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Prevalence of Various Antiphospholipid Antibodies in Pregnant Women

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Summary

Antiphospholipid antibodies (APAs) are characterized as a heterogeneous population of autoantibodies directed against different target antigens, predominantly anionic phospholipids or phospholipid-containing structures. The presence of APAs has been strongly associated with a variety of clinical disorders including adverse pregnancy complications such as spontaneous abortions, pregnancy-induced hypertension, preeclampsia and intrauterine growth retardation. The purpose of this study was to compare the prevalence of anticardiolipin antibodies (ACAs), which are routinely examined, with APAs directed against phosphatidylserine (APS), phosphatidylinositol (API), phosphatidylethanolamine (APE) and phosphatidylcholine (APC) in the sera of pregnant women. We examined 410 serum samples of pregnant women hospitalized in the department for pathological pregnancies. They underwent prenatal biochemical screening of fetal congenital abnormalities in the first and the second trimester of gravidity. Anticardiolipin IgG and IgM were measured using commercial ELISA kits (ImmuLisa^{TM} Anti-Cardiolipin Antibody), whereas APS, APE, API and APC were determined by our modified ELISA kit. Among 410 pregnant women we found 21 patients (5.1 %) positive for ACA IgG (>20 GPL) and 30 patients (7.3 %) positive for ACA IgM (>10 MPL). It was found that 7.8 % of pregnant women had at least one high-titer APA IgG and 9.8 % high-titer APA IgM. One third of ACA IgG or IgM positive sera contained polyspecific autoantibodies reactive to at least two various phospholipids. In the group of IgG ACA positive women, 28.6 % patients were positive for APS, 28.6 % were positive or moderately positive for API, 23.8 % for APC and 19 % for APE. In the group of IgM ACA positive women, 33.3 % were also positive for APS, 26.7 % for APE, 26.7 % for API and 23.3 % for APC were present. IgG and IgM ACA negative patients exhibited a significantly lower incidence of other APA than the group of ACA positive pregnant women. It still remains to clarify if the routine examination of APA reacting with other anionic and zwitterionic antigens other than cardiolipin would improve the probability of identifying women liable to adverse pregnancy complications.

Key words

Antiphospholipid antibodies • Anticardiolipin antibodies • Pregnancy • Phospholipids

Introduction

Antiphospholipid antibodies (APAs) are characterized as a heterogeneous population of antibodies directed against different target antigens, predominantly anionic phospholipids or phospholipid-containing structures. The presence of APAs has been associated

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with a variety of health disorders including adverse pregnancy complications such as spontaneous abortion, pregnancy-induced hypertension, preeclampsia and intrauterine growth retardation. Women with a high titer of IgG antiphospholipid antibodies do have an approximately 28 % probability of fetal loss. Low-titer antibodies occur in 2-5 % of healthy young women (Faux *et al.* 1989, Brown 1991, McNeil *et al.* 1991, Lynch *et al.* 1994, Matzner *et al.* 1994).

Most APAs may show various reactivities with regard to the phospholipid structure as well as to their isotype. Cardiolipin is the phospholipid most commonly used as an antigen to test APAs by ELISA. Phosphatidylserin seems to be more physiologically relevant than cardiolipin as the antigen for the detection of APA. Phosphatidylinositol and phosphatidylethanolamine can also be used to detect APAs. The association of clinical complications with APAs appears to depend on the specificity, isotype, level and probably the time during which these antibodies are present (Gharavi *et al.* 1987, Triplett *et al.* 1988, Falcon *et al.* 1990, Zima *et al.* 1998). The presence of IgG APAs appears to be of greater significance than the presence of IgM in identifying women at risk of miscarriage or thrombosis (Harris *et al.* 1987).

The purpose of this study was to compare the prevalence of anticardiolipin antibodies (ACAs), which are commonly examined, with APAs directed against phosphatidylserine (APS), phosphatidylinositol (API), phosphatidylethanolamine (APE) and phosphatidylcholine (APC) in sera of pregnant women.

Methods

We examined 410 serum samples of pregnant women who underwent prenatal biochemical screening of fetal congenital abnormalities in the first and the second trimester of gravidity. Their mean age was 28.4 years (range 19-39 years). Blood samples were withdrawn during weeks 9-18 of gestation and stored at –20 °C.

