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Immunological factors in endometriosis-associated reproductive failure: studies in fertile and infertile women with and without endometriosis

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Immunopathophysiological mechanisms in endometriosis-associated reproductive failure were studied in appropriate populations: infertile and fertile women with and without endometriosis. The incidence of sera positive for any of the autoantibodies tested among infertile women with endometriosis (*n* = 25) was similar to that observed in the three control groups [unexplained infertility patients (*n* = 25) and fertile women with (*n* = 10) and without (*n* = 25) endometriosis]. The mean volume of peritoneal fluid was significantly elevated in women with endometriosis (both fertile and infertile) as compared with patients without endometriosis (fertile or infertile). The concentration of peritoneal fluid leukocytes and the percentage of cells positive for macrophage markers were significantly increased and the percentage of T lymphocytes significantly decreased in infertile women with endometriosis but not in patients with unexplained infertility and fertile women with endometriosis, as compared with fertile controls without endometriosis. Macrophages from infertile patients with endometriosis had higher sperm phagocytosis than did those from infertile women without endometriosis or fertile subjects with or without endometriosis. Incidences of serum and peritoneal fluid samples embryotoxic to the in-vitro development of 2-cell mouse embryos were significantly higher in infertile patients with endometriosis than in unexplained infertility patients and fertile women with or without endometriosis. It is concluded that immunological mechanisms of endometriosis-associated infertility exist but that these peritoneal immunological factors in infertile women with endometriosis are related to their subfertility rather than to the presence of ectopic endometrial implants. This is supported by the lack of immunological abnormalities observed among fertile women with endometriosis. These immunological abnormalities are lacking in patients with unexplained infertility.

Key words: endometriosis/immunology/infertility/unexplained infertility

Introduction

Mechanical disruption of fertility due to the presence of structural damage and tubo-ovarian adhesions may explain reproductive failure in patients with moderate and severe endometriosis. In women with minimal and mild endometriosis, however, the aetiological basis for reduced fecundity is unclear (Hill, 1992a; Prentice and Ingamells, 1996). Numerous factors have been investigated to explain how mild endometriosis could affect fertility, but recently different studies have suggested that immunological mechanisms are involved in the pathophysiology of endometriosis and endometriosis-associated reproductive failure (Mahmood and Templeton, 1990; Christman and Halme, 1992; Hill, 1992a; Thomas and Prentice, 1992; Ramey and Archer, 1993; Berkova *et al.*, 1996; D'Hooghe and Hill, 1996; D'Hooghe *et al.*, 1996; Oral *et al.*, 1996; Prentice and Ingamells, 1996; Vinatier *et al.*, 1996). Thus, it has been postulated that both organ-specific and organ non-specific autoantibodies might affect implantation, and it is also likely that changes in peritoneal fluid might be related to the aetiology of infertility. This has been attributed to increased numbers of macrophages and cytokines that may interfere with reproduction at various levels, including gamete function, fertilization and embryo development, and implantation. However, in previous immunological studies, subfertile women with endometriosis at laparoscopy were compared with unexplained infertility patients or with fertile women without laparoscopic evidence of endometriosis.

At present, accumulating data suggest that in many women 'endometriosis' may be a paraphysiological condition, while in other patients 'the disease endometriosis' may have implications for their reproductive health and may produce symptoms (e.g. pelvic pain) (Vercellini and Crosignani, 1993; Koninckx, 1994; Thomas, 1994; Bergqvist, 1995; Balasch *et al.*, 1996a). Therefore, the present prospective study was undertaken to investigate serum and peritoneal immunological profiles in infertile women with minimal to mild endometriosis but using three different control groups: infertile women without endometriosis, fertile women with endometriosis, and fertile women without endometriosis. To the best of our knowledge, this kind of comparative study has not been previously reported.

Materials and methods

Patients

A total of 85 women aged <41 years were included in the present study. The experimental subjects were 25 primary infertile patients diagnosed as having endometriosis at laparoscopy (group 1). The 60 controls comprised the following three groups: 25 primary infertile patients with unexplained infertility (group 2), 10 healthy fertile women undergoing laparoscopy for tubal sterilization and having evidence of minimal/mild endometriosis elsewhere in the pelvis (group 3), and 25 healthy fertile women without laparoscopic evidence of endometriosis at tubal sterilization (group 4).

