Anti-endometrial antibodies in women measured by an enzyme-linked immunosorbent assay

S.Fernández-Shaw1,3, S.H.Kennedy1, B.R.Hicks1, K.Edmonds2, P.M.Starkey1 and D.H.Barlow1

1University of Oxford, Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Oxford and 2Queen Charlotte’s and Chelsea Hospital, London, UK
3To whom correspondence should be addressed at: Departamento de Ginecología y Obstetricia, Centro Médico La Zarzuela, Pleyades 25, 28023 Aravaca, Madrid, Spain

An enzyme-linked immunosorbent assay (ELISA) was developed to measure anti-endometrial antibody concentrations in the serum of women with endometriosis. Pooled cytosolic protein extracts from the endometrial gland cells of 10 women were used as an antigen source. Serum samples were obtained from women with endometriosis before (n = 51) and after 6 months treatment with danazol or nafarelin (n = 30). Control sera came from women with a normal pelvis at laparoscopy, performed for sterilization (n = 23) or the investigation of pain and/or infertility (n = 22), 13 women with Rokitansky syndrome, and 10 umbilical cord bloods and adult males. There were no significant differences in serum anti-endometrial antibody concentrations before and after treatment, or between women with endometriosis and without endometriosis. Concentrations were lower in male and cord blood serum than in female’s serum (P < 0.0001). We conclude that the ELISA is not a useful diagnostic tool for endometriosis unless more specific antigens can be isolated.

Key words: anti-endometrial antibodies/ELISA/endometriosis/endometrium

Introduction

Anti-endometrial antibodies have been detected in the serum of women with endometriosis using immunofluorescence (Mathur et al., 1982; Wild and Shivers, 1985), immunodiffusion (Badawy et al., 1984), passive haemagglutination (Chihal et al., 1986; Badawy et al., 1990), enzyme-linked immunosorbent assay (ELISA) (Kennedy et al., 1990b) and Western blots (Mathur et al., 1988, 1990). Most studies show a higher prevalence of autoantibodies in women with endometriosis than in controls. However, Switchenko et al. (1991), failed to demonstrate consistently higher concentrations of anti-endometrial antibodies in women with endometriosis using immunofluorescence, haemagglutination, ELISA, or Western blotting, and concluded that the case for specific anti-endometrial antibodies remains unproven.

Whole endometrial extracts (Badawy et al., 1990; Mathur et al., 1990), isolated endometrial epithelial cells (of unspecified purity) (Switchenko et al., 1991) and endometrial carcinoma cell lines (Wild et al., 1991b) have all been used as antigenic sources to detect anti-endometrial antibodies but it is unclear which is the best. Using a double-labelling immunohistochemical technique, we previously reported that anti-endometrial antibodies are more common in the serum of women with endometriosis than of controls and they are directed against the cytoplasm of glandular and surface endometrial epithelium (Kennedy et al., 1990a; Fernández-Shaw et al., 1993). An ELISA was developed to quantify concentrations of anti-endometrial gland antibodies (Kennedy et al., 1990a). Preliminary results suggested that anti-endometrial antibody concentrations were significantly higher in women with endometriosis compared with a control group composed of adult males and cord blood samples (P < 0.001), and that concentrations were lowered after 6 months treatment with danazol or nafarelin (P < 0.001). These results implied that the ELISA had potential as a diagnostic test for endometriosis. Using male controls, Switchenko et al. (1991) failed to replicate these results. These authors prepared the antigenic gland extract in the same way as Kennedy et al. (1990b) but they used a protein coating concentration of 100 µg/ml, with serum and anti-human immunoglobulin (Ig)G concentrations of 1:10 and 1:500 respectively, which might account for the different results.

The experiments described in this paper were designed to re-evaluate and improve the use of the ELISA for the diagnosis of endometriosis. In addition, sera from women without endometriosis and women with Rokitansky–Kuster–Hauser syndrome (46XX; total or partial agenesis of the vagina; non-canalized rudimentary uterus) were used as controls.

Materials and methods

A total of 30 serum samples from women with endometriosis was collected as part of a double-blind randomized study comparing danazol and nafarelin in the treatment of endometriosis (stage I, n = 11; stage II, n = 11; stage III, n = 5; stage IV, n = 3) (Kennedy et al., 1990a,b). Disease severity was categorized according to the revised American Fertility Society (AFS) classification (1985). Serum samples were also collected from 21 women with endometriosis diagnosed at laparoscopy for pain or infertility (stage II, n = 5; stage III, n = 13; stage IV, n = 3). None of these 51 patients had received treatment before diagnosis. We compared pre- and post-treatment antibody concentrations in the 30 patients’ sera collected for the original ELISA. Antibody concentrations were measured at the end of clinical treatment, not later.

