Antiphospholipid Antibodies Associated with Implantation Failure After IVF/ET

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Purpose: Our purpose was to determine the specific antiphospholipid antibodies (APAs) that should be evaluated to identify individuals at risk for implantation failure associated with reproductive autoimmune failure syndrome (RAFS).

Methods: The prevalence of APAs among 312 women with implantation failure was compared with that of 100 fertile control women. To be included in the implantation failure group, each woman had to have had at least 12 embryos transferred without subsequent positive pregnancy test. Enzyme-linked immunosorbent assay was used to measure IgG, IgM, and IgA anticardiolipin, antiphosphatidyl ethanolamine, antiphosphatidyl inositol, antiphosphatidic acid, antiphosphatidyl glycerol, antiphosphatidyl choline, and antiphosphatidyl serine.

Results: When the values for each of the seven APAs in three isotypes were compared between women with implantation failure and the control population, all of the APAs tested had a significantly higher frequency among women with implantation failure. Positive APAs were detected in 69 (22%) of the 312 women with implantation failure compared with 5 (5%) of the 100 control women ($P < 0.0001$). Anticardiolipin antibodies were found in 13 (4%) of the 312 women with implantation failure and none of the controls. Fifty-six (18%) of the 312 with implantation failure were negative for anticardiolipin antibodies but had positive values of other APAs.

Conclusions: A complete APA panel using seven isotypes is necessary for diagnosing implantation failure associated with RAFS. If only anticardiolipin antibody is measured, 4% (13/312) of the positive APAs are detected, and 81% (56/69) of women with implantation failure associated with RAFS will have the diagnosis missed.

KEY WORDS: antiphospholipid antibodies; implantation failure; in vitro fertilization; embryo transfer.

INTRODUCTION

Antiphospholipid antibodies (APAs) have been widely described in association with recurrent pregnancy loss (1–12) since the first report in 1975 of association with APAs and adverse pregnancy outcome (13). A decade later, Gleicher *et al.* (12) defined the association of APAs with not only recurrent pregnancy loss but also unexplained infertility and endometriosis. The presence of APAs in women who experience recurrent pregnancy loss, unexplained infertility, or endometriosis allows the diagnosis of reproductive autoimmune failure syndrome (RAFS) (14). More recently, several investigators have reported an increased frequency of APAs among women undergoing in vitro fertilization (IVF) for treatment of infertility (15–28). However, controversy has developed regarding the significance of the increased APAs and outcome of the IVF cycle (17–21,24–28). Explanations for the controversial results include differences in populations of women studied, differences in methodology of performing APA testing, and differences in interpretation of results.

To address one of the questions of methodology and interpretation of performing the APA assay, the present study was performed. Even though other autoimmune diseases have been shown to involve polyclonal rather than monoclonal B cell activation (29), most studies of RAFS associated with IVF have included immunoglobulin G (IgG) and IgM antibodies to cardiolipin alone or cardiolipin and phosphatidyl serine (16–18,21,22,24,28). Several investigators have advocated the use of a panel of APAs to screen for RAFS (5–8,12). This panel of tests includes not only anticardiolipin but also antiphosphatidyl ethanolamine, antiphosphatidyl

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inositol, antiphosphatidyl acid, antiphosphatidyl glycerol, antiphosphatidyl choline (5), and antiphosphatidyl serine (6–8,12). To evaluate specific APAs that should be tested to identify individuals at risk for implantation failure associated with RAFS after IVF/ET, sera from 312 women were studied for the prevalence of seven APAs and the results were compared with those for 100 women with no reproductive problems.

MATERIALS AND METHODS

Sera from 312 women with a history of failure of implantation after IVF/embryo transfer (ET) were evaluated for the presence of elevated APAs. A reproductive history was obtained from each of the women and the number of years of infertility, the cause of infertility, and the number of previous clinical, ectopic, and biochemical pregnancies were recorded. The numbers of oocytes retrieved and fertilized, and of embryos transferred during previous IVF/ET attempts, were noted. An implantation failure was defined as no signs of implantation after transfer of 12 or more embryos (95th percentile for women achieving pregnancy after IVF/ET) (30) or the occurrence of two or more pregnancy losses during previous transfer attempts. All women had unexplained IVF/ET failure without obvious ovulatory, male, or uterine factors.