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The mean age was similar in the four groups (31.5 ± 3.7, 32.2 ± 3.3, 34.1 ± 2.5 and 34.8 ± 5.7 years respectively). The mean parity for patients in groups 3 and 4 was 2.4 (range 1–4) and 2.2 (range 1–3) respectively. All fertile women were asymptomatic and had delivered at term within one year of testing. Four (40%) patients in group 3 and 13 (52%) patients in group 4 breastfed for 1–3 months and all underwent laparoscopy >6 months later; one (10%) patient in group 3 and one patient (4%) in group 4 were still breastfeeding when tubal sterilization was performed 4–6 months after delivery (*P* was not significantly different between groups 3 and 4). No patient had been treated with hormonal therapy for endometriosis and none had received exogenous hormones for at least 3 months before laparoscopy. Infertility was defined as the inability to achieve pregnancy after a minimum of 1 year of unprotected intercourse. Unexplained infertility included patients with no discernible cause after complete infertility workup (n = 21; Balasch *et al.*, 1996b) or with unexplained failure to conceive following appropriate treatment such as donor insemination (n = 3) or bromocriptine for mild hyperprolactinaemia associated with regular menstrual cycles (n = 1).

A systematic laparoscopic evaluation of all pelvic peritoneal surfaces and structures was carried out in all patients during the midluteal phase, when peritoneal fluid volume and macrophage activation are higher (Drake *et al.*, 1980; Halme *et al.*, 1984). Endometriosis was staged according to the revised American Fertility Society (AFS, 1985) scoring. Superficial ovarian and peritoneal endometriosis was classified as recently suggested by Brosens *et al.* (1993). Biopsies of suspicious lesions were taken when the visual diagnosis of endometriosis was in doubt (six cases). Biopsy of the endoscopically suspected endometriosis revealed the presence of endometrial glands and stroma in five cases, while the remaining biopsy showed fibrosis with haemosiderin-laden macrophages. The latter case was considered as negative for endometriosis.

For the specific purpose of this study, which was approved by the Ethics Committee of the Hospital Clínic i Provincial, Barcelona, Spain, all the consecutive women in each study group were screened for the following: (i) autoimmune serological tests [antismooth muscle, antimitochondrial and antinuclear antibodies and antiphospholipid antibodies (aPL), i.e. lupus anticoagulant (LA) and anticardiolipin antibodies (aCL)]; (ii) serum embryotoxicity on 2-cell mouse embryo development *in vitro*; (iii) peritoneal fluid characteristics (volume, total leukocytes and profiles of leukocyte subpopulations) and (iv) effects of peritoneal fluid on in-vitro models of reproductive processes (sperm phagocytosis by peritoneal fluid macrophages and embryotoxic effect of cell-free heat-treated peritoneal fluid on in-vitro cleavage of 2-cell mouse embryos). In order to obtain a comprehensive immunological investigation, only patients for whom all of these immunological parameters could be appropriately evaluated were included in our study. Three cases in group 1, one in group 2, two in group 3, and two in group 4 were discarded because certain of the proposed experiments could not be performed for technical problems. Inclusion of these partial data, however, did not influence results.

Sample collection

Blood (50 ml) was obtained from each woman and serum samples were aliquoted and stored at –70°C. At laparoscopy and before placement of the patient in the Trendelenburg position, peritoneal fluid was aspirated from the anterior and posterior cul-de-sacs with a hollow canule. The peritoneal fluid was then placed in sterile heparinized tubes to avoid macrophage adherence to glass and immediately transported to the laboratory and its volume determined. After centrifugation at 600 *g* for 10 min the supernatant was removed, aliquoted and stored at –70°C until assay. The cell pellet was then resuspended in phosphate-buffered saline (PBS, 0.01 M, pH 7.2), layered onto a Ficoll-Hypaque gradient (Ficoll–sodium diatrizoate 1077; Sigma Chemical Co., St. Louis, MO, USA) and centrifuged at 600 *g* for 25 min to remove contaminating erythrocytes and cellular debris. The leukocyte band was isolated and washed twice in PBS. The leukocytes were then resuspended in 1 ml of PBS and counted under an optical microscope in a Neubauer chamber.

Table I. Monoclonal antibodies used to identify leukocyte subpopulations

Antibody	CD number	Specificity
Anti-HLe-1	45	all leukocytes
Leu 4	3	T lymphocytes
Leu®-12	19	B lymphocytes
Leu®-3a+3b	4	T-helper lymphocytes
Leu®-2a	8	T-suppressor/cytotoxic lymphocytes
Leu®-M3	14	macrophages

Autoantibody tests

Antismooth muscle, antimitochondrial and antinuclear antibodies were determined by direct immunofluorescence (Simón *et al.*, 1992) using rat stomach, rat kidney and rat liver as a substrate. Antiphospholipid antibodies were determined as described previously (Balasch *et al.*, 1990, 1996b).

