Humoral immune response to membrane components of *Chlamydia trachomatis* and expression of human 60 kDa heat shock protein in follicular fluid of in-vitro fertilization patients

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Recent evidence suggests that *Chlamydia trachomatis* can persist in the female upper genital tract in an unculturable state. Since unsuspected *C. trachomatis* infection has been associated with adverse in-vitro fertilization (IVF) outcome we sought to detect further evidence of *C. trachomatis* in the genital tracts of women undergoing IVF. The prevalence and distribution of antibodies to the major structural proteins of *C. trachomatis* in paired follicular fluid and sera of women undergoing IVF were examined. Sera and follicular fluid samples from 149 women were assayed for immunoglobulin (Ig)G and IgA antibodies to two *C. trachomatis* antigens, the major outer membrane protein (MOMP) and a recombinant lipopolysaccharide (rLPS) fragment. Additionally, the expression of human 60 kDa heat shock protein (hsp 60) in follicular fluid was determined. All cervical and follicular fluid samples were negative for *C. trachomatis* by polymerase chain reaction, ligase chain reaction and DNA probe. Sera from 60% of the subjects were positive for anti-chlamydia lPS IgG; 36% were positive for anti-MOMP IgG. Similarly, rLPS-directed and MOMP-directed IgA were detected in sera of 34 and 14% of the subjects respectively. IgG antibodies to MOMP and rLPS were detected in 42 and 41% of the follicular fluid examined respectively. Anti-MOMP IgA was identified in 8.7% of the follicular fluid while 27.5% were positive for anti-rLPS IgA. Human hsp 60 expression was documented in 11.6% of the follicular fluid tested. IgA antibodies to both MOMP (*P* = 0.03) and rLPS (*P* = 0.02) in follicular fluid were associated with a failure to become pregnant after embryo transfer. IgG antibodies in sera and follicular fluid and IgA antibodies in sera were unrelated to IVF outcome. Similarly only anti-MOMP IgA (*P* = 0.02) and anti-rLPS IgA (*P* = 0.04) in follicular fluid were correlated with human hsp 60 expression in follicular fluid. The unique association between IgA antibodies to two chlamydial antigens in follicular fluid and both hsp 60 expression and IVF failure provides further support for the possibility that a persistent upper genital tract chlamydial infection contributes to IVF failure in some women.

**Key words: Chlamydia trachomatis/follicular fluid/human heat shock protein infertility/IVF**

**Introduction**

Evaluating infertility retrospectively, most women with tubal obstruction do not report a history of acute salpingitis, suggesting that their pelvic infection occurred in an asymptomatic or silent form. The prominent role of *Chlamydia trachomatis* in these silent infections is supported by the known association between tubal infertility and circulating chlamydial antibodies (Moore *et al.*, 1982; Meikle *et al.*, 1994). *C. trachomatis* constitute a unique group of inclusion-forming bacteria. Immune responses are most prominent among infected persons, but as obligate intracellular parasites members of the *Chlamydiae* can evade immune defences and persist within their host for prolonged periods. In fimbrial and peritubal adhesions of patients with tubal infertility, for example, chlamydial DNA can be detected even after antibiotic treatment (Patton *et al.*, 1994). *C. trachomatis* can ascend to the Fallopian tubes and induce an inflammatory reaction without clinical symptoms. The pathogenic events of chronic chlamydial inflammation are not well understood, but the persistence of this organism in tissue has been identified as a key element in the initiation and maintenance of chronic disease. Viable *C. trachomatis* can also persist in macrophages and this may provide an alternative target cell for chlamydial replication in a number of infections (La Verda and Byrne, 1994). Since follicular fluid contains tissue macrophages in large amounts (Loukides *et al.*, 1990; Lachapelle *et al.*, 1996) as well as T and B lymphocytes (Castillo *et al.*, 1990), it was of interest to evaluate follicular fluid for evidence of infection and its relation to in-vitro fertilization (IVF) outcome.

The human 60 kDa heat shock protein (hsp 60) is a highly conserved protein that shares a 48% amino acid sequence identity with the chlamydial hsp 60 (Cerrone *et al.*, 1991). Under conditions of rapid cell growth or differentiation or following environmental stress such as inflammation, host hsp 60 synthesis is induced. Initiation of heat shock gene transcription results in inhibition of transcription of genes coding for proinflammatory cytokines (Schmidt and Abdulla, 1988). Thus, hsp 60 expression may indicate a down-regulatory response to inflammation. It has also been suggested that, in women previously sensitized to a conserved epitope of a microbial hsp 60, the reactivation of hsp 60-reactive lymphocytes by expression of human hsp 60 may interfere with...
implantation or early embryo development (Witkin et al., 1996).

