

Gülinnaz ALPER¹
Ege Nazan TAVMERGEN²
Yasemin KABASAKAL³
Taner ONAT¹

Lupus Anticoagulant and Anticardiolipin Antibodies in Unexplained Fetal Losses

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Abstract: Lupus anticoagulant (LA) and anticardiolipin antibodies (ACAs) are acquired antiphospholipid antibodies (APAs), which are considered to be important markers for pregnancy losses and intrauterine fetal demise. LA and ACAs have anticoagulant effects in vitro and thrombotic effects in vivo and are considered to be the cause of recurrent pregnancy losses (RPLs), resulting from placental vascular thrombosis and infarction. The aim of this study was to identify the most sensitive and specific method of determining APA positivity and to evaluate the prevalence of APA in patients with unexplained RPL.

The experiment consisted of 25 women with unexplained RPL, the patient group, and 15 healthy women with successful pregnancies, the control group. ACA positivity was determined with ELISA and LA activity with phospholipid dependent coagulation tests (PDCTs): prothrombin time (PT), activated

partial thromboplastin time (APTT), kaolin clotting time (KCT) and a platelet neutralization procedure (PNP).

LA activity was detected in 5 of the 25 women in the experimental group (20%), but in none of the 15 women in the control group. Increased ACA levels were observed in 8 of the experimental group (32%) and in one of the control group subjects (7%).

These results provide quantitative evidence of the association between APA and RPL. LA was best identified through KCT and should be specifically confirmed by PNP. Screening for APAs, both with ACAs for sensitivity and LA for specificity, is indicated in patients with adverse pregnancy outcomes.

Key Words: Lupus anticoagulant (LA), anticardiolipin antibodies (ACAs), antiphospholipid antibodies (APAs), pregnancy losses.

Departments of ¹Biochemistry, ²Obstetry and Gynecology, ³Internal Medicine, Bornova, Izmir-Turkey

Introduction

Lupus anticoagulant (LA) and anticardiolipin antibodies (ACAs) are acquired antiphospholipid antibodies (APAs) characteristically found in patients with SLE or related autoimmune diseases and considered to be important markers for pregnancy losses and intrauterine fetal demise (1, 2).

LA is an antibody (IgG and/or IgM) that prolongs phospholipid dependent coagulation tests (PDCTs) by binding to epitopes on the phospholipid portion of prothrombinase (a complex of FXa, FVa, PL and Ca⁺²) (3-5). Closely related to LA, ACAs are antiphospholipid autoantibodies (IgG, IgM and IgA) which have specificity for negatively charged phospholipids such as cardiolipin and are detected by immunoassays that use cardiolipin as the solid phase (1, 6, 7). It has been suggested recently that LA and ACAs form two distinct but related subgroups (8, 9).

APAs are found in different clinical situations, such as autoimmune diseases (including SLE and rheumatoid arthritis), infections (including syphilis and AIDS), malignancy and drug exposure (chlor promazine, procainamide, phenothiazines, hydralazine, quinidine, dilantin, etc.) (1, 7, 10-18). APAs are also sometimes found in healthy people (2).

In recent years, it has been suggested that LA and ACAs are strongly associated with a diverse set of clinical manifestations, including venous and arterial thrombosis, neuropsychiatric disorders, thrombocytopenia and RPL, which together constitute the APA syndrome (2, 4, 10, 19-21). It is thought that patients who have LA or ACAs have a greater risk of developing these complications and that these associations are largely independent of the underlying disease (2, 5, 22).

Recently, LA and ACAs have been recognized as having a role in RPL, even in women with no clinically diagnosed

autoimmune disease (3). LA and ACAs having anticoagulant effects *in vitro* and thrombotic effects *in vivo* (23) are considered to be the cause of RPL, resulting from placental vascular thrombosis and infarction (1, 2, 4, 24).