Characterization of leukocyte subpopulations in the peritoneal fluid

The leukocyte subpopulations were characterized using monoclonal antibodies identifying selective cellular surface markers and flow cytometry. Indirect immunofluorescence measurements were carried out with the following monoclonal fluorescein-isothiocyanate labelled antibodies: Anti-HLe-1, Leu-4, Leu®-12, Leu®-3a+3b, Leu®-2a, and Leu®-M3 (Becton Dickinson, Mountain View, CA, USA). Their antigenic specificities are given in Table I. Immunofluorescence analysis was performed as previously described (Reinherz *et al.*, 1979). Flow cytometry was performed on a FACScan (Becton Dickinson Immunocytometry Systems) and analysis of data was made with the software Lysis II (Becton Dickinson Immunocytometry Systems) on a Hewlett-Packard computer. Calibration of the flow cytometer parameters for forward scatter, side scatter and fluorescence was always the same and analysis was done over 6000 cells in each event. The proportion of cells positive for each monoclonal antibody was expressed with reference to the proportion of CD45+ (all leukocytes) in order to avoid a bias for the potential presence of contaminating particles.

Sperm phagocytosis

Sperm phagocytosis assay was performed as previously reported (Awadalla *et al.*, 1987). Spermatozoa were obtained from a single fertile donor who participated in an artificial insemination programme and cryopreserved. Sperm motility was 65%. The spermatozoa were washed twice with Ham's F-10 medium (GIBCO, Grand Island, NY, USA) containing 10% fetal calf serum (FCS) and adjusted to a concentration of 5×10^6 spermatozoa/ml. A portion of the purified leukocytes of each patient was resuspended at a concentration of 2.25×10^6 cells in the same medium and 0.1 ml of this suspension was placed in a microtitre chamber (Microtest II; Falcon Plastics, Barcelona, Spain). The macrophages were allowed to adhere to the plastic for 1 h. Each well was then washed twice with Ham's F-10 medium to remove non-adherent cells, which consisted primarily of lymphocytes and non-viable macrophages, leaving a monolayer consisting of >95% macrophages. Next, 200 ml of sperm suspension (10^6 spermatozoa) was added to each of two macrophage-coated

chambers and to each of two empty chambers (controls). The plates were incubated for 24 h at 37°C in humidified 5% carbon dioxide/95% air. The supernatant media were carefully removed by pipetting and the spermatozoa counted in a Makler chamber. The percentage phagocytosis was expressed as: [(no. SR control – no. SR case)/no. SR control]×100, where 'no. SR' is the number of spermatozoa in the supernatant. All assays were done in duplicate. The intra-assay variability was <7%.

Mouse embryo assay

Mature female F_1 (C57B1/6J×CBa/Ca) mice (Facultad de Ciencias Biológicas de la Universidad Autónoma de Barcelona, Barcelona, Spain) were superovulated with 5 IU of pregnant mare serum gonadotrophin (study day 1) followed 48 h later by 5 IU of human chorionic gonadotrophin (HCG) (study day 3), given intraperitoneally. At the time of HCG injection, each female mouse was placed with a stud male and allowed to mate overnight. Mating was confirmed by the presence of a vaginal plug on study day 4. On day 5, 44 h after the HCG injection, the mice were killed by cervical dislocation, and the oviducts flushed with Ham's F-10 medium under a dissecting microscope. Normal 2-cell embryos selected from several mice were placed in a common pool and divided randomly into 15–20 embryo lots among the different medium preparations. Samples of serum and peritoneal fluid from both cases and controls were heat inactivated for 45 min at 56°C, sterilized using 0.2 μm filters (Millipore Co, Madrid, Spain), diluted to 15% concentration in Ham's F-10 medium and placed in duplicate in four-well Nunclon plates (Nunc, Delta, Denmark). The total volume in each well was 1 ml and the osmolality and pH were within physiological ranges. Four to five embryos were placed in each well and incubated in 5% CO_2 in air at 37°C for 72 h. Control embryos in Ham's F-10 alone were cultured simultaneously. Embryos were examined under the microscope at 24-h intervals. In Ham's F-10 medium, ≥50% of 2-cell embryos developed to the blastocyst stage by 72 h, which is used in in-vitro fertilization (IVF) programmes as a quality control for the medium. Thus, a serum or peritoneal fluid sample was defined as toxic if <50% of the embryos reached the blastocyst stage at 72 h (Damewood *et al.*, 1990; Simón *et al.*, 1992; Abu-Musa *et al.*, 1992a,b).

Statistical analysis

The χ^2-test with the Yates correction when necessary, Fisher's exact test and the Mann–Whitney *U*-test were used for statistical analysis, as appropriate.

