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Anti-Endometrial Autoantibodies in Women with a Diagnosis of Infertility

J.R. Palacio, A. Iborra, J.M. Gris, P. Andolz, and P. Martínez

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PROBLEM: The purpose of this study was to investigate the frecuency of anti-endometrial antibodies (AEA) in infertile women.

METHOD OF STUDY: Sera from fertile women (n = 6), and from patients with ovulatory dysfunction (n = 11), tubal obstruction (n = 9) and unexplained infertility (n = 5) were investigated for the presence of anti-endometrial membrane antibodies. We used two human endometrial cancer cell lines and human endometrial cells from gynecological biopsies as an antigenic source for analysis. The immunoenzymatic assay (ELISA) was performed with cultured endometrial cells in monolayers. Immunoblot analysis was performed with these two cell lines.

RESULTS: A good correlation between the response with each cell line and with human endometrial cells was obtained, indicating that the antigens analyzed were probably similar. Endometrial antibodies were detectable in a high percentage of women with tubal obstruction (77.8 and 66.7%, respectively) and ovulatory dysfunction (54.5 and 45.5%, respectively). Unexplained infertility showed anti-endometrial immunological response (40 and 60%, respectively). Some endometrial antigens in infertile women are the target for autoimmune response. The serum from a patient with tubal obstruction and ovulatory dysfunction showed two antigens by immunoblot, with molecular weights of 97 and 50 kDa.

CONCLUSION: The presence of anti-endometrial antibodies, detected by ELISA, is associated with infertility, mainly with ovulatory dysfunction and tubal obstruction. Some endometrial antigens may be involved in these two pathologies.

Key words:
Anti-endometrial antibodies, tubal obstruction, ovulatory dysfunction, female infertility, autoantibodies

J.R. PALACIO
A. IBORRA
P. MARTINEZ
Unidad de Inmunología, Departamento de Biología Celular y Fisiología, Instituto de Biología Fundamental, Universidad Autónoma de Barcelona, Barcelona, Spain

J.M. GRIS
P. ANDOLZ
Departamento de Obstetricia y Ginecología, Hospital Universitario del Mar, Barcelona, Spain

Address reprint requests to Dr. Paz Martínez, Instituto de Biología Fundamental "V. Villar i Palasí" Universidad Autónoma de Barcelona, Campus de Bellaterra 08193, Barcelona, Spain.

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INTRODUCTION

Reproductive failure may be induced by autoantibodies to organs or cells involved in fertilization or implantation such as gametes, ovaries, Fallopian tubes, or endometrial cells. The presence of anti-endometrial antibodies has been detected by several techniques in patients with endometriosis: passive hemaglutination,[1] indirect immunofluorescence,[2,3] enzyme-linked immunoadsorbent assay (ELISA),[4,5] or immunoblot.[6,7]

The presence of autoantibodies in serum is often associated with inflammatory response. Some authors coined the term reproductive autoimmune failure syndrome (RAFS) after the fact that abnormal autoimmune processes can affect reproduction at various stages.[8] How these anti-endometrial antibodies lead to infertility remains to be clarified.[9] Patients with tubal obstructions or ovulatory dysfunctions would potentially have abnormal autoimmune function associated with decreased fertility.

Several reproductive organs could be the target for immune response such as the

ovary, Fallopian tubes, and endometrium, thus inducing impaired function. It has been established that an inflammatory mechanism is critical for autoimmune ovarian inflammation (oophoritis). Antibodies bind to ovarian target antigens during the development of oophoritis and are responsible for spontaneous infertility.[10] An increased association between autoimmune diseases has been reported.

A critical review in reference to autoantibodies associated with reproductive failure has been published,[11] in which phospholipid antibodies, anti-sperm antibodies, and the specificity of these antibodies and glucocorticoid therapy are discussed. Some other markers have been proposed and compared to anti-endometrial antibodies. Wild et al.[12] showed that antibodies were a more sensitive test than CA-125 for detecting endometriosis. They used endometrial carcinoma cell lines for the analysis of the antibodies, and the reactivity of the sera were determined by indirect immunofluorescence. This encouraged us to search for a marker for the diagnosis of immunological reproductive failure. The purpose of this study was to determine whether the antibody anti-endometrium is a good immunological sensor of the pathologies related to the reproductive organs (ovulatory dysfunction, tubal obstruction, and in some cases of unexplained infertility) for a correct therapy to be given to the patients.

For the detection of anti-endometrial antibodies (AEA) we used an ELISA described previously for the analysis of autoantibodies that show good reproducibility and sensitivity.[13] We used two types of antigenic source: a) human endometrial cells from biopsies, and b) human endometrial cancer cell lines. A well-established endometrial cancer cell line would be a good source of antigens for a noninvasive method of diagnosis of any disease that would correlate with the presence of AEA.

