SLE. The vast majority of the primary APS patients did not develop overt signs and symptoms compatible with SLE along the mean 45-month follow-up period. As an exception, however, the one patient with reactivity in the anti-NCS/chromatin assay presented lymphopenia, polyarthritis, optic neuritis, white matter demyelinating lesions suggestive of multiple sclerosis, and an autoantibody profile highly suggestive of SLE.

The presence of anti-NCS/chromatin antibodies in patients with primary APS has been subject of some controversy in the recent literature. Since anti-NCS/chromatin antibodies are considered as highly specific for SLE, the occurrence of these autoantibodies in a patient with current diagnosis of primary APS might indicate the possibility of progression towards SLE. In fact, it has been shown by one group that the prevalence of anti-NCS/chromatin antibodies in primary APS is low[9] and other groups associated their presence in APS patients with subsequent development of SLE features.[21,22] Although the development of manifestations of APS in SLE patients with APL antibodies has been reported in 50%–70% of cases within twenty years of follow-up,[1,29] the opposite is not usually expected. Actually, a complete picture of SLE may evolve in 4%–10% primary APS patients, and the presence of a family history of SLE, Raynaud phenomenon, migraine, multiple sclerosis-like features, hemolytic anemia, low C3 and C4, and Coombs' test positivity are recognized risk factors.[30] The present finding suggests that anti-NCS/chromatin antibodies might be included as an additional risk factor for SLE development in patients with a diagnosis of primary APS.

The antigenic substrate used in the anti-NCS/chromatin assays is relevant to the heterogeneity in results obtained by different authors. The commercial assay used in the present study was H1-stripped chromatin devoid of non-histone proteins (mainly oligonucleosomes) derived from calf thymus. This is a widely used antigen for anti-NCS/chromatin assays and contains most nucleosome epitopes recognized by polyclonal and monoclonal anti-NCS antibodies.

The lack of standardization of serologic tests is a major problem for autoantibody determination.[31] The antigens currently used for determination of anti-NCS/chromatin antibodies include H1-stripped chromatin, polynucleosomes devoid of H1, and mononucleosomes with or without H1. In addition, there is variation in the biochemical conditions for antigen purification and in the raw source used for antigenic preparation. Finally there is heterogeneity in the conditions established in the various immunoassays. This overall heterogeneity implies that the different assays do not detect exactly the same autoantibody population and this may contribute to the partially conflicting results observed in the literature.[32]

In conclusion, the present study showed a low frequency of antibodies against NCS/chromatin in patients with primary APS and those autoantibodies may be associated with the development of SLE features in patients with a diagnosis of primary APS. Further multicentric and longitudinal studies should be performed to confirm this finding.

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