Results

Table II shows the AFS stage and the laparoscopic appearances of the condition in the two groups of patients with endometriosis. The majority of women (68 and 70% in groups 1 and 3 respectively; not significantly different) had stage I according to the revised AFS (1985) classification of endometriosis. The incidence of the distinctive appearances of the lesions was similar in the two groups of patients (Table II).

Incidences of detection of autoantibodies in sera of patients in the four groups studied are presented in Table III. Antimitochondrial antibodies were detectable in none of the 85 subjects studied. Of the other autoantibodies tested, the incidence of positive sera among infertile women with endometriosis (group 1) was similar to that observed in the three control groups (unexplained infertility patients and fertile women with and without endometriosis).

Table II. American Fertility Society (AFS) stage and laparoscopic appearance of endometriosis in groups 1 and 3[a,b]

Endometriosis	No. (%) of patients at each stage	
	Group 1 (n = 25)	Group 3 (n = 10)
AFS stage[b]		
I	17 (68)	7 (70)
II	8 (32)	3 (30)
Laparoscopic appearance[b]		
Red lesion	7 (28)	2 (20)
Black lesion	22 (88)	9 (90)
White lesion	10 (40)	6 (60)

[a] Group 1 = infertile patients with endometriosis; group 3 = fertile women with endometriosis.
[b] P = not significant between the two groups for AFS stage or laparoscopic appearance.

As shown in Table IV, the mean volume of peritoneal fluid was significantly elevated in women with endometriosis (both fertile and infertile) as compared with patients without endometriosis (fertile or infertile). Peritoneal fluid leukocyte population data are presented in Table IV. The concentration of peritoneal fluid leukocytes was significantly higher in group 1 patients than in group 4. When leukocyte subpopulation profiles were examined, the percentage of cells positive for the macrophage markers was significantly increased and the percentage of T lymphocytes significantly decreased in infertile women with endometriosis, but not in women in groups 2 and 3, as compared with fertile controls without endometriosis (group 4). B lymphocyte, T-helper lymphocyte and T-suppressor/cytotoxic lymphocyte concentrations, as well as the T-helper-to-T-suppressor lymphocyte ratio were similar in the four groups studied (Table IV).

Results of sperm phagocytosis mediated by peritoneal macrophages from the four groups of patients are displayed in Table V. Macrophages from infertile patients with endometriosis (group 1) had higher sperm phagocytosis than did those from infertile women without endometriosis (group 2) or fertile subjects with or without endometriosis (groups 3 and 4). Incidences of serum and peritoneal fluid samples embryotoxic to the in-vitro development of 2-cell mouse embryos were significantly increased in group 1 versus groups 2, 3 and 4 (Table V).

Discussion

Endometriosis is one of the most important gynaecological disorders affecting the fertility potential of women. Estimates suggest that 25–50% of infertile women have evidence of endometriosis, and 30–50% of women with endometriosis are infertile (Mahmood and Templeton, 1990; Christman and Halme, 1992). Although the anatomical distortion often associated with advanced stages of endometriosis appears to be a major aetiological factor of infertility, in women with minimal to mild endometriosis and no apparent structural damage the pathogenesis of the infertility remains obscure.

Endometriosis is associated with a relative reduction in fertility potential, but not all women with endometriosis are infertile. In fact, whether minimal and mild endometriosis

Table III. Autoantibodies in sera from patients in the four groups studied. Values in parentheses are percentages

Patient group[a]	Autoantibody-positive women[b]						
	Antinuclear		Antismooth muscle		LA	aCL	Overall
	1/50[c]	>1/50[c]	1/50	>1/50			
1 (n = 25)	9 (36)	1 (4)	4 (16)	1 (4)	1 (4)	3 (8)	12 (48)
2 (n = 25)	2 (8)	3 (12)	4 (16)	1 (4)	0	0	8 (32)
3 (n = 10)	2 (20)	0	0	0	0	0	2 (20)
4 (n = 25)	8 (32)	1 (4)	3 (12)	0	1 (4)	1 (4)	10 (40)

LA = lupus anticoagulant; aCL = anticardiolipin antibodies.
[a] Group 1 = infertile patients with endometriosis; group 2 = unexplained infertility; group 3 = fertile women with endometriosis; group 4 = fertile women without endometriosis.
[b] No significant differences between groups.
[c] Values are sera dilution in phosphate-buffered saline.

