

Antibodies to phosphatidylethanolamine and phosphatidylserine are associated with increased natural killer cell activity in non-male factor infertility patients

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Antiphospholipid antibodies (APA) have been identified in patients with recurrent pregnancy loss and IVF failure. Of these, antiphosphatidylethanolamine (aPE) and antiphosphatidylserine (aPS) may have special significance. A link between increased natural killer cell activity (NKa+) and trophoblast cell apoptosis has also been reported. This study was undertaken to determine how the APA profile was associated with peripheral NK cell activity. We evaluated 197 female WIT candidates for APA and NKa. Eighty-nine patients (45%) were APA+ and of these, 51 (57%) were aPE/aPS+. Fifty-four patients (27%) had increased NK cell activity. Some 51% of APA+ and 78% of aPE/ aPS+ patients had increased NK cell activity compared with 8 % and 13 % when APA and aPE/aPS tested negative respectively (P < 0.0001). Non-male factor infertility patients were APA+ and NKa+ in 57% and 34% of cases respectively, compared with 19 % and 13 % if a pure male factor was present. Some 88% of aPE/aPS+, non-male factor patients had increased NK cell activity, compared with 12% who tested aPE/aPS negative (P < 0.0001) and 25% of aPE/aPS+, isolated male factor patients (P < 0.0001). These findings establish a direct relationship between APA (specifically aPE/aPS) and increased peripheral NK cell activity among non-male factor infertility patients. It is possible that APA do not directly cause reproductive failure but rather function as markers or intermediaries for an underlying, abnormal activation of cellular immunity.

Key words: antibodies/in-vitro fertilization/natural killer cells/phosphatidylethanolamine/phosphatidylserine

Introduction

Numerous investigators have shown an increased prevalence of antiphospholipid antibodies (APA) among infertile women. However, the exact relationship between APA and infertility

in general, and IVF specifically, remains an enigma (Coulam, 1999). Four studies suggest that APA exert an adverse influence on IVF outcome (Birkenfeld *et al.*, 1994; Geva *et al.*, 1994; Sher *et al.*, 1994, 1998a; Dmowski *et al.*, 1995), while five others show no such relationship (Gleicher *et al.*, 1994; Birdsall *et al.*, 1996; Denis *et al.*, 1997; Kowalick *et al.*, 1997; Kutteh, 1997). Possible explanations for the discrepancies include: (i) absence of standardization in the assays used to measure APA; (ii) varying cut-off points used to define positive versus negative results; (iii) differences in the populations of patients studied; and (iv) the fact that IVF, by its very nature, involves so many sensitive and complex steps, as to render assessment of the influence of any single variable on outcome, virtually impossible.

The authors have previously reported a correlation between APA positivity and decreased IVF pregnancy rates in cases of organic female and unexplained infertility, which could not be established in cases of isolated male factor infertility (Sher *et al.*, 1994, 1998b). The IVF outcome in these patients was significantly improved through administration of mini-dose heparin/ aspirin (H/A) therapy (Sher *et al.*, 1994, 1998b). However we noted that, in contrast to other phospholipid epitopes, in the presence of IgG or IgM class antibodies against phosphatidylethanolamine (PE) and/or phosphatidylserine (PS), H/A therapy alone was not found to be beneficial (Sher *et al.*, 1998b). In these patients, the addition of empiric treatment with intravenous immunoglobulin G (IVIG) was able to improve outcome in a subsequent IVF cycle (Sher *et al.*, 1998a,b). The therapeutic role of IVIG for treating reproductive failure is controversial (Balasch *et al.*, 1996; Christiansen, 1998; Daya *et al.*, 1998; Stephenson *et al.*, 1998). However, proponents of its use for both immunological spontaneous abortion and IVF failure have suggested that a possible mechanism of action may be through down-regulation of NK cell cytotoxicity (activity), thereby converting a hostile Th1 endometrial milieu to a trophoblast-friendly Th2 environment (DePlacido *et al.*, 1994).

The present study had two objectives. The first was to evaluate the prevalence of APA and increased peripheral NK cell activity (NKa) in IVF candidates with organic female indications (i.e. endometriosis, pelvic adhesions) or unexplained infertility, compared with a similar group of patients with isolated male factor infertility. Second, given our previous experience of IVIG being beneficial for IVF outcome in aPE/ aPS+ patients, as well as its reported down-regulatory effect on NK cell activity, we attempted to evaluate the association of the presence of antibodies against these specific phospholipid epitopes with increased peripheral NK cell activity.