MATERIALS AND METHODS

Patients and Clinical Diagnosis

Serum samples were obtained at cycle days 20 to 24 from 38 women (age 23–45 years) that consulted for infertility in the couple. Hysterosalpingography and laparoscopy have been used in conjunction to evaluate uterine anomalies and permeability of the Fallopian tubes as an indication of tubal obstruction. We have been studying the ovulatory periods by hormonal effects on target organs such as the thermogenic shift in basal body temperature and changes in the consistency and biophysical properties of the cervix. Moreover, we did other hormonal evaluations such as the test for luteinizing hormone in urine and a mild luteal phase level of serum progesterone. At the end of the study of the couples, if we had not found a cause of infertility, the final diagnosis was infertility of unknown cause, or unexpained infertility. All the infertile patients in our study had no endometriosis, as diagnosed by laparoscopy. In 22 couples, the infertility was of female origin: 9 diagnosed with tubal obstruction, 11 with hormonal ovulatory dysfunctions, and 5 were diagnosed with unexplained infertility. Three cases had more than one diagnosis (tubal obstruction and ovulatory dysfunction). In 16 infertile couples, the infertility was diagnosed as being of male origin. Six fertile women without any pathology were used as controls. Blood samples of all women were obtained during the luteal phase.

ELISA

The samples of human endometrial cells obtained on the luteal phase were washed with medium, disgregated mechanically, and then were digested enzymatically with collagenase. After washing by centrifugation, cells were cultivated in a flask for 4–5 days until 70% of confluence. On the other hand, two human adenocarcinoma endometrial cell lines (line 1671 and line 113 from ATCC USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with growth factors [bovine insulin (0.05 µg/ml), transferrin (0.05 µg/ml), glutation (0.1 µg/ml), and hydrocortisone (0.05 µg/ml)], with antibiotics (1% of a mixture of penicilin, streptomycin, and fungizone), and with 10% fetal calf serum (FCS).[14] Cells were cultured in 96-well flat-bottomed tissue culture-treated plates [Costar, Cambridge, MA; 100 µl/well (1×10^5 cells/well)] for 48 hr at 37°C and 10% CO_2.

The ELISA procedure used was described by Benet-Rubinat et al.[13] For this assay, cells in culture plates were fixed with 100 µl/well of 0.25% glutaraldehyde solution. After a 20-min incubation at 4°C, the fixing solution was discarded and the wells were washed with 200 µl/well of phosphate-buffered saline with 0.05% Tween-20 (PBS-Tween-20). Washings with PBS-Tween-20 were repeated three times at 5-min intervals after each step of the immunoassay. All incubations were made in a moist chamber.

Before adding the serum, a blocking solution was added to eliminate nonspecific background reactions; the blocking solution consisted of 10% goat serum and 1% bovine serum albumin in PBS-Tween-20. The culture plate was incubated for 90 min at 37°C. Then the wells were washed with PBS-Tween-20 and a 1/128 dilution of the serum was added to each well in duplicate and incubated overnight at 4°C.

After incubation and washing of the culture plate, 100 µl of sheep anti-human immunoglobulin G (IgG) conjugated with horseradish peroxidase (The Binding Site, Birmingham, U.K.) at a 1/1500 dilution was added to each well and was incubated for 90 min at 37°C. The culture plates were washed and then developed for 15 min, at room temperature, with a chromogenic substrate [3-methyl-2-benzothiazoline hydrazone hydrochloride (MBTH) and 3-dimethylaminobenzoic acid (DMAB)] with 30%H_2O_2. The reaction was stopped with 50 µl/well of 2

M sulfuric acid (H_2SO_4), and the optical density (A) at 620 nm was automatically determined with a multiscan plate reader.

Endometrial Antigens

Antigen extracts were prepared from two tumoral endometrial cell lines (line 1671 and line 113). For this purpose, tumoral cells cultured in falcons (75 cm^2) were washed with sterile PBS and were processed as follows: 7 ml of a pre-heated lysis solution [10 mM Tris-HCl, 1% sodium dodecyl sulfate, pH 7.4 (2×)] was added to the culture for 2–3 min. The solubilized cells were removed with a sterile scraper and then centrifuged at 16,000*g* for 15 min. The clear supernatants were heated for 5 min at 100°C, were divided into 100-µl aliquots, and were stored frozen at –20°C until used.