Antiphospholipid Antibodies

Antiphospholipid antibodies (IgG, IgM, and IgA) were detected by ELISA against seven phospholipids—cardiolipin (CL), phosphatidic acid (PA), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl glycerol (PG), and phosphatidyl choline (PC) (all from Sigma Chemical)—as described previously (5). Briefly, all phospholipids were dissolved in methanol:chloroform (3:1) at a final concentration of 50 μ l/ml, and 30 μ l was applied to each well. The ELISA plates were dried in an O₂-free chamber and blocked by 100 μ l per well of 10% adult bovine serum (ABS) in PBS for 1 hr. After being washed three times with phosphate-buffered saline (PBS), 50 μ l of patient sera diluted 1:100 in adult bovine serum (ABS; 1:10) was added in triplicates and incubated for 1 hr. After washing times with PBS buffer, 50 μ l of goat anti-human IgG, IgM, or IgA, alkaline phosphatase-conjugated antibodies were added and incubated for 1 hr. The plates were washed three times, and *p*-nitrophenyl phosphate in diethano-

lamine buffer was used as a developer. The reaction was stopped with 3 *M* NaOH, and the color optical density was detected with a Bio-Kinetics EL312e ELISA reader. Each plated contained negative and positive controls. The threshold for a positive result for each phospholipid and each isotype was established based on results from 100 healthy male and female individuals.

Statistical Analysis

Statistical analyses were performed using the two-tailed Fisher's exact test and chi-square analysis. A significant difference was *P* less than 0.05.

RESULTS

Overall, 69 (22%) of the 312 women experiencing implantation failure were positive for APAs compared with 5 (5%) of the 100 control women (*P* < 0.0001). These 69 women had 166 positive APAs. The frequencies of multiple APAs measured in an individual are shown in Table I. The prevalence of each antibody to antiphospholipid among the 312 women experiencing implantation failure is shown in Fig. 1. Among the 312 women experiencing implantation failure, 13 (4%) had anticardiolipin and 16 (5%) had antiphosphatidyl serine antibodies (Fig. 2). Fifty-six (18%) of the 312 women were negative for anticardiolipin antibodies but had positive values for other APAs. Among the 166 positive APAs, 13 (8%) were anticardiolipin and 16 (10%) were antiphosphatidyl serine antibodies. The prevalence of anticardiolipin antibodies was 19% (13/69) and that of antiphosphatidyl serine antibodies was 23% (16/69) among women with positive antibodies.

The proportions of IgG, IgM, and IgA isotypes among the 166 positive APAs are illustrated in Fig. 2.

Table I. Frequency of Women with One or More Positive APA Among 312 Women Experiencing Implantation Failure After IVF/ET

No. of positive tests	No. of women	% (<i>n</i> = 312)
At least 1	69	22
At least 2	27	9
At least 3	14	4
At least 4	7	2
At least 5	6	2
At least 6	4	1
At least 7	1	0.3

Fig. 1. Frequency of specific antiphospholipid antibodies among 312 women experiencing implantation failure after IVF/ET. CL, cardiolipin; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PA, phosphatidic acid; PG, phosphatidyl glycerol; PC, phosphatidyl choline; PS, phosphatidyl serine.

Fig. 2. Proportion of IgG, IgM, and IgA isotypes among 312 women experiencing implantation failure after IVF/ET who had 50 positive antiphospholipid antibodies.

IgG antibodies comprised 44% (73/166), IgM 33% (54/166), and IgA 23% (39/166).