Materials and methods

Patient population

All patients evaluated between December 1998 and June 1999 for treatment with IVF-embryo transfer who were aged under 40 years, and had cycle day 3 FSH concentrations < 10 mIU/ml, were included in this retrospective analysis. All of these patients were screened for immunological abnormalities as part of a standard work-up. Indications for IVF treatment included male factor, endometriosis, pelvic adhesions and unexplained infertility with previous treatment failure. Patients with a male factor and other female factors were classified in the female factor group. Endometriosis patients encompassed all stages of disease, but were mainly stages I and II. Patients classified as unexplained infertility were documented by laparoscopy to have patent tubes, were free of pelvic adhesions and endometriosis, and had a normal uterine cavity by hysteroscopy or hysterosalpingography. They had normal ovulation, and there was no evidence of male factor or antisperm antibodies. Not all patients evaluated subsequently underwent treatment and therefore, no attempt was made in this study to correlate the presence of APA or NKa with IVF outcome.

Laboratory evaluation

Assays were performed by Reproductive Immunology Associates (Van Nuys, CA, USA) and by University Health Sciences Laboratory (Chicago, IL, USA). Patients were screened for the presence of antiphospholipid antibodies, using an enzyme-linked immunosorbent assay (ELISA) for IgM, IgG and IgA isotypes to six phospholipid epitopes [cardiolipin (CL), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylglycerol (PG) and phosphatidylinositol (PI)], as described previously in detail (Matzner *et al.*, 1994).

The control group for the APA assays consisted of 40 non-infertility patients, aged between 25 and 45 years, who had no history of clinical or subclinical autoimmune disease, or recurrent pregnancy loss. Using the central limit theorem, the sampling distribution of the sample mean was approximated by a normal probability distribution as the sample size became 'large' (defined as $n > 30$). Based upon this theorem, borderline positives were defined as >2 SD above the mean of normal controls, and positive values were defined as >3 SD above the mean for normal controls. As is standard in the field of rheumatology, and as defined by the American Society of Reproductive Immunology (Coulam *et al.*, 1999), a positive assay in the presence of the proper clinical history was used to define the autoimmune reproductive failure syndrome in these patients.

Each time an ELISA assay was performed, both known negative and positive controls were run simultaneously for each isotype of every epitope. Positive controls were obtained from APL Diagnostics (Louisville, KY, USA), and from serum samples in the radioimmunoassay laboratory that were consistently over 2.0 optical densities (OD). This was important to assess the performance of the antigen coated on each plate, the antibody conjugates, the pipetting technique, the washing method, the incubation times, the incubation temperatures and the substrate. Intra-assay variation was addressed by running each sample in duplicate with the final reported value being the average of the two. The inter-assay assay coefficient of variation was 2.12%.

The determination of natural killer cell function was performed by flow cytometry using a previously described technique (Kane *et al.*, 1996). Briefly, K562 cells were grown as stationary cultures at 37°C in 5% CO₂. The cells were subcultured for 3 days before the assay, to be certain that they were in log phase. Before use in the assay, cells were incubated with 10 μl of 30 mmol/l diiodoacetylcarboxyanine perchlorate (DiO) per ml for 20 min at 37°C, 5% CO₂. Effector cells

Table I. Patient distribution and incidence of antiphospholipid antibodies (APA) and increased natural killer cell activity (NKa) by diagnosis

	All patients	APA+	NKa+
All patients ^a	197	89 (45)	54 (27)
Pure male factor	63 (32)	12/63 (19)	8/63 (13)
Other diagnoses	134 (68)	77/134 (57) ^b	46/134 (34)
Endometriosis	54 (27)	35/54 (65)	24/54 (44)
Pelvic adhesions	55 (28)	31/55 (56)	15/55 (27)
Unexplained	25 (13)	11/25 (44)	7/25 (28)

^aMean age = 35.7 years.

^bP < 0.004 compared with male factor patients who were APA+.

Values in parentheses are percentages.

were isolated from the buffy coat of heparinized blood using the FicolHypaque centrifugation. Target cells at the standard concentration and effector cells at various dilutions (1:1, 1:2, 1:4, 1:8) were added to create effector/target ratios from 50:1 down to 6.25:1. A total of 130 μl of propidium iodide (PI) was added to the tubes, and the mixture was centrifuged for 30 s at 1000 g in order to pellet target, effector cells and PI. Either interleukin-2 (IL-2) or various concentrations of IVIG were added to the assay, and the mixture was incubated overnight at 37°C, 5% CO₂. Data were collected for analysis on the Becton-Dickinson FACScan flow cytometer, using the Consort30 (Becton-Dickinson Immunocytometry systems; BDIS) program and Lysis software (BDIS). The spontaneous lysis was subtracted from the actual lysis for each sample. Based upon the control population (noted above), increased NK activity was defined as > 10% killing, with increased killing activity in the presence of IL-2, and decreased activity of at least 50% from the natural state in the presence of IVIG.

Statistical methods

Data were placed in two-by-two tables. Analyses of differences within and between groups were performed using the chi-square and Fisher's exact tests for significance where appropriate. A P-value < 0.05 was considered statistically significant.

