Humoral immune response to the chlamydial heat shock proteins hsp60 and hsp70 in Chlamydia-associated chronic salpingitis with tubal occlusion

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The aim of this study was to evaluate the prevalence of serum immunoglobulin (Ig) G and IgA antibodies to recombinant chlamydial 60 kDa heat shock protein (C-hsp60) and to assess the prevalence of serum IgG antibodies to recombinant chlamydial 70 kDa heat shock protein (C-hsp70) in Chlamydia-associated chronic salpingitis and/or salpingitis isthmica nodosa with tubal occlusion. Infertile patients (n = 34) with Chlamydia-associated, histologically documented chronic salpingitis and/or salpingitis isthmica nodosa and bilateral tubal occlusions (group I) were compared with infertile patients (n = 19) without tubal occlusions (group II). The prevalence of chlamydial antigen in endocervical, urethral and urine samples was low in both groups. The median chlamydial serum IgG and IgA antibody titres were significantly higher in group I than in group II (P < 0.0001 and P = 0.0002 respectively). Serum IgG antibodies to C-hsp60 and C-hsp70 were detected in 24 out of 34 patients (71%) in group I compared with 10 out of 19 (53%) and nine out of 19 (47%) patients in group II (not significantly different). There was a significant difference (P = 0.035) between the prevalences of serum IgA antibodies to C-hsp60 in groups I (seven out of 34 patients; 21%) and II (none of the 19 patients). The association between the presence of serum IgA antibodies to C-hsp60 and Chlamydia-associated chronic salpingitis and/or salpingitis isthmica nodosa with tubal occlusion underlines the significance of chlamydial 60 kDa heat shock protein in the pathogenesis of tubal infertility.

Key words: Chlamydia trachomatis infection/chlamydial antibodies/heat shock protein/salpingitis/tubal infertility

Introduction

Chlamydia (C.) trachomatis is one of the most frequent sexually transmitted pathogens. This organism can cause symptomatic and asymptomatic infections of the female urogenital tract, including cervicitis, urethritis, endometritis and pelvic inflammatory disease (PID). Repeated PID can lead to tubal occlusions (Weström, 1975), but tubal infertility is not necessarily related to a history of symptomatic PID (Cates et al., 1993). Asymptomatic infections of the Fallopian tubes can be caused by C. trachomatis (Marana et al., 1990). Repeated tubal infections with C. trachomatis can lead to distal tubal obstructions in monkeys (Patton et al., 1987). The significance of C. trachomatis in tubal infertility is supported by the association between tubal infertility and the presence of chlamydial serum antibodies indicating a past or present chlamydial infection (Jones et al., 1982; Moore et al., 1982). The pathogenic mechanism is as yet unclear.

C. trachomatis is an intracellular organism which is able to remain viable in cells in an uncultivable state (Campbell et al., 1993). A persistent infection with C. trachomatis can result in the relatively increased production of 60 kDa heat shock protein (hsp60) (Beatty et al., 1993). Heat shock proteins are produced by prokaryotic and eukaryotic organisms in response to stress factors. Because of their amino acid sequence homology, exposure to chlamydial heat shock protein may lead to genetically determined cross-reactive immune responses (for review see Brunham and Peeling, 1994). Hsp60 can induce a delayed hypersensitivity response in animals, characterized by a submucosal cellular infiltrate of lymphocytes and mononuclear macrophages (Morrison et al., 1989; Patton et al., 1994). The presence of serum immunoglobulin (Ig) G antibodies to recombinant chlamydial hsp60 (C-hsp60) has been associated with PID (Wagar et al., 1990), tubal infertility (Brunham et al., 1985; Toye et al., 1993; Arno et al., 1995) and ectopic pregnancy (Wagar et al., 1990; Brunham et al., 1992). A cell-mediated immune response to conserved epitopes of chlamydial and human hsp60 was found in women with salpingitis (Witkin et al., 1993, 1994a). Little is known about the role of 70 kDa heat shock protein (hsp70) in the immune response to C. trachomatis infections. Because hsp70 concentrations were significantly higher in endometrial samples from infertile women than in specimens from a fertile control group, hsp70 may also be involved in the pathogenesis of infertility (Nip et al., 1994). The objective of this study was to examine the humoral immune responses to recombinant C-hsp60 and to recombinant C-hsp70 in Chlamydia-associated chronic salpingitis and/or salpingitis isthmica nodosa with tubal occlusion.