Western Blot Analysis

The method was essentially as outlined earlier.[7] Proteins in extracts from different tumoral cell lines were separated by 10% polyacrylamide gel electrophoresis by standard methods. Protein concentration in the extracts from different tumoral cell lines was adjusted to 0.5 mg of protein/ml by the method of Lowry. Protein bands were analyzed by silver staining. Protein extracts were transferred to a nitrocellulose membrane (Bio-Rad, Richmond, CA) in a solution of 25 mM Tris, 150 mM glycine, and 20% methanol. The nitrocellulose membranes were washed with 25 ml of Tris-buffered saline (TBS: 0.05 M Tris, 2 M NaCl, pH 7.5) with 0.05% Tween-20 (Tris-Tween-20), and they were incubated overnight in a blocking solution containing PBS, 20 mM Tris, 3% bovine serum albumin (BSA), and 10% heat-inactivated goat serum (pH 7.5) to saturate all protein binding sites. The membrane was washed with 25 ml of TBS-Tween three times at room temperature. The membrane was cut in two, and each half was incubated at 4°C overnight with different sera: one with a positive serum from an infertile woman (1/200) and the other with a negative control serum (1/200). Membranes were washed with 25 ml of TBS, and then they were incubated with anti-IgG conjugated with alcaline phosphatase (1/2000) (The Binding Site) for 60 min at room temperature. The substrate was added [1 ml of nitro blue tetrazolium (NBT; 30 mg/ml in 70% dimethylformamide) and 1 ml of 5-bromo 4-cloro 3-indolil phosphate (BCIP; 15 mg/ml in 70% dimethylformamide)] in a 100-ml buffer (0.1 M Tris HCl, 0.5 mM $MgCl_2$, pH 9.5). The membranes were soaked in this solution for 15 min until the highest band was clearly visible, rinsed with distilled water, and allowed to air-dry.

RESULTS

The antigenic source for the ELISA assay (human endometrial cells and human endometrial cancer cell lines, 1671 and 113) were grown for 48 hr in a 96-well flat bottomed microplate until confluency. When we compared the ELISA results obtained with human endometrial cells and with the two endometrial adenocarcinoma cell lines, we observed a high correlation between all the antigen sources. For this reason we used the 113 and 1671 cell lines for our purpose (Fig. 1).

All control sera (n = 6) were analyzed at various serum dilutions, from 1/32 to 1/2,048; the standard deviations (2 SD) were calculated for every dilution, as shown in Figure 2. For further analysis,1/128 of the serum dilution was used, because an optimal difference was observed at this concentration, when comparing the control and some positive sera.

The mean value (A_{620}) for cell line 1671 was 0.492 ± 0.104 (1 SD) and 0.500 ± 0.120 (1 SD) for cell line 113. Sera with values higher than the mean value plus 1 SD were considered positive. Each serum sample was analyzed in two separate experiments. In every experiment samples were examined in duplicate. For every sample the mean value of two experiments was calculated. The ELISA described showed high reproducibility among experiments. Figure3 shows the complete analysis of the sera classified after diagnosis of the patients; tubal obstruction and ovulatory dysfunction have many positive sera for the presence of AEA.

Endometrial antigens from both tumoral endometrial cell lines were tested against the serum of a patient with ovulatory dysfunction and tubal obstruction. This showed the presence of two antigenic bands from both extracts (1671 and 113), at 97 and 50 kDa, that were not present in the control (Fig. 4).

From the sera of 38 women, we established the presence of AEA in cell line 1671 and cell line 113 related to

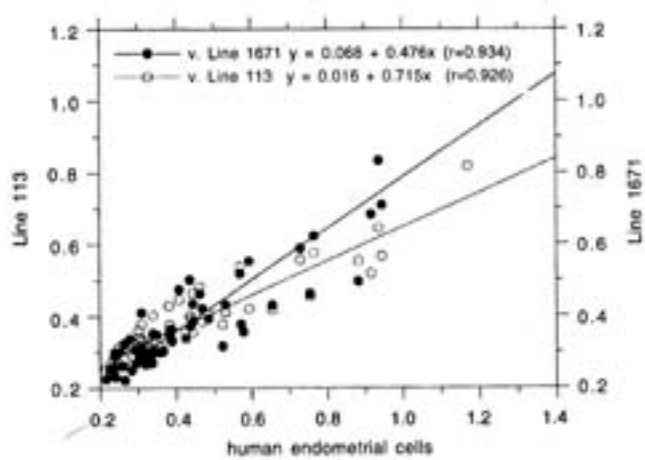

Fig. 1. Correlation between values ($A_{620\,nm}$) of anti-endometrial antibodies (AEA) by ELISA, in sera from infertile women: ●, human endometrial cells versus cell line 1671; ○, human endometrial cells versus cell line 113.