To elucidate further the possible cryptic presence of C. trachomatis in IVF patients and its possible contribution to IVF failure we examined the distribution of immunoglobulin (Ig)A and IgG antichlamydial antibodies in follicular fluid and sera of asymptomatic women undergoing IVF. Additionally the expression of human hsp 60 in follicular fluid was determined. Results were compared with the cause of infertility and subsequent pregnancy outcome.

Materials and methods

Patients and sample collection

A total of 149 women undergoing a cycle of IVF at the Women’s Hospital of the University of Tübingen, Germany between January 1994 and December 1995 participated in this study. Informed written consent was obtained from each individual patient. All women had negative results for cervical C. trachomatis by DNA probe (Genprobe®, San Diego, CA, USA) 2 days prior to oocyte retrieval. Vaginal cultures for microbial pathogens, mycoplasmas and bacterial vaginosis were routinely performed 7 days after onset of stimulation for IVF and were uniformly negative. Follicular fluid and sera were obtained at the time of oocyte retrieval by vaginal aspiration and venipuncture respectively. None of the samples was visibly contaminated with blood. The samples were stored at 4°C and transferred to the laboratory within 6 h. Follicular fluid was centrifuged and the resulting pellet was used for polymerase chain reaction (PCR) and ligase chain reaction (LCR). Tubal occlusion was diagnosed by laparoscopy and hysterosalpingosonography.

In-vitro fertilization

Ovarian stimulation was performed using a similar protocol for all patients. Gonadotrophin-releasing hormone analogue was continuously administered from the midluteal phase of the previous cycle. After complete down-regulation gonadotrophins [follicle stimulating hormone (FSH) or human menopausal gonadotrophin (HMG)] were given from the second day of the stimulation cycle until human chorionic gonadotrophin (HCG; 10 000 IU) administration. The dosage of gonadotrophins was adapted according to oestradiol concentration and follicle size as determined by transvaginal sonography. When at least three follicles reached 17 mm in diameter by ultrasonound, ovulation was induced. Transvaginal oocyte retrieval was performed 34–36 h after ovulation induction and pooled follicular fluid free of visible blood contamination was collected.

Immunological analyses

Antibodies to chlamydial major outer membrane protein (MOMP) were detected by the Immunocombi® Chlamydia Bivalent (Organics, Israel) assay. Chlamydial anti-lipopolysaccharide (LPS) antibodies were determined by enzyme-linked immunosorbert assay (ELISA) using a recombinant Chlamydia-specific LPS fragment (rELISA®, medac Hamburg, Hamburg, Germany). Samples were evaluated according to the instructions of the manufacturer. A titre of 1:200 was considered positive for recombinant (rLPS) IgA in follicular fluid. A recombinant antibody to human hsp 60 (SPA 804, StressGen, Victoria, BC, Canada) was diluted to 10 µl/ml in 0.1 M carbonate buffer, pH 9.8, and 0.1 ml added separately to wells of a microtitre plate. After an overnight incubation at 4°C the wells were washed four times with PBS–Tween. Aliquots (0.1 ml) of follicular fluid diluted 1:4 were added to the wells and the plate floated on a 37°C water bath for 60 min. The wells were then washed and a 1:500 dilution of rabbit anti-hsp60 polyclonal antibody (SPA 804, StressGen) was added. After 1 h incubation and another 4-fold washing, an alkaline phosphatase (AP)-conjugated goat antibody to rabbit IgG (Kierkegaard and Perry, Gaithersburg, MD, USA; dilution 1:400) in PBS–Tween was added. Following an additional 60 min incubation at 37°C, the wells were washed as above and the colourless AP substrate, p-nitrophenylphosphate, in 10% diethanolamine buffer (Pierce, Rockford, IL, USA) was added. After a 30–60 min room temperature incubation the appearance of a yellow colour in the wells was quantified at 405 nm. Known positive and negative controls were always assayed in parallel to the test samples. Inter- and intra-assay variations were <10%. A positive sample was defined as one yielding an optical density value that was at least 2SD above the mean value of known negative samples.

PCR/LCR

PCR was performed employing two sets of plasmid PCR primers (KL1–KL2 and T1–T2) as described by Mahony et al. (1994). Additionally the commercially available Amplicor® system (Roche Molecular Systems, Branchburg, NJ, USA) employing primers specific for the chlamydial cryptic plasmid DNA was used. Positive controls were derived from C. trachomatis grown in McCoy cell cultures. The Abbott–LCx® system (Abbott, Wiesbaden, Germany) was utilized for LCR (Lee et al., 1995).

Statistics

Fisher’s exact test and Student’s t-test were utilized to examine differences in discrete variables among the groups of subjects. P < 0.05 was considered significant.