Table 1. Occurrence of APA with various phospholipid specificity in ACA IgG positive patients

Patient	Week of pregnancy	Age	ACA IgG	APS IgG	APE IgG	API IgG	APC IgG
1	16+1	35	*		±		
2	16+2	33	*			±	*
3	15+6	30	*				
4	17+5	25	*				
5	16+1	32	*				
6	16+0	34	*				
7	16+2	29	*				*
8	15+6	36	*				
9	15+3	39	*	*			
10	16+1	31	*	*		±	
11	18+1	34	*			*	
12	15+1	22	*	*		*	*
13	16+1	32	*		*	±	
14	16+0	30	*				
15	16+1	32	*	*			
16	11+3	35	*				
17	11+2	39	*		*		*
18	8+4	28	*	*			
19	16+0	32	*				
20	15+3	36	*				
21	12+3	28	*	*	*	*	*

* *positive results,* ± *moderately positive results*

ACA IgG and IgM were measured using commercial ELISA kits: ImmuLisa™ Anti-Cardiolipin Antibody ELISA (IMMCO Diagnostics, Buffalo, NY). The results were expressed in units /ml – GPL for IgG isotype and MPL for IgM isotype. One unit was defined as the cardiolipin binding activity of 1 μg/ml of an affinity-purified Ig anticardiolipin preparation, i.e. IgG and IgM. ACA IgG values >19 GPL and ACA IgM values >10 MPL were considered to be positive. APS, APE, API, and APC were determined by the modified ELISA method (Harris 1990). Briefly, polystyrene microtiter plates (Polysorp-Nunc) were coated with 1 μg/well of phospholipid L-α-phosphatidyl-L-serine, L-α-phosphatidylethanolamine, L-α-phosphatidylinositol or L-α-phosphatidylcholine (Sigma Chemicals) dissolved in ethanol or a mixture of methanol-chloroform. The plates were allowed to dry overnight at 4 °C and then saturated with 10 % adult bovine serum in phosphate buffered saline (PBS). The serum samples diluted 1:50 in 10 % bovine serum in phosphate buffered saline were added to the wells and incubated for 2 h at 37 °C and then washed 3 times. After washing, the horseradish peroxidase-conjugated goat antihuman IgG or IgM (Sevapharma, CR), diluted 1:5000 in PBS, was added to the appropriate wells and left to stand for 1.5 h at room temperature. After three washings, color development was induced by adding 0.1 ml of an ortho-phenylendiamine solution (Sevapharma, CR) with H_2O_2 for 15 min at room temperature. The reaction was stopped by adding 2 mol/l H_2SO_4. Antiphospholipid assays other than ACA have not yet been standardized and there is no standard material available for calibration. Known positive and negative sera and our own internal standard were run on each plate.

Table 2. Occurrence of APAs with various phospholipid specificity in ACA IgM positive patients.

Patient	Week of pregnancy	Age	ACA IgM	APS IgM	APE IgM	API IgM	APC IgM
1	15+5	28	*				*
2	17+5	25	*				
3	17+2	23	*				
4	17+4	38	*				
5	16+1	32	*	*			
6	16+0	34	*	*		*	*
7	15+4	36	*		*		*
8	15+6	36	*				
9	16+5	30	*		*		±
10	16+4	35	*				
11	15+3	39	*		*	±	
12	16+1	31	*	*	*	*	
13	17+3	34	*	*		*	
14	18+1	34	*	±	±	*	
15	16+6	33	*				
16	16+4	30	*				
17	17+6	22	*				
18	16+2	24	*				
19	15+1	22	*	*			
20	18+0	38	*	*		*	
21	16+4	33	*	*	*		*
22	16+3	30	*				
23	8+6	31	*				
24	16+0	30	*				
25	16+1	32	*				
26	11+3	35	*				
27	11+2	39	*	*	*	*	*
28	16+6	33	*				*
29	16+1	37	*	*	*	*	
30	8+4	28	*				

** positive results, ± moderately positive results*

Evaluation of four antiphospholipid antibodies (APS, APE, API, APC) was carried out by direct comparison of the optical density (OD) of each patient sample with the optical density of our internal standard. A sample was considered positive if its respective OD was greater than the cut off + 10 %. A sample was considered negative if its respective OD was less than the cut off + 10 % (cut off = OD of our internal standard). Specimens exhibiting OD values between these limits (cut off ± 10 %) were considered moderately positive.