DISCUSSION

In this study of 312 women experiencing implantation failure after IVF/ET, 22% had positive antiphospholipid antibodies. This prevalence is consistent with the frequency of antiphospholipid antibodies reported in women undergoing IVF cycles (17–20,24–28) as well as in those in whom IVF treatment failed (23,24) and is significantly higher than that in normal pregnant (31) and nonpregnant (Fig. 1) (32) controls. Although increased antibody prevalence has been uniformly demonstrated in all studies of women undergoing IVF, the magnitude of increase varies (Table II). Sher *et al.* (19) found a frequency of 45 to 66% among women with organic pelvic pathology and 14% of women undergoing IVF for an indication of male factor. Organic pelvic pathology included endometriosis, a history of pelvic inflammatory disease, and pelvic adhesions. Others have found no difference in the prev-

alence of autoantibodies among women with tubal-factor, male-factor, and unexplained infertility (28). The explanation for the difference in these findings is not clear but could be explained by differences in patient populations studied or by different methods used to detect or interpret the APAs assays.

The increase in the prevalence of APAs in the current study compared with that in some of the previous studies (Table III) could reflect the method of patient selection. The current study selected women who had not become pregnant after 12 embryos had been transferred. Most of the previous studies (17–21,24–28) evaluated all women undergoing IVF treatment. One of the reports describing the prevalence of APAs among patients with implantation failures used the definition of three embryo transfers (24). In this study, only anticardiolipin and antiphosphatidyl serine antibodies were investigated (24).

Different assays have been used to detect APAs. Published studies are, therefore, difficult to compare because they report different autoantibody profiles and have different definitions of a positive result. Enlarging the APA panel could account for some of the differences by increasing the prevalence of antibodies detected (Table II). Eighteen percent of women experiencing implantation failure after IVF/ET were negative for anticardiolipin antibodies but positive for at least one of the other APAs. This value of 18% correlates with 10% among women with a history of recurrent pregnancy loss (33). If only anticardiolipin antibodies were tested, 4% of women experiencing implantation failure after IVF/ET with positive APAs would be included. And if antiphosphatidyl serine was added to anticardiolipin antibodies, only 14% of women with positive antibodies would be detected. Why would women with RAFS be expected to have only anticardiolipin or antiphosphatidyl serine antibodies?

The prototype of autoimmune disease, systemic lupus erythematosus, is characterized by a large variety of autoantibody abnormalities, not just anticardiolipin antibodies (34). In most autoimmune diseases, the resultant B cell abnormality is so broad that the term polyclonal B cell activation has been used to describe the variety of autoantibody abnormalities (29). There is no reason to suggest that RAFS is different from other autoimmune diseases with regard to polyclonal B cell activation. In fact, a large body of literature has accumulated suggesting a broad B cell abnormality in these women. In women who experience recurrent pregnancy loss, an increased prevalence of antiphospholipid, antinuclear (1,2,12), antipolynucleotides (12,14), and antihistones (12,14) has been reported.

Table II. Summary of the Prevalence of APAs Among Women Undergoing IVF/ET Reported in the Literature

Ref. No.	N	% APA		No. of phospholipids tested	IVF failure only
		Pt.	Control		
18	21	14	0	1	No
24	240	15	1	2	No
28	570	18	5	2	No
26	191	19	5	5	No
15	26	40	2	6	No
19	383	53	17	6	No
20	105	28	2	6	No
25	90	48	3	6	No
17	139	30	0	1	Yes
23	42	26	5	7	Yes
Current study	312	22	5	7	Yes

The strategy of evaluating only anticardiolipin antibodies in women experiencing recurrent pregnancy loss carries the obvious danger that patients with reproductive autoimmune failure associated with other (than cardiolipin) autoantibody abnormalities are overlooked. The evaluation of other phospholipid antibodies (in addition to anticardiolipin antibodies), therefore, makes sense.