Results

Distribution of chlamydial antibodies in sera and follicular fluid

The distribution between serum and follicular fluid of all antibodies tested is shown in Table I. Sera from 90 (60.4%) and 54 (36.2%) subjects were positive for antichlamydial IgG utilizing the rLPS and the MOMP assays respectively. rLPS-directed and MOMP-directed IgA were detected in sera from 50 (33.6%) and 21 (14.1%) of the subjects respectively. In follicular fluid, IgG antibodies to MOMP were detected in 63 (42.3%) cases while anti-MOMP IgA was present in 13 (8.7%) subjects. IgG anti-rLPS antibodies were present in follicular fluid from 50 (41%) women and 41 (27.5%) women were IgA anti-rLPS positive. There was not a complete concordance between antibodies in serum and follicular fluid. This was most striking for anti-MOMP IgA where 46% of women with this antibody in their follicular fluid were seronegative.

Polymerase and ligase chain reaction

All cervical samples and follicular fluid samples were negative for C. trachomatis by PCR and LCR.

IVF outcome

No fertilization occurred with oocytes from 25 (17%) women. Most of these cases were due to severe male infertility. Embryo transfers were performed in 124 (83%) women. Of these, 25 (20%) were delivered of viable term infants, six (5%) ended in spontaneous abortion, four (3%) in ectopic pregnancy and
Follicular immune response to *Chlamydia* and IVF outcome

### Table I. Distribution of chlamydial antibodies in sera and follicular fluids

<table>
<thead>
<tr>
<th>Antibody</th>
<th>No. positive/no. tested (%)</th>
<th>Sera</th>
<th>Follicular fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOMP IgA</td>
<td>21/149 (14.1)</td>
<td>13/149 (8.7)</td>
<td></td>
</tr>
<tr>
<td>MOMP IgG</td>
<td>54/149 (36.2)</td>
<td>63/149 (42.3)</td>
<td></td>
</tr>
<tr>
<td>rLPS IgA</td>
<td>50/149 (33.6)</td>
<td>41/149 (27.5)</td>
<td></td>
</tr>
<tr>
<td>rLPS IgG</td>
<td>90/149 (60.4)</td>
<td>50/122 (41.0)</td>
<td></td>
</tr>
</tbody>
</table>

MOMP = major outer membrane protein; rLPS = recombinant lipopolysaccharide; Ig = immunoglobulin.

### Table II. Antichlamydial antibodies in sera and pregnancy outcome following embryo transfer

<table>
<thead>
<tr>
<th>Antibody</th>
<th>No. positive (%)</th>
<th>Pregnant (n = 35)</th>
<th>Not pregnant (n = 89)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOMP IgA</td>
<td>3 (8.5)</td>
<td>17 (19.1)</td>
<td></td>
</tr>
<tr>
<td>MOMP IgG</td>
<td>12 (34.2)</td>
<td>38 (42.6)</td>
<td></td>
</tr>
<tr>
<td>rLPS IgA</td>
<td>13 (37.1)</td>
<td>35 (39.3)</td>
<td></td>
</tr>
<tr>
<td>rLPS IgG</td>
<td>25 (71.4)</td>
<td>60 (67.4)</td>
<td></td>
</tr>
</tbody>
</table>

See Table I for abbreviations.

### Table III. Antichlamydial antibodies in follicular fluid and pregnancy outcome after embryo transfer

<table>
<thead>
<tr>
<th>Antibody</th>
<th>No. positive (%)</th>
<th>Pregnant (n = 35)</th>
<th>Not pregnant (n = 89)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOMP IgA</td>
<td>0 (0.0)</td>
<td>11 (12.3)a</td>
<td></td>
</tr>
<tr>
<td>MOMP IgG</td>
<td>18 (51.4)</td>
<td>46 (51.6)</td>
<td></td>
</tr>
<tr>
<td>rLPS IgA</td>
<td>6 (17.1)</td>
<td>35 (39.3)b</td>
<td></td>
</tr>
<tr>
<td>rLPS IgG</td>
<td>10 (28.5)</td>
<td>32 (35.9)</td>
<td></td>
</tr>
</tbody>
</table>

*P = 0.03 vs pregnant; bP = 0.02 vs pregnant. See Table I for abbreviations.

89 (72%) showed no evidence of pregnancy after embryo transfer. There was no significant difference in outcome according to patients’ age. The number of transferred embryos was the same in women with or without antichlamydial antibodies.

### Antichlamydial antibodies and pregnancy outcome

In sera, no association between pregnancy outcome and antichlamydial antibodies could be demonstrated (Table II). In marked contrast, however, the presence of IgA antibodies to both MOMP and rLPS in follicular fluid was associated with a negative pregnancy outcome after embryo transfer (P = 0.03 and P = 0.02 respectively, Table III). MOMP IgA was not present in any of the follicular fluid from the 35 women who became pregnant, as opposed to its detection in 11 of 89 (12.3%) women who did not conceive (P = 0.03). Similarly, rLPS IgA was present in 6 of 35 (17.1%) follicular fluids of women who became pregnant versus 35 of 89 (39.3%) of patients whose IVF cycle did not result in pregnancy (P = 0.02). There was no relationship between IgG antichlamydial antibodies in follicular fluid and IVF outcome.