Control sera were obtained from women with a normal pelvis at laparoscopy, performed for sterilization (n = 23) or the investigation of pain and/or infertility (n = 22), and 13 women with Rokitansky syndrome (46XX; total or partial agenesis of the vagina; non-canalized rudimentary uterus) were used as controls.
Anti-endometrial antibodies

syndrome. A further 10 control serum samples were collected from umbilical cord blood (n = 7; three male and four female) and adult males (n = 3), as in the original ELISA. All sera were stored at -20°C until tested.

Endometrial tissue was collected at hysterectomy from seven premenopausal women with regular but heavy menstrual cycles (four proliferative, three secretory phase). Histological reports confirmed that the endometria were normal. Purified endometrial glands were isolated as described before (Femandez-Shaw et al., 1992) and resuspended in 1 ml of 1:10 phosphate-buffered saline (PBS)/H2O containing 1 mM phenyl-methyl-sulphonyl-fluoride (PMSF; Sigma Chemical Co. Ltd, Poole, UK) to inhibit any tissue serine proteinases. The cell suspension was homogenized 10 times in a glass homogenizer (Uniform mini-glass homogenizer 1 ml; Jencons Scientific Ltd., Bedfordshire, UK). The homogenate was ultracentrifuged at 40 000 g for 30 min to separate the soluble proteins in the supernatant from membrane micelles. From this step onwards the extract was kept at 4°C to minimize the risk of proteolysis. The gland extract was run through a protein G sepharose column (Protein G sepharose 4 Fast Flow; Pharmacia, Milton Keynes, UK) which binds the Fc portion of immunoglobulin (IgG) to remove endogenous immunoglobulins.

The protein concentration of the samples freed from IgG was measured by absorbance at 280 nm (A280)- Gland extracts were concentrated by centrifugation at 5000 g.

Concentrations of anti-endometrial antibodies in the serum (n = 51) were compared with each of the control groups with a Kruskal–Wallis one-way analysis of variance.

Results

There was no statistical difference in antibody concentration between women treated with danazol or nafarelin and therefore the results for these two groups were pooled. The purity of the gland fractions used as antigenic source was assessed immunohistologically as previously described (Femandez-Shaw et al., 1992) and the amount of protein obtained from each tissue measured. Cells were 84% (range 68–95%) pure cytokeratin-positive gland cells contaminated with 13% (range 1–23%) thy-1-positive stromal cells. Protein concentrations of crude extracts were 55 μg/10^6 gland cells (range 30–246).

There was no relationship between the amount of protein extracted and the phase of the menstrual cycle.

ELISA confirmed that IgG had been removed by the protein G column. Protein concentrations after the column were 24 μg/10^6 gland cells (range 12–148). An anti-endometrial antibody ELISA, using two serum samples, showed that specific binding was clearly higher using a gland extract after the column than before, while non-specific binding (B – C in the formula described in the materials and methods section) was significantly reduced (data not shown). All the gland cytosolic extracts obtained after the protein G column were pooled and used for the ELISA.

The anti-endometrial antibody ELISA had been previously optimized by titrating the sera and the gland extract. The lowest level of non-specific binding of serum to the plastic (A – C) was observed with a 1/100 serum dilution. Coating the plates with a gland extract protein concentration of 2.5 μg/ml gave the highest binding. The use of a blocking solution reduced non-specific serum binding and the second antibody binding to plastic (A – C).

The gland extract separated into two fractions of proteins above and below 30 000 mol. wt. Total autoantibody binding was similar against the two gland fractions, suggesting that more than one antigen was recognized by the autoantibodies.

There were no significant differences in anti-endometrial antibody concentrations between women with endometriosis before [mean (SD) = 84.6 (18.7)] and after medical treatment [mean = 90.1 (28.8)] (n = 30) (Figure 1). There were no significant differences in anti-endometrial antibody concentrations between women with endometriosis at any disease stage (Table I and Figure 2). Anti-endometrial antibody concentrations in women with endometriosis were not different from concentrations in any other control group, except for the control including cord blood and male sera, where the difference was highly significant (P < 0.0001) (Table II and Figure 2).

Discussion

We have changed the original ELISA protocol for the detection of serum anti-endometrial antibodies (Kennedy et al., 1990b) aiming at improving the assay's sensitivity. The main changes
Figure 1. Concentrations of anti-endometrial antibodies in sera from women with endometriosis before and after medical treatment (Tx) (n = 30), in percentages, taking as 100% the concentration of anti-endometrial antibodies in control serum.

Figure 2. Concentrations of anti-endometrial antibodies in sera from women with endometriosis (stages I—IV, solid symbols) and in controls (open symbols), in percentages, taking as 100% the concentration of anti-endometrial antibodies in a given serum.

Table I. Anti-endometrial antibody concentrations at different stages of endometriosis

<table>
<thead>
<tr>
<th>Endometrial stage</th>
<th>No. patients</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11</td>
<td>88.2</td>
<td>10.7</td>
</tr>
<tr>
<td>II</td>
<td>16</td>
<td>76.3</td>
<td>22.1</td>
</tr>
<tr>
<td>III</td>
<td>18</td>
<td>81.5</td>
<td>17.3</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>85.6</td>
<td>24.2</td>
</tr>
</tbody>
</table>


*Expressed as percentage of concentration in control serum.