Materials and methods

Patients

Group I consisted of 34 infertile women (mean age ± SD, 28.3 ± 4.1 years) with Chlamydia-associated chronic salpingitis and/or salpingitis isthmica nodosa and bilaterally occluded Fallopian tubes. All patients underwent reconstructive tubal infertility surgery. Biopsies of the occluded parts of the Fallopian tubes were collected and examined histologically. In this study, only patients with histologically documented chronic salpingitis and/or salpingitis isthmica nodosa...
Humoral immune response to hsp60 and hsp70

Figure 1. Western immunoblot for the detection of serum antibodies to recombinant chlamydial 70 kDa heat shock protein (hsp70; lanes A–C) and C-hsp60 (lanes D–F). Lane A, negative control using C-hsp70 as antigen; lane B, representative positive serum sample; lane C, positive control (serum from a patient with Chlamydia-induced reactive arthritis and reactivity to C-hsp70); lane D, negative control using C-hsp60 as antigen; lane E, representative positive serum sample; lane F, positive control; m.w. = molecular weight markers.

Figure 2. Chlamydial serum immunoglobulin G antibody titres.

Methods

Indirect immunofluorescence assay

Endocervical smears were stained with fluorescein isothiocyanate-conjugated monoclonal antibodies to C. trachomatis major outer membrane protein (MOMP; Syva MicroTrak DFA; Syva Company, San Jose, CA, USA), according to the instructions of the manufacturer. A sample was considered to be positive if at least five elementary bodies were detected.

Enzyme immunoassays

Endocervical and urethral swabs and first-void urine specimens (20 ml) were examined by an enzyme immunoassay for chlamydial lipopolysaccharide (LPS; Syva MicroTrak Chlamydia EIA; Syva Company), according to the instructions of the manufacturer.

Serum samples were tested for chlamydial serum IgG and IgA antibodies using an indirect immunoperoxidase assay (IPAzyme; Savyon Diagnostics, Israel). Serum IgG antibodies were evaluated starting at a dilution of 1:64 and titrated to the end-point. Serum IgA antibodies were tested, beginning with a dilution of 1:16, and titrated to the end-point.

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western immunoblots

Antibodies against C-hsp60 (a homologue of the GroEL heat shock protein of Escherichia coli) and C-hsp70 (a homologue of the DnaK heat shock protein of E.coli) were detected by a Western immunoblot (Figure 1). Recombinant C-hsp60 and recombinant C-hsp70 were kindly provided by Dr D.T.Y.Yu (University of California, Los Angeles, CA, USA). C-hsp60 and C-hsp70 (equivalent to 1 µg protein per slot) were resuspended in 62.5 mM Tris, 6% (w/v) SDS, 30% (v/v) glycerol and 15% (v/v) mercaptoethanol, and separated by SDS–PAGE with an 11.5% separating gel. The antigens were then transferred to an Immobilon P nitrocellulose filter (Millipore, Eschborn, Germany), using a transfer buffer containing 48 mM Tris-base, 39 mM glycine, 1.3 mM SDS, 30% (v/v) glycerol and 15% (v/v) mercaptoethanol, and separated by SDS–PAGE with an 11.5% separating gel. The antigens were then transferred to an Immobilon P nitrocellulose filter (Millipore, Eschborn, Germany), using a transfer buffer containing 48 mM Tris-base, 39 mM glycine, 1.3 mM SDS 10% and 20% (v/v) methanol. A strip of the nitrocellulose filter was stained with anidro black, and the rest of the nitrocellulose filter was blocked by incubation for 60 min at 4°C with 5% (w/v) blocking milk (Bio-Rad, Munich, Germany), 10 mM Tris, pH 7.5 and 150 mM NaCl. The antigen-containing slots were then cut into 5 mm wide strips.

Serum samples were diluted 1:500 in antibody buffer containing 10 mM Tris, pH 7.5, 5% (w/v) milk and 150 mM NaCl. They were incubated with the nitrocellulose strips on a rocker for 1 h at 20°C. The strips were then washed five times with washing buffer containing 10 mM Tris, pH 7.5, 0.05 mM Tween and 150 mM NaCl. Bound
antibodies were detected by incubation with horseradish peroxidase-conjugated goat antibody to human IgG or IgA (heavy and light chains, bovine IgG or IgA absorbed; Bio-Rad) and, after washing, with horseradish peroxidase colour development reagent 3,3'-diaminobenzidine (Bio-Rad), according to the instructions of the manufacturer.