### Antichlamydial antibodies and cause of infertility

Of our patients, 64 (43%) had occluded Fallopian tubes. The remaining 85 patients (non-tubal infertility group) had multiple causes of infertility, including poor sperm quality (48%), endometriosis (10%), endocrine disorders leading to anovulation (20%), unexplained factors (17%) and miscellaneous other causes (6%). The presence of antichlamydial IgG and IgA MOMP and rLPS antibodies in sera were each associated with a diagnosis of tubal infertility (Table IV). Similarly in follicular fluid, anti-MOMP IgG (P < 0.0001), anti-MOMP IgA (P = 0.005) and rLPS IgA (P = 0.01) antibodies were all related to a diagnosis of tubal infertility (Table V). In 13 (20%) patients diagnosed with occluded Fallopian tubes no antichlamydial antibody was present in sera or follicular fluid.
Chlamydial antibodies and human heat shock protein expression in follicular fluid

Due to limited sample volumes, human hsp 60 was analysed in only 60 follicular fluid samples. Of these, seven (11.6%) were positive. The presence of MOMP IgA (P = 0.02) and rLPS IgA (P = 0.04) in follicular fluid and the expression of human hsp 60 were significantly related (Table VI). Interestingly, all follicular fluid positive for human hsp 60 were derived from patients with tubal infertility who did not become pregnant after embryo transfer. However, due to the small number of cases the relationship between hsp 60 and poor IVF outcome was not statistically significant and further exploration is needed to draw final conclusions.

Discussion

The overwhelming majority of women who are infertile due to occluded Fallopian tubes have never been diagnosed as having a sexually transmitted disease (Cates et al., 1993). However, the association between tubal factor infertility and antimyeloid antibodies (Jones et al., 1982) strongly implicates asymptomatic infections by this organism as a major cause of this condition. Studies from several laboratories have further provided evidence that C. trachomatis infections can persist for long periods of time in an unculturable state. The presence of chlamydial DNA and/or protein and persistence of localized inflammation, in the absence of a positive chlamydial culture, have been reported for occluded Fallopian tubes (Patton et al., 1994), trachoma (Holland et al., 1992) and reactive arthritis (Keat et al., 1987).

Many women with occluded Fallopian tubes now turn to IVF for treatment of infertility. Whether C. trachomatis still persists in some form in the genital tracts of a number of these women, and whether its presence affects IVF outcome, is still under investigation. A relationship between tubal factor infertility combined with hydrosalpinx and poor IVF outcome has been reported (Katz et al., 1996). We have recently shown that women with cervical IgA antibodies to C. trachomatis surface antigens or to the chlamydial hsp 60, but who were negative for C. trachomatis in the cervix by PCR, had an increased prevalence of unsuccessful IVF outcomes (Witkin et al., 1994).

The distribution pattern of antibodies to C. trachomatis in paired follicular fluid and sera from IVF patients has not previously been examined. Detection of IgG and IgA anti-MOMP and anti-rLPS antibodies in both sera and follicular fluid suggest that these antibodies can enter the follicular fluid by transudation from the circulation. However, the unique relationship between follicular fluid IgA anti-MOMP and anti-rLPS antibodies and both expression of human hsp 60 and IVF failure after embryo transfer strongly suggest that local production of IgA antibodies in response to C. trachomatis was also being induced within the genital tract.

C. trachomatis infections of the male genital tract have also been associated with local IgA antibody production in the absence of systemic anti-chlamydial immunity (Witkin et al., 1995). A recent study identified an association between IgA antibodies, but not IgG antibodies, to the chlamydial hsp 60 and chronic salpingitis (Dieterle and Wollenhaupt, 1996). This suggested the predominance of a mucosal, rather than a systemic, immune response to C. trachomatis in women with upper genital tract infections. The absence of C. trachomatis in follicular fluid, as determined by PCR and LCR, indicates that active Chlamydia replication and release of elementary bodies into the extracellular fluid was not occurring at this location. The antibody observations, plus the findings that all women whose follicular fluid was positive for anti-MOMP IgA, anti-rLPS IgA and human hsp60 had laparoscopy-verified tubal occlusion and who were unsuccessful at IVF, suggest the possibility of a persistent chlamydial infection in these patients.

In conclusion, this study provides another link between C. trachomatis-associated tubal infertility and subsequent IVF failure. Women with a history of occluded Fallopian tubes who were positive for chlamydial IgA antibodies and human hsp 60 in follicular fluid had a poor IVF outcome. We would suggest that a persistent upper genital tract chlamydial infection might contribute to the IVF failure observed in these women.

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