

MULTIPLE ANTIPHOSPHOLIPID TESTS DO NOT INCREASE THE DIAGNOSTIC YIELD IN ANTIPHOSPHOLIPID SYNDROME

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SUMMARY

The family of antiphospholipid antibodies (aPL) includes a heterogeneous population of autoantibodies whose specificity is directed against not only phospholipids, but their complex with plasma proteins. Anticardiolipin antibodies (aCL) and lupus anticoagulant (LA) tests are widely performed to screen the aPL family which is associated with thrombotic complications in patients with systemic lupus erythematosus (SLE) or antiphospholipid syndrome (APS). The clinical significance of other aPL tests, including antibodies against phosphatidylserine (aPS), phosphatidylinositol (aPI), phosphatidic acid (aPA), phosphatidylcholine (aPC) and phosphatidylethanolamine (aPE), has not been established. The purpose of this study was to evaluate whether multiple aPL tests have enhanced diagnostic value for APS. We tested IgG/M/A aPS, aPI, aPA, aPC and aPE by ELISA using 10% bovine serum as blocking and sample diluent in 26 SLE patients with clinical manifestations of APS, but negative for both aCL and LA (Group 1). The results were compared with 32 SLE patients without any features of APS (Group 2) and 24 SLE patients with APS (aCL and/or LA positive) (Group 3). In Group 1, 1/26 (4%) was positive for IgA aPE, less frequent than in other groups, and none of the patients had any other aPL. In Group 2, 1/32 (3%) was positive for aPS, two (6%) for aPI, one (3%) for aPA and four (12.5%) for aPE. None was positive for aPC. In the third group, 13/24 (54%) were positive for aPS, 11 (46%) for aPI, 15 (63%) for aPA, four (17%) for aPC and seven (29%) for aPE. Since aPE was found in some patients, we extended the study, including 207 SLE patients, and tested aPE. IgG/M/A aPE was found in six (3%), 10 (5%) and 21 (10%), respectively, but no association was found between aPE and any clinical features of APS. This study suggests that screening by multiple aPL tests does not increase the diagnostic yield in APS.

KEY WORDS: Anticardiolipin antibodies, Antiphosphatidylethanolamine, Lupus anticoagulant, Systemic lupus erythematosus, Thrombosis.

THE family of antiphospholipid antibodies (aPL) includes a heterogeneous population of autoantibodies directed against different phospholipids or their complex with plasma proteins. The better known aPL are anticardiolipin antibodies (aCL) and lupus anticoagulant (LA), both associated with thrombotic complications in systemic lupus erythematosus (SLE) or antiphospholipid syndrome (APS). It has been demonstrated that these antibodies are directed to plasma proteins bound to anionic phospholipids. The phospholipids may induce some conformational changes in protein structure; thus, many of the antibodies against phospholipid binding proteins can be detected basically in the presence of phospholipids [1–3]. So far, β 2-glycoprotein I (β 2GPI) and prothrombin are the best known and characterized antiphospholipid cofactors [4]. In recent years, attention has been focused toward other aPL, such as phosphatidylserine (aPS), phosphatidylcholine (aPC), phosphatidylinositol (aPI), phosphatidic acid (aPA) and phosphatidylethanolamine (aPE), and their relationship with thrombotic events [5–8]. As multiple phospholipids may use heterogeneous cofactors (phospholipid binding proteins) for aPL binding, multiple aPL ELISAs might increase the sensitivity of aPL family detection and improve the diagnostic yield of APS. However, findings

have been varied and their clinical significance has not been well established. We evaluated whether multiple tests would have better diagnostic value, testing IgG/M/A aPS, aPC, aPI, aPA and aPE in three selected groups of consecutive patients.