The statistical significance was evaluated using Fisher's exact χ^2 test.

Table 3. Occurrence of APA with various phospholipid specificity in ACA negative patients.

Patient	Week of pregnancy	Age	APS IgG	APS IgM	APE IgG	APE IgM	API IgG	API IgM	APC IgG	APC IgM
1	16+5	33				*				
2	15+3	35	*				*			
3	15+6	35			*	*				
4	17+4	33	*							
5	8+6	31						*		
6	15+0	31	*							
7	16+6	30	*				*		*	*
8	16+2	35					*			
9	16+1	32				*				
10	16+2	33			*					
11	16+5	31			*					
12	15+5	34		*						
13	16+2	33			*				*	
14	16+0	29				*				*
15	11+1	31		*				*		
16	11+4	37					*			
17	16+2	26			*	*				
18	15+4	28		*		*		*		*

* *positive results*

Results

Among 410 pregnant women, we found 21 patients (5.1 %) positive for ACA IgG (>20 GPL/ml) and 30 patients (7.3 %) positive for ACA IgM (>10 MPL/ml). The serum samples were divided on the basis of ACA results into 4 groups:

ACA IgG positive patients (group 1, n=21)
ACA IgM positive patients (group 2, n=30)
ACA IgG negative patients (group 3, n=389)
ACA IgM negative patients (group 4, n=380).

We found that 7.8 % of the pregnant subjects had at least one high-titer IgG APA and 9.8 % high-titer IgM APA. Tables 1 and 2 summarize the presence of APA with various phospholipid specificity in groups 1 and 2. As can be seen, 28.6 % of patients in group 1 were also positive for APS, 28.6 % were positive or moderately positive for API, 23.8 % for APC and 19 % for APE. In the IgM ACA positive group of pregnant women (group 2), 33.3 % had higher titers of APS, 26.7 % of APE, 26.7 % of API and 23 % of APC. One third of ACA IgG or IgM positive sera contained polyspecific autoantibodies reactive to at least two various phospholipids. The incidence of IgG and IgM APS, APE, API, APC in the group of IgG and IgM ACA negative patients (groups 3 and 4) is given in Table 3. IgG and IgM ACA negative patients demonstrated a significantly lower incidence of APS, APE, API and APC than did the group of ACA positive pregnant women (Tables 4 and 5).

Table 4. Comparison of APA IgG with various phospholipid specificity in women ACA IgG positive (group 1) and ACA IgG negative (group 3).

	ACA IgG positive (n =21)	ACA IgG negative (n=389)	p
APS IgG positive	6	4	< 0.0001*
APE IgG positive	4	5	0.0005*
API IgG positive	6	4	< 0.0001*
APC IgG positive	5	3	< 0.0001*

** Fisher's exact test - highly significant*

Table 5. Comparison of APA IgM with various phospholipid specificity in ACA IgM positive (group 2) and ACA IgM negative (group 4) women.

	ACA IgM positive (n =30)	ACA IgM negative (n=380)	p
APS IgM positive	10	4	< 0.0001*
APE IgM positive	8	6	< 0.0001*
API IgM positive	8	4	< 0.0001*
APC IgM positive	7	3	< 0.0001*

** Fisher's exact test - highly significant*

Discussion

Antiphospholipid antibodies are an extremely heterogeneous group of autoantibodies and may be associated with the clinical disorders. The spectrum of diseases related to APAs leads to the description of the antiphosholipid antibody syndrome, characterized by the presence of arterial and venous thrombosis, autoimmune thrombocytopenia, fetal loss and moderate to high levels of APAs (Hughes *et al.* 1986).

Antiphospholipid antibodies include anticardiolipin antibodies, the lupus anticoagulant and antibodies to other phospholipids such as phosphatidylserine, phosphatidylinositol, phospatidyl-ethanolamine, phosphatidylcholine and antibodies to some phospholipid binding proteins. Gharavi *et al.* (1987) found that IgG, IgM and IgA ACAs bound the negatively charged phospholipids – phosphatidylserine and phosphatidylinositol, but not the zwitterionic phospholipid phosphatidylcholine. Phosphatidylserine is a negatively charged phospholipid, which is located at the inner side of plasma membranes, but it is also found on the outer surface of plasma membranes of platelets and endothelial cells (McNeil *et al.* 1991). Phosphatidylserine has a role in clot formation and participates in the coagulation cascade (Bevers *et al.* 1982). Triplett *et al.* (1988) detected a higher incidence of complications in APS positive patients, although it did not reach statistical significance.