The mechanisms by which APAs contribute to implantation failure and pregnancy loss are of interest. Proposed theories include inhibition of prostacycline generation by vascular tissues with resultant thromboxane predominance, increased platelet activation, and decreased activation of antithrombotic action of placental vessels, resulting in placental insufficiency (35–37). All of these actions lead to thrombosis of placental vessels resulting in placental insufficiency (35–37). More recent evidence suggests that direct binding of APAs to the trophoblast may inhibit cytotrophoblastic conversion to syncytiotrophoblast (36,38), trophoblastic invasion (12,36,38), hCG production (38), and implantation (39). To have a biological effect the APAs must

bind to its antigen. The predominant phospholipid on the cell surface is phosphatidyl choline; other principal phospholipid components of plasma membranes include phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and phosphatidyl glycerol (40–42). Cardiolipin has restricted, if not exclusive distribution to the inner mitochondrial membrane (43). Cardiolipin is not accessible on the cell membrane of trophoblast and therefore would not be expected to affect its biological activity. The most frequently positive APA among women experiencing implantation failure is antiphosphatidyl choline, followed by phosphatidyl glycerol, phosphatidyl ethanolamine, and phosphatidyl inositol (Fig. 1). Studies that did not include these phospholipids (16–22,24–28) would likely show no effect on implantation or pregnancy outcome after IVF/ET.

While two studies (17,19) have shown an adverse effect of the presence of APAs on pregnancy rates after IVF/ET, a number of studies (20,24–26,28) have shown an increase in the frequency of APAs among women undergoing IVF but no correlation between the presence of APAs and the implantation or pregnancy outcome after ET (Table III). Kowalik *et al.* (28) evaluated only anticardiolipin and antiphosphatidyl serine antibodies, which represent 14% of the positive APAs in the present study (Fig. 1). Gleicher *et al.* (20) and Kutteh *et al.* (26) studied six and five APAs, both not testing for antiphosphatidyl choline, the most frequent APA in the current study. Denis *et al.* (27), however, showed no effect of APAs on pregnancy outcome after IVF/ET when all seven phospholipid antibodies were assayed. Therefore an explanation other than the specific antiphospholipid responsible for implantation failure must be sought.

Table III. Summary of Reports of Pregnancy Rates After IVF/ET in Women with (+) and Without (–) APAs Who Did or Did Not Receive Heparin and Aspirin Treatment (H/A)

Ref. No.	APA (+)		APA (–)		P value
	H/A+	H/A–	H/A+	H/A–	
26	53	47	75	50	NS
25	51	—	—	50	NS
19	47	15	—	27	<0.05
28	—	59	—	58	NS
24	—	40	—	28	NS
20	—	50	—	48	NS

Annexin V binds to the trophoblastic cell surface and acts as an anticoagulant by preventing the formation of the prothrombinase complex and inhibiting procoagulant activity (44,45). The expression of annexin V on the trophoblast surface is affected by the presence of APAs (38,44,45). In pregnancies complicated by APAs, less annexin V is bound to the surface of the placenta (44). Rote *et al.* (38) have shown that APAs can remove annexin V from the trophoblast surface and increase the binding of prothrombin (46). Annexin V binds to human preembryos in vitro (47). The presence of APAs could remove annexin V binding from preembryos and prevent normal implantation. APAs are not present in media used for embryo culture during IVF. It is possible that the IVF procedure itself obviates the effect of APAs on implantation by protecting the preembryos from the effects of APAs. We have previously shown that APAs recognize fertilized eggs and have a detrimental effect on preembryonic development in vitro (48). That pregnancy loss rates after IVF/ET are also unaffected by the presence of APAs can be explained by the high prevalence of chromosomal abnormalities among these abortuses (49).

The diagnosis of an autoimmune disease is based on a combination of clinical and laboratory findings. For example, the diagnosis of SLE is based on a list of clinical and laboratory criteria established through revised American Rheumatism Association classification (50). These criteria take into account the polyclonal nature of autoantibody abnormalities in SLE. Reproductive autoimmune failure syndrome has also been defined based on both clinical and laboratory criteria (14). These include one clinical criterion (recurrent pregnancy loss, unexplained infertility, or endometriosis) and one laboratory criterion (positive lupus anticoagulant or positive APA). If only anticardiolipin antibody is measured, 4% of the positive APAs are detected and 81% of women with implantation failure associated with RAFS will have the diagnosis missed.

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