The association between APAs and RPL is well established (25, 26). What is still unclear is the actual prevalence of APAs in women with unexplained RPL. The aim of this study was to identify the most sensitive and specific method of determining APA positivity and to evaluate the prevalence of APAs in patients with unexplained RPL.

Materials & Methods

Materials: A case-control study of the association between RPL, LA and ACAs was conducted in the hospital of Ege University School of Medicine. The experimental subjects were 25 women (median age 30 years, range 19-41) with no clinical or laboratory evidence of SLE or related autoimmune diseases, and whose only pregnancies had resulted in two or more consecutive unexplained spontaneous or missed abortions or IUMF. The control group consisted of 15 women (median age 34, range 26-49) who had had one or more normal pregnancies without previous spontaneous abortion. These women were admitted to the same hospital for acute conditions that were not immunologic, infective, neoplastic, gynecologic or cardiovascular. In the patient group the following parameters were screened in order to detect immunologic etiology and to exclude the endocrine, metabolic, anatomic, infectious and genetic factors that can lead to RPL:

- Normal standard medical and gynecologic examination.
- Hysterosalpingogram (HSG), ultrasonogram (US), luteal phase endometrial biopsy.
- Hormonal profile (FSH, LH, progesterone, oestradiol, prolactin, free testosterone, TSH, T3, T4)
- Biochemical profile (including oral glucose tolerance test (OGTT), protein electrophoresis, urine analysis, haemogram, blood grouping, thrombocyte count).
- TORCH group tests, Group Agglutination tests, hepatitis markers, VDRL, TPHA, RPR, ICT.
- IgG, IgA, IgM, C3, C4, ANA, Anti-DNA Ab, LE cells, CRP, Latex, Antisperm Ab.
- Maternal and paternal karyotype determination from peripheral leucocytes.

Based on these data, cases with known etiology such as SLE were excluded from the study. All the experimental cases and controls were tested for LA and ACAs.

Methods used in APA determination: ACA values were determined using an ELISA test (Asserachrom^R APA, Diagnostica Stago). LA activity was determined through PDCTs: prothrombin time (PT), Neoplastine Ca 12 (Diagnostica Stago); activated partial thromboplastin time (APTT), Activated Thrombofax (Ortho Diagnostic Systems); kaolin clotting time (KCT), using Exner's method (27), and also with a commercial kit of kaolin; APTT (C.K.Prest, Diagnostica Stago); and a platelet neutralization procedure (PNP), using the method of Triplett et al. (18).

We prepared platelet poor plasma (PPP) and looked for any prolongation in the PDCT. In order to determine ACAs and PDCT (not including KCT), after ruling out recent heparin treatment, we collected blood on trisodium citrate (9 parts blood to 1 part 0.109 M trisodium citrate as the anticoagulant) and centrifuged at 2500 g for 15 minutes at 20°C to obtain PPP, which was stored at -20°C until use. Blood for the KCT tests, using Exner's method (27), was collected through venipuncture into polyethylene tubes containing one-tenth (final) 3.8% w/v sodium citrate pH 6.5. Pooled PPP from 20 normal individuals was prepared for use in mixing tests.

PDCTs were considered positive when the ratio of the subject clotting time to the mean of the control clotting time exceeded 1:3 (baseline test) and when this ratio remained greater than 1:2 upon 1:1 mixture of experimental and normal PPP (performed to exclude clotting factor deficiency). The diagnosis of the LA was based on a prolonged PDCT not corrected with a mixing test, together with a positive PNP result (baseline test + mixing test + PNP) (7, 18).

In determining ACAs, the positive control, the negative control and the cutoff (included in the ready-to-use kit) by comparison enabled a semi-quantitative evaluation. The results were considered positive when the resultant OD readings at 492 nm on a Titertek Multiscan were above the cutoff value and negative when the OD values were below the cutoff value.

Statistical analysis: The significance of the difference between the mean values of the parameters were evaluated using the Mann-Whitney non-parametric test.