Table IV. Peritoneal fluid volume, total leukocytes and leukocyte subpopulations in the four groups studied. Values are mean ± SEM

Peritoneal fluid variable	Groups of patients[a]			
	1 (n = 25)	2 (n = 25)	3 (n = 10)	4 (n = 25)
Volume (ml)	10.3 ± 0.59[b]	7.8 ± 0.63	9.8 ± 0.71[b]	7.6 ± 0.76
All leukocytes (10^3 cells/ml)	918 ± 115.98[c]	818 ± 163.01	735 ± 77.1	532 ± 64.0
T lymphocytes (% positive cells)	11.3 ± 1.42[c]	12.7 ± 2.06	12.0 ± 2.8	18.4 ± 2.34
B lymphocytes (% positive cells)	1.9 ± 0.34	2.2 ± 0.41	1.2 ± 0.23	2.1 ± 0.32
T-helper lymphocytes (% positive cells)	4.4 ± 0.76	5.8 ± 0.88	3.2 ± 1.11	6.7 ± 0.9
T-suppressor/cytotoxic lymphocytes (% positive cells)	4.1 ± 0.62	5.5 ± 1.24	4.2 ± 1.19	7.5 ± 1.32
T-helper/T-suppressor ratio	1.4 ± 0.27	1.6 ± 0.17	0.8 ± 0.17	1 ± 0.12
Macrophages (% positive cells)	61 ± 2.35[c]	53 ± 3.13	54 ± 4.3	48 ± 3.36

[a] Group 1 = infertile patients with endometriosis; group 2 = unexplained infertility; group 3 = fertile women with endometriosis; group 4 = fertile women without endometriosis.
[b]$P < 0.05$, when compared with groups 2 and 4.
[c]$P < 0.05$, when compared with group 4.

Table V. Percentage sperm phagocytosis by peritoneal macrophages and incidence of embryotoxic sera and peritoneal fluids in the four groups studied

Patient group[a]	% Spermatozoa phagocytosis[b]	Embryotoxic sera (n, %)	Embryotoxic peritoneal fluids (n, %)
1 (n = 25)	79.3 ± 2.95[c]	17 (68)[c]	21 (84)[c]
2 (n = 25)	62.8 ± 4.62	6 (24)	11 (44)
3 (n = 10)	59.1 ± 6.3	2 (20)	3 (33.3)
4 (n = 25)	53.7 ± 5.09	5(20)	6 (24)

[a]Group 1 = infertile patients with endometriosis; group 2 = unexplained infertility; group 3 = fertile women with endometriosis; group 4 = fertile women without endometriosis.
[b] Values are mean ± SEM.
[c]$P < 0.05$, when compared with groups 2, 3 and 4.

adversely affects fecundity remains controversial (Hill, 1992a; Thomas, 1994; Prentice and Ingamells, 1996). The origins of this controversy may lie in the assumption that all women with the same stage of endometriosis have the same disease. Endometriosis, however, may be a heterogeneous disease, and the mechanisms underlying reproductive failure in these women may likewise be heterogeneous (Hill, 1992a). Thus, in a recent report we found that the prevalence of endometriosis by revised AFS (1985) classification and the diagnosis of appearances of the condition were similar in asymptomatic infertile and fertile women (Balasch *et al.*, 1996a), which is in agreement with the findings in the present report. As discussed below, however, immunological studies showed some notable differences between infertile and fertile women with endometriosis.

There is substantial evidence that immunological factors play a role in the pathogenesis of endometriosis and endometriosis-associated reproductive failure. Both humoral and cellular immune changes have been reported in endometriosis, and adverse reproductive performance has been attributed to either type of immune change. Thus, a broad range of antibodies to cell components in patients with endometriosis has been reported but the results are controversial (Gleicher *et al.*, 1987; El-Roeiy and Gleicher, 1988; Kennedy *et al.*, 1989; Confino *et al.*, 1990; Kilpatrick *et al.*, 1991; Taylor *et al.*, 1991; Simón *et al.*, 1992). As recently stressed by D'Hooghe and Hill (1995), it is difficult to interpret the weight of these data, since most of these studies either lacked control patients (El-Roeiy *et al.*, 1988; Kilpatrick *et al.*, 1991) or used as controls infertile patients without endometriosis (Simón *et al.*, 1992) or 'healthy men and/or undefined healthy women', mostly blood donors, without previous laparoscopic exclusion of endometriosis (Gleicher *et al.*, 1987; El-Roeiy and Gleicher, 1988; Kennedy

et al., 1989; Taylor *et al.*, 1991). In their recent review on the subject, D'Hooghe and Hill (1995) concluded that 'current data show the presence of autoantibodies in patients with endometriosis, but do not offer sufficient proof that their prevalences are significantly higher than values in women with a normal pelvis or that they are correlated with infertility. Further studies are required to document prevalence and levels of autoantibodies in fertile and infertile women with a normal pelvis, and these patient groups must be used as controls when endometriosis patients are investigated'. A remarkable feature of our study is that, in addition to infertile women with a normal pelvis, fertile women both with and without endometriosis were used as controls. The present report, where the incidence of autoantibody-positive sera found among infertile women with endometriosis was similar to that found in the three control groups, adds further evidence against an endometriosis-associated autoimmune reproductive failure.