API was observed to be the main marker of the antiphospholipid syndrome in some patients. It has been reported that the antibodies against phosphatidylinositol may be associated with thrombocytopenia in patients with systemic lupus erythematosus (Falcon *et al.* 1990). Autoantibodies against zwitterionic phospholipids have not been studied intensively in the course of various diseases. Antibodies reacting with phosphatidylethanolamine are known. APE has been found in patients with thrombotic disease and has even been reported as the sole APA in serum of some patients (Boffa *et al.* 1996). These antibodies are of interest because phosphatidyl-ethanolamine is a major component of both the outer and inner leaflet of plasma membranes. Cabral *et al.* (1990) described a patient with the antiphospholipid syndrome and hemolytic anemia, who had high serum titers of IgM antiphosphatidylcholine antibodies that cross-reacted with cardiolipin.

The presence of antiphospholipid antibodies (APAs), especially anticardiolipin antibodies (ACAs) has been widely reported as indicators of risk pregnancies. Many studies reported the occurrence of APAs in women with recurrent abortion and other pregnant complications (Brown 1991, Out *et al.* 1991, Matzner *et al.* 1994).

Matzner *et al.* (1994) studied APAs against various phopholipids in the serum of 352 women with recurrent pregnancy losses. Of these women, 59.1 % had either IgG or IgM antibodies to one of the six phospholipids – cardiolipin, phosphatidylserine, phospho-glycerol, phosphatidylethanolamine, phosphatidic acid or phosphatidylinositol, compared to only 4.6 % in the control group. The most common phospholipid epitope was phosphatidylserine. However, phosphatidyl-ethanolamine was the most common epitope in patients with antibodies to only one phospholipid.

Branch *et al* (1997) found that APAs other than lupus anticoagulant and anticardiolipin but including antibodies against phosphatidylserine, phosphatidyl-inositol and phosphatic acid are not independently

associated with recurrent pregnancy loss and therefore have no clinical significance. However, Aoki *et al.* (1995) concluded that a broadly based panel of conventional antiphospholipid antibodies, especially if it includes anticardiolipin, antiphosphatidylserine and antiphosphatidylinositol, appears to be suitable for the detection of autoantibody-associated conditions of reproductive failure. The combination of at least two conventional antibodies such as anticardiolipin, antiphosphatidylserine or antiphosphatidylinositol, as assayed by the standard ELISA method, disclosed a significant difference between suspected reproductive autoimmune failure syndrome and control group. APAs were also studied in the group of infertile patients. It was demonstrated that in these women, serum levels of ACA, APS, and APC were significantly higher than the corresponding values of the control group (Fisch *et al.*1995).

In our study we found sera positive for ACA IgG and IgM in 5.1 % and 7.3 %, respectively. Our finding is in agreement with most studies which have shown that the prevalence of ACA IgG or ACA IgM in the serum of pregnant women is less than 6 %, ranging from 1 % to 5.15 % (Lynch *et al.* 1997). We found that 7.8 % of pregnant women had at least one positive APA IgG and 9.8 % positive APA IgM. One third of ACA positive sera contained polyspecific autoantibodies reactive with at least two various phospholipids, but some antibodies were monospecific. Similarly, Lynch *et al.* (1997) reported that 24.4 % of pregnant females had an abnormal APA level. The findings in our patients show the heterogeneity of APA.

The occurrence of antiphospholipid antibodies binding to phospholipids other than cardiolipin was significantly lower in the group of ACA negative patients than in the ACA positive group. It remains to be clarified if the routine examination of APA reacting with anionic and zwitterionic antigens other than cardiolipin would improve the ability to identify patients with adverse pregnancy prognosis. Further studies aiming to elucidate the association between the levels of fetoplacental antigens and the occurrence of APAs are currently in progress in our laboratory.

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References

AOKI K, DUDKIEWICZ AB, MATSUURA E, NOVOTNY M, KABERLEIN G, GLEICHER N: Clinical significance of beta-2-glycoprotein I-dependent anticardiolipin antibodies in the reproductive autoimmune failure syndrome: correlation with conventional antiphospholipid antibody detection systems. *Am J Obstet Gynecol* **172:** 926-931, 1995.