Results

LA activity was detected in 5 of the 25 experimental subjects (20%), but in none of the 15 controls. Increased

Table 1. ACA and PDCT Results of APA (+) Experimental and Control Groups.

APA (+) Experimental	ACA OD ₄₉₂	ACA	PT	APTT*	APTT**	KCT*	KCT**	CK.P*	CK.P**	PNP	SC	LA
1	229	+	18"	33"	30"	120"	120"	65"	51"	39"	53"	+
2	209	+	13"	27"	-	100"	85"	35"	35"	-	-	-
3	170	+	13"	32"	28"	85"	90"	47"	58"	37"	49"	+
4	382	+	14"	32"	28"	150"	145"	50"	50"	39"	52"	+
5	513	++	14"	36"	37"	200"	180"	60"	65"	32"	60"	+
6	1809	++	15"	37"	36"	130"	125"	41"	39"	37"	41"	+
7	183	+	13"	27"	-	65"	65"	30"	30"	-	-	-
8	357	++	14"	28"	-	110"	100"	35"	35"	-	-	-
Control	178	+	13"	27"	-	90"	90"	35"	35"	-	-	-

APA Cutoff: OD₄₉₂=151, APA (+): OD₄₉₂= 152-300, APA (++) : OD₄₉₂ ≥ 301, LA (+): PNP ≤ 39", LA (-): PNP > 39"

*Baseline Test, **Mixing Test, SC: Saline Control

ACA levels were observed in 8 of the 25 subjects (32%) and in one of the 15 controls (7%).

The ACA and PDCT results of the APA (+) experimental and control groups are presented in Table 1.

Table 2 shows the mean±SEM values of each method used in APA determination (ACA, KCT, CK.P, APTT and PT).

Table 3 shows the significance of the difference between the mean values of the parameters (Mann-Whitney non-parametric test) in the control and the experimental groups.

Discussion

In the general population, the prevalence of LA and ACAs ranges from 0% to 2% and 7.5%, respectively (2, 3, 20, 22, 28-31). In reports relating to APA prevalence in RPL cases with no apparent etiology, a prevalence of 3-48% is stated for LA and 8-50% for ACA (2, 3, 7, 19, 25, 32). The results of 16 studies of 827 RPL cases show that the frequency of APA positivity is 29% (25% for LA and 37% for ACAs) (7). This wide range of prevalence values is probably due to the use of different criteria in patient selection, different methods used in determining APA and the lack of international uniformity for the definition of the LA. PDCTs used to determine LA vary widely in sensitivity (7, 32, 33). Methods of determining ACAs vary according to antibody subclass or class and cutoff values used for positivity (2, 7, 25).

In this study, LA activity was detected in 5 of the 25 experimental subjects (20%), but in none of the 15 controls. Increased ACA levels were observed in 8 of the 25 experimental subjects (32%) and in one of the 15 controls (7%). These results offer quantitative evidence of the association between APAs and RPL. While the LA prevalence of the control group shows concordance with the studies of El-Roeiy A (28) and Miliez J (30), the ACA prevalence (7%) of our controls are also in concordance with the ones of Love et al. (2) and al-Momen et al. (26). The results (prevalence) of our RPL cases (32% ACA and 20% LA) are in concordance with the data of McNeil et al. (7) and al-Momen et al. (26), who observed 30% APA positivity in idiopathic RPL cases.

In spite of the fact that LA and ACAs are observed together, it is interesting to note that in most of the studies there is no correlation in their levels (7). In accordance with the findings of McNeil et al. (7, 34) and Triplett et al. (8), in our study no correlation between the levels of ACA and LA was found (Table 1 and Table 3).

It has been well documented that APTT is a test with low sensitivity and is insufficient for detecting weak inhibitors (35, 36). In agreement with these data, it was observed in this study that APTT had the lowest sensitivity of the PDCT tests applied to determine LA activity (Table 2).