Peritoneal fluid represents a combined peritoneal and ovarian exudate, and its volume fluctuates normally throughout the menstrual cycle (for review, see Ramey and Archer, 1993; D'Hooghe and Hill, 1996). It has been reported that the mean volume of peritoneal fluid is elevated in infertile women with endometriosis compared with fertile women without the disease, and an association between subfertility and increased peritoneal fluid volume has been suggested (Ramey and Archer, 1993; D'Hooghe and Hill, 1996). Our study, in which peritoneal fluid volume was higher in women with endometriosis than in those without evidence of the disease irrespective of whether they were fertile or infertile, shows that an increased peritoneal fluid volume is associated with the presence of endometriosis but is unrelated to the fertility status of the woman. In addition, our results show that peritoneal fluid volume is not increased in patients with unexplained infertility.

There is compelling evidence for increased white blood cell populations in the peritoneal fluid of subfertile women with endometriosis as compared with fertile women without endometriosis (Hill, 1992a; D'Hooghe and Hill, 1996). The majority (50–70%) of peritoneal fluid cells are macrophages, and there is general agreement that the total number of peritoneal fluid macrophages is elevated in infertile women with endometriosis as compared with fertile women without the disease. Some authors have reported similar findings regarding T cells, CD4+ T-helper cells and the T4/T8 ratio (Hill, 1992a; Ramey and Archer, 1993; D'Hooghe and Hill, 1996). The present report confirms those previous studies with respect to the total number of peritoneal fluid leukocytes and macrophages but not T cells, B lymphocytes and T subpopulations. A striking feature of our study is the suggestion that these peritoneal immunological factors in infertile women with endometriosis are a function more of their subfertility than of the presence of ectopic endometrial implants, which is supported by the lack of immunological abnormalities observed in group 3 (fertile women with endometriosis). This is further stressed by experimental studies showing that peritoneal white blood cells and macrophage concentrations were elevated in baboons with spontaneous endometriosis as compared with primates with a normal pelvis or those having induced disease (D'Hooghe and Hill, 1996). In addition, our results disagree with previous studies reporting an increased number of total T cells, T-helper cells and macrophages in the peritoneal fluid from unexplained infertility patients as compared with fertile women with a normal pelvis (Hill *et al.*, 1988).

The observation that women with minimal and mild endometriosis are as successful following IVF as women with tubal disease (FIVNAT, 1993) favours the concept that early reproductive events in women with minimal and mild endometriosis may be adversely affected (e.g. sperm phagocytosis, impaired fertilization, embryotoxicity against early embryonic development; Hill, 1992a). However, subfertility persists even after embryo transfer in patients with endometriosis who undergo an IVF procedure. Hence, alterations in early embryonic development must be invoked (Damewood *et al.*, 1990; Simón *et al.*, 1992). In-vitro incubation of mouse embryos with heat-inactivated serum from patients with endometriosis has been found to impair development (Damewood *et al.*, 1990; Abu-Musa *et al.*, 1992b; Simón *et al.*, 1992) compared with serum from patients with tubal infertility (Damewood *et al.*, 1990; Simón *et al.*, 1992) and infertile women without evidence of endometriosis (Abu-Musa *et al.*, 1992b). In-vitro incubation of mouse embryos in peritoneal fluid from infertile patients with endometriosis resulted in impaired (Morcos *et al.*, 1985; Prough *et al.*, 1990) or similar development (Awadalla *et al.*, 1987) when compared with peritoneal fluid from fertile controls (Prough *et al.*, 1990) and unexplained infertility patients (Morcos *et al.*, 1985; Awadalla *et al.*, 1987).

As thoughtfully stressed by Hill (1992a,b), conflicting reports concerning adverse effects of serum and peritoneal fluid on reproductive processes may be explained in part by the inadequate controls used. Many investigators have not included fertile women in their studies, but rather have compared subfertile patients with endometriosis with subfertile women without endometriosis. Inclusion of a fertile control population should be a fundamental component of the basic study design of infertility and endometriosis studies because it has been suggested that similar peritoneal factors may be operable in subfertile women with and without evidence of endometriosis (Gleicher, 1992). On the other hand, the possibility exists that women with endometriosis may not represent a homogeneous population, and this is another variable potentially confounding studies investigating endometriosis-associated reproductive failure (Hill, 1992a). Hence, study populations should ideally consist of fertile and subfertile women with and without endometriosis (Hill, 1992b). This has been done in the present study, where we found that both serum and peritoneal fluid from infertile women with endometriosis are embryotoxic to in-vitro development of 2-cell mouse embryos, whereas sera and peritoneal fluids from patients with unexplained infertility and fertile women (with or without laparoscopic evidence of endometriosis) were not embryotoxic when studied in the in-vitro mouse embryo system.