**Statistical analysis**

Groups were compared by a \( \chi^2 \) analysis with Yates' correction factor, Fisher's exact test and the Mann–Whitney \( U \)-test.

**Results**

Chlamydial LPS antigen was detected in one out of 29 urine specimens in group I and in two out of 16 urine specimens in group II. All endocervical and urethral samples were negative for chlamydial LPS or MOMP antigen.

All 34 patients with chronic salpingitis and/or salpingitis isthmica nodosa and tubal occlusions had chlamydial serum IgG antibody titres \( \geq 1:512 \) (Figure 2), with a geometric mean titre of 1:4096. Chlamydial serum IgA antibody titres \( \geq 1:16 \) were detected in 33 of these patients (97%), with a geometric mean titre of 1:256 (Figure 3). All 19 infertile patients without tubal occlusions had chlamydial serum IgG antibody titres \( \geq 1:128 \), with a geometric mean titre of 1:256 (Figure 2). Chlamydial serum IgA antibody titres \( \geq 1:16 \) were found in seven infertile patients without tubal occlusions (37%) (Figure 3). The median chlamydial serum IgA antibody titre was <1:16 in group II. There were significant differences between the median chlamydial serum IgG antibody titres \( P < 0.0001 \) and between the median chlamydial serum IgA antibody titres \( P = 0.0002 \) for both groups.

Serum IgG antibodies to C-hsp60 were detected in 24 out of 34 patients (71%) with chronic salpingitis and/or salpingitis isthmica nodosa and tubal occlusions compared with 10 out of 19 infertile patients (53%) without tubal occlusions (not significant; Table I).

Serum IgA antibodies to C-hsp60 were found in seven out of 34 patients in group I (21%), but in none of the 19 patients in group II \( (P = 0.035 \), Fisher's exact test). If only patients with chlamydial serum IgA antibody titres \( \geq 1:16 \) are included, seven out of 33 patients in group I (21%), but none of the seven patients in group II, had serum IgA antibodies to C-hsp60 (not significant; Table II).

Serum IgG antibodies to C-hsp70 were detected in 24 out of 34 patients in group I (71%) compared with nine out of 19 patients in group II (47%) (not significant; Table III).

With the exception of serum IgA antibodies to C-hsp60, the prevalences of serum IgG antibodies to C-hsp60 and to C-hsp70 were not significantly different between the two groups of patients. However, to detect significant differences at \( P < 0.05 \), a minimum of 54 patients would have been required in each group.

**Discussion**

Chlamydial LPS or MOMP antigen could not be detected in endocervical or urethral samples from infertile patients with chronic salpingitis and/or salpingitis isthmica nodosa and occluded Fallopian tubes or in infertile patients without tubal occlusions. However, chlamydial LPS antigen was found in one of 29 urine specimens in group I and in two out of 16 urine specimens in group II. Other bacterial urinary tract infections were excluded. The examination of first-void urine specimens was reported to be a sensitive and specific alternative to urethral swabs (Chernesky et al., 1990; Leonardi et al., 1992). Our results support the significance of urine testing for *Chlamydia*.

In both groups, endocervical and urethral chlamydial infections did not occur more frequently than in patients attending a German gynaecological clinic (Degen et al., 1990) or in randomly selected pregnant women in Germany (Hoyne, 1992). In previous studies, *C. trachomatis* could not be detected by culture in endocervical and/or urethral samples from infertile patients with tubal occlusions (Moore et al., 1982; Kane et al., 1984; Anestad et al., 1987; Sellors et al., 1988). Prevalences of 6% (Shepard and Jones, 1989) and 19% (Henry-Suchet et al., 1987) were reported for patients with tubal infertility. However, these patients did not necessarily present with tubal occlusions.