Secondly, we focused our study on aPE. Phosphatidylethanolamine is a zwitterionic phospholipid present in both internal and external sides of normal cell membranes, thought to be involved in the development of thrombotic events via its role in the protein C pathway and the consequent inactivation of factor Va by activated protein C [7]. Antibodies directed to PE (aPE) could play an important role by inhibiting activated protein C [9]. Our purpose was to study the prevalence of aPE in a large number of SLE patients, to search for relationships between the presence of IgG/M/A aPE and thrombotic events, and to evaluate the clinical significance of aPE in a large cohort of SLE patients.

PATIENTS AND METHODS

Patients

The first part of this study comprised 82 patients with SLE from the Lupus Clinic at St Thomas' Hospital, who fulfilled the revised criteria for the classification of SLE [10]. Patients were divided into three groups according to their laboratory and clinical features: Group I [$n = 26$, mean age 40.77 yr (range 17–57), 24 females]: SLE patients with clinical features of APS (arterial thrombosis, venous thrombosis, recurrent pregnancy loss and/or thrombocytopenia), but

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both aCL and LA negative; Group II [$n = 32$, mean age 40.59 yr (27–72), all females]: SLE patients without any feature of APS; Group III [$n = 24$, mean age 40.6 yr (range 20–64), 22 females]: SLE patients with secondary APS (aCL and/or LA positive).

For the second part of the study, IgG/M/A aPE were tested in 207 patients with SLE [mean age 39 yr (range 10–72), 199 females].

The medical records of all patients were carefully reviewed retrospectively. One hundred and thirteen healthy individuals were included as controls.

Methods

Multiple aPL ELISA. Microtitre ELISA plates (Immulon 1, Dynatech Inc., Virginia, USA) were coated with each of the following phospholipids: phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (all from Sigma, USA), in chloroform:methanol (1:4) solution and dried. After blocking with 10% adult bovine serum (Sigma) in phosphate-buffered saline (10% ABS-PBS), serum diluted 1:100 in 10% adult bovine serum (10% ABS) was added in duplicate. After incubation for 3 h and three washes with PBS, alkaline phosphatase-conjugated goat anti-human IgG, IgM or IgA was added in the appropriate dilution. Colour was developed by adding 100 μ l of 1 mg/ml of *p*-nitrophenylphosphate disodium in 1 M diethanolamine buffer (pH 9.8). Plates were incubated until the optical density (OD) of a high-binding serum (positive control) reached 1.0. Results were expressed as a binding index, calculated as follows: $BI = [OD(\text{sample}) - OD(\text{blank})] / [OD(\text{positive control}) - OD(\text{blank})]$.

Values >5 s.d. above the mean of healthy controls were considered as positive.

Anticardiolipin antibodies and lupus anticoagulant. aCL were determined according to the standardized aCL ELISA [11].

Because many of the patients were on warfarin at the time of the study, data regarding LA were those historically present in the patients' clinical records before starting anticoagulation therapy. A prolonged dilute Russell's viper venom time and its platelet neutralization was taken as evidence of LA.

Statistical analysis. All statistical analysis was performed using StatView II software (Apple Macintosh software). Categorical analysis was determined by χ^2 test.

RESULTS

Table I shows the prevalence of IgG/M/A aPS, aPA, aPE, aPC and aPI in Group 1, Group 2 and Group 3. No significant difference in the prevalence of each aPL was found between Group 1 and Group 2.

Since some patients had positive aPE in Group 3 (secondary APS to SLE), IgG/M/A aPE were tested in 207 patients with SLE and their clinical significance was evaluated in a larger number of SLE patients. However, no association was found between any isotypes of aPE and any clinical features of APS [thrombosis, pregnancy loss (Table II) and thrombocytopenia] or SLE.