In a recent prospective study (Balasch *et al.*, 1996a), we showed a high prevalence (45–50%) of endometriosis (including microscopic forms) in asymptomatic women, both fertile and infertile, thus supporting the modern concept that in many women with patent Fallopian tubes, endometriosis,

rather than being a 'disease', may be a normal consequence of uninterrupted menstrual periods. In some patients, however, small amounts of endometriosis may have implications for their reproductive health and therefore should be defined as a 'disease'. The present report adds further evidence favouring this new concept, now from an immunological point of view. In addition, our study contradicts the proposed immunological similarity between patients with unexplained infertility and minimal to mild endometriosis.

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References

Abu-Musa, A., Takahashi, K. and Kitao, M. (1992a) The effect of serum obtained before and after treatment for endometriosis on in vitro development of two-cell mouse embryos. *Fertil. Steril.*, **57,** 1098–1102.

Abu-Musa, A., Takahashi, K. and Kitao, M. (1992b) Effect of serum from patients with endometriosis on the development of mouse embryos. *Gynecol. Obstet. Invest.*, **33,** 157–160.

American Fertility Society (1985) Revised American Fertility Society classification of endometriosis 1985. *Fertil. Steril.*, **43,** 351–352.

Awadalla, S.G., Friedman, C.I., Haq, A.U. *et al.* (1987) Local peritoneal factors: their role in infertility associated with endometriosis. *Am. J. Obstet. Gynecol.*, **157,** 1207–1214.

Balasch, J., Font, J., López-Soto, A. *et al.* (1990) Antiphospholipid antibodies in unselected patients with repeated abortion. *Hum. Reprod.*, **5,** 43–46.

Balasch, J., Creus, M., Fábregues, F. *et al.* (1996a) Visible and non-visible endometriosis at laparoscopy in fertile and infertile women and in patients with chronic pelvic pain: a prospective study. *Hum. Reprod.*, **11,** 387–391.

Balasch, J., Creus, M., Fábregues, F. *et al.* (1996b) Antiphospholipid antibodies and human reproductive failure. *Hum. Reprod.*, **11,** 2310–2315.

Bergqvist, A. (1995) The relationship between endometriotic lesions and the disease endometriosis. *Hum. Reprod.*, **10,** 11–12.

Berkova, N., Lemay, A., Boutin, M. *et al.* (1996) Decreased amounts of antibodies to 22 and 18 kDa antigens in the peritoneal fluid of patients with endometriosis. *Hum. Reprod.*, **11,** 861–867.

Brosens, I., Donnez, J. and Benagiano, G. (1993) Improving the classification of endometriosis. *Hum. Reprod.*, **8,** 1792–1795.

Christman, G.M. and Halme, J.K. (1992) Pathophysiology of endometriosis-associated symptoms. *Infertil. Reprod. Clin. N. Am.*, **3,** 551–564.

Confino, E., Harlow, L. and Gleicher, N. (1990) Peritoneal fluid and serum auto-antibody levels in patients with endometriosis. *Fertil. Steril.*, **53,** 242–245.

Damewood, M.D., Hesla, J.S., Schlaff, W.D. *et al.* (1990) Effect of serum from patients with minimal to mild endometriosis on mouse embryo development *in vitro*. *Fertil. Steril.*, **54,** 917–920.

D'Hooghe, T.M. and Hill, J.A. (1995) Autoantibodies in endometriosis. In Kurpisz, M. and Fernandez, N. (eds), *Immunology of Human Reproduction*. Bios Scientific Publishers, Oxford, pp. 133–162.

D'Hooghe, T.M. and Hill, J.A. (1996) Immunobiology of endometriosis. In Bronson, R.A., Alexander, N.J., Anderson, D. *et al.* (eds), *Reproductive Immunology.* Blackwell Science, Cambridge (USA), pp. 322–356.

D'Hooghe, T.M.D., Hill, J.A., Oosterlynck, D.J. *et al.* (1996) Effect of endometriosis on white blood cell subpopulations in peripheral blood and peritoneal fluid of baboons. *Hum. Reprod.*, **11,** 1736–1740.

Drake, T.S., Metz, S.A., Grunert, G.M.O. and Brien, W.F. (1980) Peritoneal fluid volume in endometriosis. *Fertil. Steril.*, **34,** 280–281.