In a previous study, it was shown that chronic salpingitis and salpingitis isthmica nodosa with tubal occlusion are associated with the presence of chlamydial serum antibodies, indicating a past or present chlamydial infection (Dieterle et al., 1994). The pathogenic mechanism has yet to be elucidated. A lymphocyte proliferative response to conserved epitopes of human and chlamydial hsp60 was detected in patients with salpingitis (Witkin et al., 1993, 1994a). A humoral
immune response to chlamydial hsp60 was found to be associated with tubal infertility (Brunham et al., 1985; Toye et al., 1993; Arno et al., 1995). In this study, serum IgG antibodies to C-hsp60 and to C-hsp70 were detected more frequently in infertile patients with chronic salpingitis and/or salpingitis isthmic nodosa and tubal occlusion than in infertile patients without tubal occlusions. However, these differences were not statistically significant. The prevalence of serum IgG antibodies to C-hsp60 in our patients with chronic salpingitis and/or salpingitis isthmic nodosa and tubal occlusion was comparable with the results of three other studies using species-specific microimmunofluorescence to detect serum IgG antibodies to *C. trachomatis* MOMP in patients with tubal infertility (Table IV). In a study by Brunham et al. (1985), 11 out of 13 seropositive patients with tubal infertility versus two out of six patients with non-tubal infertility presented with serum IgG antibodies to hsp60. In a study by Toye et al. (1993), serum IgG antibodies to C-hsp60 were found in 26 out of 32 seropositive women (81%) with tubal obstructions, but in none out of nine seropositive patients with other causes of infertility. Arno et al. (1995) demonstrated serum IgG antibodies to C-hsp60 in 16 out of 21 seropositive patients (76%) with tubal infertility compared with two out of nine seropositive patients (22%) with non-tubal infertility. In contrast, the prevalences of serum IgG antibodies to C-hsp60 and C-hsp70 in our infertile patients without tubal occlusions were 53 (10/19) and 47% (9/19) respectively. However, it has to be considered that infertile patients without tubal occlusions but with serum IgG antibodies to C-hsp60 may have laparoscopically undetectable damages of the tubal mucosa contributing to their infertility. This variability of the immune response to chlamydial heat shock proteins may be influenced by genetic factors (Zhong and Brunham, 1992).

The presence of serum IgA antibodies to C-hsp60 has not been examined previously. Cervical IgA antibodies to C-hsp60 were found to be associated with an unsuccessful outcome after in-vitro fertilization (Witkin et al., 1994b). In this study, the prevalence of serum IgA antibodies to C-hsp60 was significantly higher in women with *Chlamydia*-associated chronic salpingitis and/or salpingitis isthmic nodosa and tubal occlusion than in infertile women without tubal occlusions (*P* = 0.035). Thus, the presence of serum IgA antibodies to C-hsp60 may reflect an immune response which could be involved in the pathogenesis of chronic salpingitis and/or salpingitis isthmic nodosa with tubal occlusion.

Patients with chronic salpingitis and/or salpingitis isthmic nodosa and tubal occlusion present with significantly higher chlamydial serum IgG and IgA antibody titres than infertile patients without tubal occlusions. Therefore, the presence of serum antibodies to C-hsp60 and C-hsp70 may not be independent of the antibody response to chlamydial structural antigen. In this study, there was no relationship between serum IgG antibody titres to chlamydial structural antigen and the presence of serum IgG antibodies to C-hsp60 (*P* = 0.299) or C-hsp70 (*P* = 0.150). This result corresponds with the study by Toye et al. (1993) who found no correlation between serum IgG antibody titres to *C. trachomatis* MOMP and serum IgG antibody concentrations to C-hsp60. However, a significant association was found in this study between the presence of serum IgA antibodies to C-hsp60 and serum IgA antibody titres to chlamydial structural antigen (*P* = 0.002). Thus, the possibility remains that the presence of serum IgA antibodies to C-hsp60 is not an independent marker of the humoral immune response.

Chlamydial heat shock proteins were suggested to be involved in the development of *Chlamydia*-associated reproductive sequelae (Brunham et al., 1985, 1992; Wagar et al., 1990; Toye et al., 1993). The association between the presence of serum IgA antibodies to C-hsp60 and chronic salpingitis and/or salpingitis isthmic nodosa with tubal occlusion supports the significance of chlamydial hsp60 in the pathogenesis of *Chlamydia*-related chronic inflammatory tubal disease.

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### References


S. Dieterle and J. Wollenhaupt