KCT is a specific type of APTT independent of tissue thromboplastine. It has been suggested that this is the most sensitive test for detecting LA activity (7, 35), an

	Control G. n=15	APA(+) EG n=8	APA(-) EG n=17	Total EG n=25
ACA	112±9	481±194	101±7	222±70
KCT	75±3	120±15	71±2	87±7
CK.P	33.4±0.4	45.4±4.4	33.1±05	37.0±1.8
APTT	26.9±0.1	31.5±1.4	26.6±0.2	28.2±0.6
PT	13±0	14.3±0.6	13±0	13.4±0.2

Table 2. Mean±SEM values of methods used in APA determination

	ACA	APTT	KCT	CK.P	PT
APA(+) EG-APA(-) EG	p<0.01	p<0.01	p<0.01	p<0.01	p<0.05
APA(+) EG-CG	p<0.01	p<0.01	p<0.01	p<0.05	p<0.05
APA(-) EG-CG	NS	NS	NS	NS	NS
TOTAL EG-CG	NS	NS	NS	NS	NS

Table 3. The significance of the difference between the mean values of the parameters in the control (CG) and experimental groups (EG).

(Mann-Whitney non-parametric test) p>0.05=NS:Not significant.

observation supported by many authors in recent years (36, 37). The procedure of eliminating the inhibitor effect by the addition of a platelet suspension, proposed by Triplett et al. (38), is called PNP and was reported by Kornberg et al. in 1989 to be the most specific method for confirming the diagnosis of LA activity (7). Similarly, we also observed that LA activity was best identified using KCT and should be specifically confirmed by PNP (Table 1).

In direct determination of APAs using the solid-phase ELISA immunoassay method, 8 out of the 25 experimental subjects with RPL (32%) exhibited APA positivity while only 5 (20%) exhibited APA positivity when a combination of KCT and PNP tests were used in order to determine the LA activity. In this situation, the ACA solid-phase immunoassay seems to be more sensitive. However, in the control group consisting of 15 healthy women with no clinical or laboratory evidence of APA positivity, one woman exhibited ACA positivity (7%) while none exhibited LA activity. This implies that, although highly sensitive, ACA determination is not sufficiently specific. From this point of view, the screening of APAs both with ACAs for sensitivity and LA for specificity would reinforce the diagnosis.

While, in previous studies, in cases of RPL, fetal losses

occurred in 88-100% of pregnancies, in this study, a RPL rate of about 93% was detected both in APA (+) and (-) patients. Abortions occurring during the first trimester were the most prevalent in RPL cases (78%). The incidence of this was 72% in the APA (+) group, while it was 83% in the APA (-) group. However, it was detected that abortions occurring during the second trimester were more common in the APA (+) group (14%) than the APA (-) group (5%). In only 13% of the cases, IUMF occurred in the third trimester.

It is noteworthy that missed abortions were more common (36%) in the APA (+) group than the APA (-) group (22%). In particular, the missed abortion prevalence in the second trimester was 11% in the APA (+) group, while no such case was detected in the APA (+) group than the APA (-) group (22%). In particular the missed abortion prevalence in the second trimester was 11% in the APA (+) group, while no such case was detected in the APA (-) group. Nevertheless, no significant correlation between APA positivity and RPL rate could be detected. In addition, no significant difference was observed between primary and secondary aborters or between fertile and subfertile groups.

In the experimental group, no positivity was detected for the indirect Coombs test (ICT), antinuclear antibodies

(ANAs), anti-DNA antibodies, LE cells or a biologically false positive serologic test for syphilis (BFP-STs). There was no decrease in the serum C₃ or C₄ complement or thrombocyte levels.

It is not yet clear whether APAs are an epiphenomenon associated with underlying thrombotic diathesis or an early marker of a developing autoimmune

disease. Until this question is answered, women with unexplained RPL should be screened for the presence of APAs. Since treatment with corticosteroids and anti-aggregants, high-dose intravenous immunoglobulins and plasmapheresis can lead to successful pregnancies (21, 24, 31), it would be wise to monitor closely cases with APA positivity.

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