Table II. Anti-endometrial antibody concentrations in women with endometriosis and in control groups

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometriosis patients</td>
<td>51</td>
<td>81.8</td>
<td>18.7</td>
</tr>
<tr>
<td>Infertile patients</td>
<td>22</td>
<td>87.6</td>
<td>32.8</td>
</tr>
<tr>
<td>Sterilized patients</td>
<td>23</td>
<td>77.3</td>
<td>30.6</td>
</tr>
<tr>
<td>Rokitansky patients</td>
<td>13</td>
<td>74.9</td>
<td>27.1</td>
</tr>
<tr>
<td>Males and cord bloods</td>
<td>10</td>
<td>35.7</td>
<td>13.3</td>
</tr>
</tbody>
</table>

*Expressed as percentage of concentration in control serum.

*Significantly different from all the other groups (P < 0.0001).

were those related to the antigenic source. Firstly, the original ELISA used an enriched gland cell fraction as antigenic source (Kennedy et al., 1990b). Our more recent studies have shown that these fractions are usually heavily contaminated by stromal cells (Fernández-Shaw et al., 1992). In the modified ELISA the isolation of gland cells was improved obtaining 84% pure cell suspensions. Secondly, the source of antigen(s) used in the original ELISA was a single endometrial gland fraction from which a protein extract was obtained. The modified ELISA pooled extracts from 10 women to decrease any effect that the variability of antigen content, observed by immunohistology (Fernández-Shaw et al., 1993), might have on the assay. Thirdly, protein extracts were also freed from contaminating membrane micelles by ultracentrifugation, since the relevant antigens appear to be cytoplasmic (Saifuddin et al., 1983; Wild et al., 1987; Kennedy et al., 1990a,b; Fernandez-Shaw et al., 1993). Finally, our results and those of others indicate the presence of endogenous IgG in glandular secretions and in the stromal interstitium of uterine endometrium (Bulmer et al., 1986; Switchenko et al., 1991; Kennedy et al., 1990a,b; Fernandez-Shaw et al., 1993).
from the extracts, which resulted in decreased non-specific binding in the ELISA. Other changes to the ELISA included diluting the gland extract in buffer instead of distilled water, coating the plates with less antigen, and using much higher serum and conjugate dilutions.

Our ELISA recognizes more than one antigen in the endometrium as demonstrated by reactivity with glandular fractions of proteins above and below 30,000 mol. wt. These results are compatible with those of Mathur et al. (1988, 1990) using Western blots and with our previous immunohistochemical study (Fernández-Shaw et al., 1993).

The number of serum samples used in our modified ELISA allowed us to detect (with two-tailed \( \alpha = 0.05 \)) differences of about 20% between means of the endometriosis group and healthy female control groups (power of 83%) and differences of about 26% between means of the endometriosis group and the Rokitansky and male and cord sera control groups (power of 80%). Differences between the means of healthy women and women with endometriosis were in fact very small, and there is no indication that we would have arrived at a different conclusion with a larger sample.

Our ELISA results showed a considerable variability in the level of individual serum reactivity with the endometrial gland extract. Anti-endometrial antibody concentrations in male and cord blood sera were much lower than in any female sera group \( (P < 0.0001) \), which confirms the results of the original ELISA and is in contrast with the results of Switchenko et al. (1991). In common with others, we observed no correlation between anti-endometrial antibody concentrations and the severity of the disease measured by the revised AFS classification (Chihal et al., 1986; Badawy et al., 1990; Kennedy et al., 1990b, Wild et al., 1991a).

The modified ELISA, however, did not detect any difference between pre- and post-treatment antibody concentrations in the sera of women with endometriosis, in contrast to our previous report using ELISA (Kennedy et al., 1990b) and that of others using passive haemagglutination (Chihal et al., 1986); neither could the modified ELISA detect any differences between women with endometriosis and any female controls. It is possible that the quantity of target antigen(s) in normal endometrial tissue is insufficient to facilitate detection by the ELISA; or, the antigen(s) may have been poorly liberated; or inadvertently, we may have selected for antigen(s) primarily recognized by control sera rather than patients’ sera. It is also possible that the source of antigen(s) chosen for the modified ELISA was not appropriate. Our previous immunohistochemical study recognized the presence of antigenic antibodies in the sera of patients with endometriosis; however, it was impossible to ascertain any antibody reaction to stromal antigens due to the presence of endogenous IgG in the endometrial stroma (Fernández-Shaw et al., 1993). Stromal cell contamination in the original ELISA gland preparation might have provided antigen(s) that are more specific for endometriosis than those isolated from gland cells, which could explain our different results. Two additional factors make antibody detection even more problematic: the low affinities that autoantibodies might have, if present at all (Switchenko et al., 1991), and the known variability in reactiv-

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References


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