DISCUSSION

In this study, we evaluated the clinical significance of different aPL, other than well-established aCL and LA, in patients with SLE. Some studies have shown a

TABLE I
Prevalence of IgG/M/A aPS, aPA, aPE, aPC and aPI in the study groups

	aPS			aPA			aPE			aPC			aPI		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
Group 1 ($n = 26$) %	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Group 2 ($n = 32$) %	1	1	0	0	0	1	1	0	4	0	0	0	0	0	2
Group 3 ($n = 24$) %	8	6	2	12	3	6	1	0	7	0	3	1	7	4	4
	33	25	8	50	12.5	25	4		29		12.5	4	29	17	17

Group 1, SLE with clinical features of APS (aCL and LA negative); Group 2, SLE without clinical features of APS; Group 3, SLE/APS. aPS, antiphosphatidylserine; aPA, antiphosphatidic acid; aPE, antiphosphatidylethanolamine; aPC, antiphosphatidylcholine; aPI, antiphosphatidylinositol.

TABLE II
Association between the presence of aPE, thrombotic events and pregnancy loss in SLE

	With thrombosis ($n = 78$)	Without thrombosis ($n = 129$)	With pregnancy loss ($n = 32$)	Without pregnancy loss ($n = 164$)	<i>P</i> *
aPE G/M or A	13	19	5	25	ns
aPE G	1	5	1	5	ns
aPE M	5	5	1	9	ns
aPE A	9	12	3	16	ns

aPE, antiphosphatidylethanolamine antibodies.

*By χ^2 test; ns, not significant.

high prevalence of autoantibodies directed against a range of anionic phospholipids in patients with SLE [5, 7, 12–14]. They also reported an association between the presence of IgG isotype of these antibodies and thrombosis or thrombocytopenia [5, 15], and between IgM isotype and haemolytic anaemia [8, 16, 17]. The correlation between the clinical features of APS and antibodies detected by 'conventional' ELISA using anionic phospholipids as antigen is not surprising. APS-associated aCL is well known to recognize cardiolipin- β 2GPI complex and the β 2GPI molecule bears their binding epitopes. β 2GPI has the property of binding to negatively charged phospholipids. Since bovine serum, which is used as a blocking agent and sample diluent in conventional ELISA, contains β 2GPI, aCL (virtually anti- β 2GPI antibodies) can bind to β 2GPI phospholipid complex and be detected by aPS, aPA and aPI assays as well as aCL ELISA. Our data showed a similar distribution of aPS, aPA and aPI between the three groups. We could not find any patients with positive aPS, aPA or aPI in Group 1 (aCL/LA negative). Thus, standard aCL (and LA) tests were sufficient to detect this population of aPL.

Phosphatidylethanolamine is a zwitterionic phospholipid present in both internal and external sides of normal cell membrane. It is thought to be involved in the development of thrombotic events through its role in the protein C pathway and the consequent inactivation of factor Va by activated protein C. Branch *et al.* [18] reported that 80% (12/15) of LA patients were aPE positive. Weidmann *et al.* [14] studied 92 SLE patients and found that the prevalence of aPE was 22%. They could not, however, demonstrate any association between aPE and thrombosis. Falcon *et al.* [19] investigated the clinical significance of aPE in a large cohort of patients with various underlying diseases (including 33 SLE), concluding that there was no association between aPE and the presence of aPL-related clinical complications. However, contradictory results were later reported by Karmochkine *et al.* [5]. aPE was present in 16.6% of patients with SLE and they suggested that aPE was an additive risk of aCL and LA for thrombosis or pregnancy loss in SLE. Sugi and McIntyre [20] showed that aPE found in patients with SLE and recurrent fetal losses required low-molecular-weight and high-molecular-weight kininogen binding proteins bound to PE for detection in ELISA. Our aPE assay may detect kininogen-dependent aPE since ABS (used as blocking agent and diluent) contains enough kininogen.

In our study, aPE was found in some patients; thus, we extended this study to include 207 patients with SLE and tested aPE in all patients. The prevalence of aPE in the SLE group was 15% (32/207). Although the prevalence was similar to other studies, we could not demonstrate any association between aPE and any features of APS. Therefore, we could not consider aPE as a marker for thrombosis or APS in SLE.

In conclusion, we suggest that screening by multiple aPL tests does not increase the diagnostic yield in APS.

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