BEVERS E, COMFRIUS P, VAN RIJN J, HEMKER C, ZWAAL R: Generation of prothrombin-converting activity and exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* **122:** 429-436, 1982.

BOFFA MC., BERARD M, SUGI T, MCINTYRE JA: Antiphosphatidylethanolamine antibodies as the only antiphospholipid antibodies detected by ELISA. II. Kininogen reactivity. *J Rheumatol* **23:** 1375-1379, 1996.

BRANCH DW, SILVER R, PIERANGELI S, VAN LEEUWEN I, HARRIS EN: Antiphospholipid antibodies other than lupus anticoagulant and anticardiolipin antibodies in women with recurrent pregnancy loss, fertile controls, and antiphospholipid syndrome. *Obstet Gynecol* **89:** 549-555,1997.

BROWN HL: Antiphospholipid antibodies and recurrent pregnancy loss. *Clin Obstet Gynecol* **34:** 17-26, 1991.

CABRAL AR, CABIEDES J, ALARCON-SEGOVIA D: Haemolytic anemia related to an IgM autoantibody to phosphatidylcholine that binds in vitro to stored and to bromelain-treated human erythrocytes. *J Autoimmun* **3:** 773-787, 1990.

FALCON, CR., HOFFER AM, CARRERAS LO: Antiphosphatidylinositol antibodies as markers of the antiphospholipid syndrome. *Thromb Haemost* **63:** 321-322, 1990.

FAUX JA, BYRON MA, CHAPEL HM: Clinical relevance of specific IgG antibodies to cardiolipin. *Lancet* **2:** 1457-1458, 1989.

FISCH B, FRIED S, MANOR Y, OVADIA J, WITZ IP, YRON I: Increased antiphospholipid antibody activity in in vitro fertilization patients is not treatment-dependent but rather an inherent characteristic of the infertile state. *Am J Reprod Immunol* **34:** 370-374, 1995.

GHARAVI AE, HARRIS EN, ASHERSON RA, HUGHES GRV: Anticardiolipin antibodies: isotype distribution and phospholipid specificity. *Ann Rheum Dis* **46:** 1-6, 1987.

HARRIS EN: Antiphospholipid antibodies. *Br J Haematol* **74:** 1-9, 1990.

HARRIS EN, GHARAVI AE, PATEL SP, HUGHES GRV: Evaluation of the anticardiolipin antibody test. *Clin Exp Immunol* **68:** 215-222, 1987.

HUGHES GRV, HARRIS EN, GHARAVI AE: The anticardiolipin syndrome. *J Rheumatol* **13:** 486-489, 1986.

LYNCH A, MARLAR R, MURPHY J, DAVILA G, SANTOS M, RUTLEDGE J, EMLEN W: Antiphospholipid antibodies in predicting adverse pregnancy outcome: a prospective study. *Ann Intern Med* **120:** 410-475, 1994.

LYNCH A, SILVER R, EMLEN W: Antiphospholipid antibodies in healthy pregnant women. *Rheum Dis Clin North Amer* **23:** 55-70, 1997.

MATZNER WM., CHONG P, GUANGHUI X, CHING W: Characterization of antiphospholipid antibodies in women with recurrent spontaneous abortions. *J Reprod Med* **39:** 27-30, 1994.

MCNEIL HP, CHESTERMAN CN, KRILLIS SA: Immunology and clinical importance of anticardiolipin antibodies. *Adv Immunol* **49:** 193-280, 1991.

OUT HJ, BRUINSE HW, CHRISTIAENS GC, VAN VLIET M, MEILOF JF, DE GROOT PG, SMEENK RJ, DERKSEN RH: Prevalence of antiphospholipid antibodies in patients with fetal loss. *Ann Rheum Dis* **50:** 553-557, 1991.

TRIPLETT D, BRAND J, MUSGRAVE K, ORR C: The relationship between lupus anticoagulants and antibodies to phospholipid. *JAMA* **259:** 550-554, 1988.

ZIMA T, FIALOVÁ L, MIKULÍKOVÁ L, MALBOHAN IM, POPOV P, NEŠPOR K: Antibodies against phospholipids and oxidized LDL in alcoholic patients. *Physiol Res* **47:** 351-355, 1998.

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