Results

During the study period, 197 patients were evaluated for the presence of APA and NK cell activity. In total, 89 patients (45%) were positive for APA, and 51 of these (57%) were positive for IgG or IgM antibodies against PEPS (Tables I and II). Fifty-four patients (27%) had increased NK cell activity (Table II). The mean patient age was 35.7 years. Isolated male factor was seen in 63 patients (32%), endometriosis in 54 (27%) and pelvic adhesions in 55 (28%), while 25 patients (13%) had unexplained infertility (Table 1). Some 65% (35/54) of patients with endometriosis were APA+, and 44% (24/54) also had increased NK cell activity (Table I). Among patients with pelvic adhesions and unexplained infertility, 56% (31/55) and 44% (11/25) were APA+, and 27% (15/55) and 28% (7/25) had increased NK cell activity respectively (Table I). Endometriosis was almost twice as likely to be associated with the presence of NK cell activity than with other diagnoses.

Forty-five of the 89 (51%) APA+ patients had increased NK cell activity compared with only 9/108 (8%) patients who tested APA negative (P < 0.0001) (Table II). Forty of 51 (78%) aPE/aPS+ patients had increased NK cell activity

Table II. Association of antiphospholipid (APA), antiphosphatidylethanolamine (aPE) and antiphosphatidylserine (aPS) antibodies with increased natural killer cell activity (NKa+) among male and female infertility patients

		NKa+ (n = 54)		NKa- (n = 143)	
		Male factor and unexplained (n = 8)	Female factor (n = 46) ^d	Male factor and unexplained (n = 55)	Female factor (n = 88)
APA+	Total (n = 89)	3 ^a	42 ^{a,c}	9	35 ^c
	aPE/aPS+ (n = 51)	2 ^b (2)	38 ^{b,e,f} (2)	6	5
	aPE/aPS- (n = 38)	1 [1]	4 (2) [1]	3	30
APA -	Total (n = 108)	5 (3)	4 (4)	46	53 (1)

^aP < 0.0001 compared with APA-, NKa+ patients.

^bP < 0.0001 compared with aPE/aPS-, NKa+ patients.

^cP < 0.0001 compared with APA+ male factor patients.

^dP < 0.002 compared with NKa+ male factor patients.

^eP < 0.0001 compared with aPE/aPS-, female factor patients.

^fP < 0.0001 compared with aPE/aPS+, male factor patients.

() = Patients with antithyroid antibodies.

{ } = Women who shared HLA DQ- α with partner

compared with 5/38 (13%) of patients who tested negative for aPE/aPS (P < 0.0001) (Table II). Some 57% (77/134) of patients with organic female or unexplained infertility were APA+, while only 19% (12/63) of patients with an isolated male factor were APA+ (P < 0.004) (Table I). In addition, 85% (46/54) of patients with increased NK cell activity had organic female infertility, compared with only 15% (8/54) with a pure male factor (P < 0.002) (Table 11). Some 88% (38/43) of aPE/aPS+, female-factor infertility patients had increased NK cell activity, compared with only 12% (4/34) who tested aPE/aPS negative (P < 0.0001) (Table 11) and 25% (2/8) of aPE/aPS+ patients with an isolated male factor (P < 0.0001) (Table II).

Discussion

Antiphospholipid antibodies are found in 5 to 17% of the general population (Matzner *et al.*, 1994). It is well known that APA are more prevalent in cases of recurrent miscarriage (Rote, 1996; Sugi *et al.*, 1999), pelvic diseases such as endometriosis (Gleicher *et al.*, 1987), chronic pelvic adhesions due to previous infection (Sher *et al.*, 1994), unexplained infertility (Roussev *et al.*, 1996), and with repeated IVF failure (Fisch *et al.*, 1991). In cases of organic pelvic disease the prevalence was ~50%, while with isolated male factor infertility, the prevalence of APA was ~15% (i.e. the same as in the general population) and had no effect on IVF outcome (Sher *et al.*, 1994). In the past, immunological reproductive failure has focused on the role of antiphospholipid antibodies, especially those directed against cardiolipin (CL) and lupus anticoagulant (LA), which have often been associated with recurrent early pregnancy loss.

The formation of APA against any specific epitope is influenced by its prevalence in a given cellular location. Antibodies to superficial cellular phospholipids (i.e. aPS and aPE) are highly prevalent, and when present alone are not likely to be associated with systemic disease, but may still be associated with subtle immunological defects affecting

implantation and early pregnancy. It is possible that less severe cellular damage is required to induce antibody production on the cell surface, and this might help to explain why aPS and aPE have not been identified by all assays.