El-Roeiy, A. and Gleicher, N. (1988) Definition of normal autoantibody levels in an apparently healthy population. *Obstet. Gynecol.*, **72,** 596–602.

FIVNAT (French In Vitro National) (1993) French national IVF registry: analysis of 1986 to 1990 data. *Fertil. Steril.*, **59,** 587–595.

Gleicher, N. (1992) Endometriosis: a new approach is needed. *Hum. Reprod.*, **7,** 821–824.

Gleicher, N., El-Roeiy, A., Confino, E. and Friberg, J. (1987) Is endometriosis an autoimmune disease? *Obstet. Gynecol.*, **70,** 115–122.

Halme, J., Becker, S. and Wing, R. (1984) Accentuated cyclic activation of peritoneal macrophages in patients with endometriosis. *Am. J. Obstet. Gynecol.*, **148,** 85–90.

Hill, J.A. (1992a) Immunologic factors in endometriosis and endometriosis-associated reproductive failure. *Infertil. Reprod. Med. Clin. N. Am.*, **3,** 583–596.

Hill, J.A. (1992b) Immunology and endometriosis. *Fertil. Steril.*, **58,** 262–264.

Hill, J.A., Faris, H.M.P., Schiff, I. and Anderson, D.J. (1988) Characterization of leucocyte subpopulations in the peritoneal fluid of women with endometriosis. *Fertil. Steril.*, **50,** 216–222.

Kennedy, S.H., Nunn, B., Cederholm-Williams, S.A. and Barlow, D.H. (1989) Cardiolipin antibody levels in endometriosis and systemic lupus erythematosus. *Fertil. Steril.*, **52,** 1061–1062.

Kilpatrick, D.C., Haining, R.E.B. and Smith, S.S.K. (1991) Are cardiolipin antibody levels elevated in endometriosis? *Fertil. Steril.*, **55,** 436–437.

Koninckx, P.R. (1994) Is mild endometriosis a condition occurring intermittently in all women? *Hum. Reprod.*, **9,** 2202–2211.

Mahmood, T.A. and Templeton, A. (1990) Pathophysiology of mild endometriosis: review of literature. *Hum. Reprod.*, **5,** 765–784.

Morcos, R.N., Gibbons, W.E. and Findley, W.E. (1985) Effect of peritoneal fluid on in vitro cleavage of 2-cell mouse embryos: possible role in infertility associated with endometriosis. *Fertil. Steril.*, **44,** 678–683.

Oral, E., Olive, D.L. and Arici, A. (1996) The peritoneal environment in endometriosis. *Hum. Reprod. Update*, **2,** 385–398.

Prentice, A. and Ingamells, S. (1996) Endometriosis and infertility. *J. Br. Fertil. Soc.*, **1,** 51–55.

Prough, S.G., Akzel, S., Gilmore, S.M. and Yeoman, R.R. (1990) Peritoneal fluid fractions from patients with endometriosis do not promote two-cell mouse embryo growth. *Fertil. Steril.*, **54,** 927–930.

Ramey, J.W. and Archer, D.F. (1993) Peritoneal fluid: its relevance to the development of endometriosis. *Fertil. Steril.*, **60,** 1–14.

Reinherz, E.L., Kung, P.C., Goldstein, G. and Schlossman, S.F. (1979) Separation of functional subsets of human T cells by monoclonal antibody. *Proc. Natl. Acad. Sci. USA*, **76,** 4061–4065.

Simón, C., Gómez, E., Mir, A. *et al.* (1992) Glucocorticoid treatment decreases sera embryotoxicity in endometriosis patients. *Fertil. Steril.*, **58,** 284–289.

Taylor, P.V., Maloney, M.D., Campbell, J.M. *et al.* (1991) Autoreactivity in women with endometriosis. *Br. J. Obstet. Gynaecol.*, **98,** 680–684.

Thomas, E.J. (1994) The relevance of asymptomatic endometriosis. In Evers, J.L.H. and Vermeiden, J.P.W. (eds), *Proceedings from the Ferring ESHRE symposium Endometriosis and Fertility 1994: What Can We Expect*. 10th Annual Meeting of the ESHRE, Brussels, pp. 30–34.

Thomas, E.J. and Prentice, A. (1992) The aetiology and pathogenesis of endometriosis. *Reprod. Med. Rev.*, **1,** 21–36.

Vercellini, P. and Crosignani, P.G. (1993) Minimal and mild endometriosis. Is there anything new under the sun? *J. Reprod. Med.*, **38,** 49–52.

Vinatier, D., Dufour, P. and Oosterlynck, D. (1996) Immunological aspects of endometriosis. *Hum. Reprod. Update*, **2,** 371–384.

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