Cardiolipin, on the other hand is predominantly located intracellularly, on the endomitochondrial membrane, rendering it less antigenic (Hatch, 1998). Accordingly, antibody formation would only be expected in cases of severe cellular insult, and could explain why aCL is more often identified with clinical disease states associated with the antiphospholipid antibody syndrome (APS). Measurement of LA is another common test of APA activity. However, LA is a conglomerate of many APA which, because of its lack of sensitivity and specificity, is limited in its clinical utility only to the diagnosis of overt autoimmune diseases such as systemic lupus erythematosus. We routinely screen patients at risk of APA for a panel of six phospholipid epitopes for each of the IgG, IgM and IgA isotypes.

We have previously reported that in the presence of APA, treatment with heparin and aspirin led to a two-fold increase in pregnancy rates from IVF among patients with organic female infertility (Sher *et al.*, 1994). However, in patients who failed to conceive in their first treatment cycle and who underwent a second IVF cycle, we noticed that similar treatment with H/A afforded no further improvement in outcome (Sher *et al.*, 1998b). Furthermore, when these patients were positive for IgG and/or IgM class antibodies against aPE/aPS, the empiric addition of IVIG was able to impact significantly on pregnancy rates in a third treatment cycle (Sher *et al.*, 1998b). This improvement in outcome with IVIG was not seen in the presence of other phospholipid antibodies.

Intravenous immunoglobulin is an anti-idiotypic that prevents antibody binding non-selectively. It is also an immunomodulator that down-regulates the activity of activated NK cells (Finberg *et al.*, 1992; Kwak *et al.*, 1996). The observations that aPE/aPS positivity in female infertility patients was associated with increased NK cell activity and decreased IVF success, while aPE/aPS antibodies in isolated male factor

infertility had no effect on IVF outcome and were not associated with NK cell activity, may argue against a direct cause-and-effect relationship between APA and poor reproductive outcome. Rather, it is likely that in some patients aPE/aPS act as markers or intermediaries for an underlying cellular immunity that may be typified by increased NK cell activity.

Natural killer cells are large granular lymphocytes that arise from the bone marrow and circulate peripherally before localizing to specific tissues. They are the most prevalent lymphocyte population in secretory endometrium and the decidua of early pregnancy (Starkey *et al.*, 1988), and are believed to play a role in regulating trophoblast invasion. Elevated NK cell activity in the peripheral blood may be a reflection of increased NK cell killing at the tissue level. Increased peripheral and endometrial NK cell activity has been demonstrated in IVF patients who experienced spontaneous pregnancy loss or IVF failure (Fukui *et al.*, 1999).

Endometrial NK cells are classically CD56⁺/16⁻, but can be readily activated by short-term exposure to cytokines such as IL-2 to become potent lymphokine-activated killer cells (LAK) (King *et al.*, 1996). These LAK (NKa) cells express the CD16⁺ marker and contain granules of Th1 cytokines such as tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) (Faust *et al.*, 1999). The release of Th1 cytokines is associated with increased decidual and trophoblast cell apoptosis, possibly through an alteration of the bcl-2:bax ratio (Lea *et al.*, 1999). Increased mean numbers of CD56⁺ cells have also been documented in the endometrium of women with recurrent early miscarriage (Clifford *et al.*, 1999; Fukui *et al.*, 1999).

Interestingly, we found that 44% of endometriosis patients had increased peripheral NK cell activity. This contrasts with previous reports that NK cell activity is decreased in the peritoneal fluid and peripheral blood of patients with endometriosis (Oosterlynck *et al.*, 1992; Ho *et al.*, 1997). This discrepancy may be explained in part by our patient population, which mainly had untreated, early-stage disease. Furthermore, we used flow cytometry to measure NK cell activity, rather than the method of detecting ⁵¹Cr release, as was done in most previous studies. In women with endometriosis, where NK cell concentrations may be decreased, increased NK cell cytotoxicity could easily be overlooked by methods that do not express NK cell activity as a percentage of target cells killed.

The immunological contribution to successful reproduction is a complex puzzle that is still being assembled, one piece at a time. However, the concept that immunological factors play an important role is undeniable. The results of this study confirmed our previously reported finding that isolated male factor is *not* associated with immunological infertility (Sher *et al.*, 1994). In addition, we demonstrated that APA (specifically IgG and/or IgM-class antibodies to PEPS) were associated with increased peripheral NK cell cytotoxicity in patients with non-male factor infertility. This suggests that APA, rather than being causally related to reproductive failure, may act as markers or intermediaries of an underlying abnormality of cellular immunity. At this point we are uncertain of the functional relationships between APA and increased NK cell

activity. However, we are convinced that NK cell activity plays a significant role in implantation. Future research should focus on whether APA directly affect Th 1 cytokine production, whether peripheral NK cell activity correlates immunohistochemically with NK cell activity in the endometrium, and whether down-regulation of NK cell activity can impact on IVF outcome.

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Received on December 13, 1999; accepted on May 25, 2000