Antibody Response to the Chlamydial Heat-Shock Protein 60 in an Experimental Model of Chronic Pelvic Inflammatory Disease in Monkeys (Macaca nemestrina)

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A primate model of chlamydial pelvic inflammatory disease was used to characterize serum antibody responses to the 60 kDa chlamydial heat shock protein (CHSP60). Forty monkeys were infected in the fallopian tubes with Chlamydia trachomatis and then were treated. Twenty-three (58%) monkeys developed antibodies against CHSP60, of whom 6 (15%) had CHSP60 responses that persisted throughout the study and 17 (42.5%) had a transient response. A persistent CHSP60 antibody response was correlated with being culture- or ligase chain reaction–positive in the fallopian tubes (P = .004), but not in the cervix pretreatment, and with being tubal-positive posttreatment (P = .02). Compared with tubal-negative monkeys, tubal-positive monkeys had more intense CHSP60 responses (P = .006) that lasted longer (P = .002). Among CHSP60 responders, an OD > 0.5 was correlated with more severe salpingeal pathology before treatment (P = .04). CHSP60 antibody response may be useful as a marker of persistent chlamydial infection in the fallopian tubes.

Genital chlamydial infection is a major public health problem worldwide. In the United States, it is estimated that 4 million cases occur annually, and 50,000 women a year become infertile as a result of chlamydial infection. Seroepidemiologic studies show a consistent association between antibody response to the 60-kDa chlamydial heat shock protein (CHSP60) and the development of pelvic inflammatory disease (PID), tubal infertility, or ectopic pregnancy [1–3]. For this reason, further study of the role of CHSP60 in the immunopathogenesis of fallopian tube damage has been pursued in animal models. In a monkey salpingeal autotransplant model, recombinant CHSP60 caused histopathologic changes characteristic of a delayed-type hypersensitivity reaction when injected into subcutaneous pockets containing salpingeal autotransplants of monkeys previously sensitized by Chlamydia trachomatis infection but not in immunologically naive monkeys [4]. However, the exact role of CHSP60 as a cause or a mediator of chronic inflammation and immunopathology remains to be defined.

CHSP60 antibody response may be a marker for persistent or repeated chlamydial infection that may lead to severe tubal damage over time. Repeated cervical chlamydial infection has been identified as a risk factor for human PID, as well as for tubal obstruction in a monkey model of salpingitis [3, 5]. In vitro studies have shown that in cell cultures persistently infected with C. trachomatis, CHSP60 is disproportionally expressed, compared with other chlamydial antigens such as the major outer membrane protein [6]. Thus, CHSP60 expressed during repeated or persistent infections may be the source of antigenic stimulation for a sustained antibody response to the protein. Other alternate, but not mutually exclusive, hypotheses include, first, that the CHSP60 antibody response is part of a highly polarized T cell response, which causes tissue fibrosis, as seen in schistosomiasis [7]. T cell responses to CHSP60 may mediate the inflammatory pathology associated with ocular and genital chlamydial infection. Studies from trachoma endemic areas showed that individuals with scarring trachoma have depressed T cell responses to chlamydial antigens, including CHSP60, compared with individuals who were able to resolve their chlamydial infections, suggesting that a predominantly Th2 response fails to clear chlamydial infection [8]. Second, it is also possible that CHSP60 or other chlamydial antigens may be involved in immune complex diseases that cause inflammation in the infected tissues [9]. Third, because HSPs are highly conserved, antibody response to CHSP60 may be a marker of autoimmune responses initiated by cross-reactivity.
between CHSP60 epitopes and host-cell HSP60 epitopes [10–12]. A recent study showed that antecedent antibody response to CHSP60 predicted a 2- to 3-fold increased risk of development of human PID, suggesting that CHSP60 itself may be a causal factor in the immunopathogenesis of chlamydial disease [13].

Several factors hamper studies to elucidate the underlying cause of immunopathology and the determinants of disease progression after a cervical chlamydial infection in humans. First, >50% of human genital chlamydial infections are asymptomatic, and even chlamydial upper genital tract infection is often clinically silent. The potential for tubal damage in women appears to be similar whether they are symptomatic or not [14]. Thus, antecedent cervical and subsequent upper genital tract infection may not be diagnosed until investigations are initiated for tubal factor infertility or ectopic pregnancy, by which time the damage is irreversible. Second, even in symptomatic infections, our ability to obtain swabs or tissue samples from the site of infection, to characterize disease progression, is limited. Third, prospective studies to follow women with untreated cervical chlamydial infection, to delineate the determinants of disease progression, are unethical. Moreover, the efficacy of various treatment regimens for PID has not been well established [15]. In particular, information on whether these treatment regimens have any significant effect on immunopathology, disease progression, and subsequent fertility is limited. In contrast, animal models of infection provide unique opportunities to study these complex interactions of microbial and host responses, with ready access to specimens at predetermined time intervals. Such samples may be difficult, if not impossible, to obtain in human studies.

In this study, we sought to characterize the CHSP60 antibody response in a well-established primate model of chronic chlamydial PID and to determine the effect of antimicrobial and antiinflammatory treatment on the antibody response to CHSP60. If the CHSP60 responses in this model are validated against data from human studies, then it should be possible to gain new insights into the role of CHSP60 in immunopathogenesis.

Materials and Methods

Animals. Forty sexually mature female pig-tailed macaques (Macaca nemestrina) were studied. The monkeys were housed in individual cages in the Regional Primate Research Center at the University of Washington. All monkeys were prescreened by cervical culture and serology, to rule out previous or current chlamydial infection, and by laparoscopy, to document normal adnexa before study enrollment.

Inoculation and treatment. Monkeys were inoculated with C. trachomatis serovar D, a human endometrial isolate, by direct inoculation through the fimbrial os by use of 0.15 ml of inoculum per tube [16]. Each inoculum contained $9 \times 10^5$ inclusion-forming units per ml in sucrose-phosphate glutamate buffer. All monkeys were inoculated 3× at 2-week intervals. One to 2 weeks after the third inoculation, the monkeys were randomized in a blinded fashion to 1 of 4 treatment groups and were treated for 10 days. The 4 treatment regimens were as follows: placebo; doxycycline (2.2 mg/kg/day orally) alone; doxycycline plus ibuprofen, a nonsteroidal antiinflammatory drug (20 mg/kg/day orally); or doxycycline plus triamcinolone, a steroidal antiinflammatory drug (0.2 mg/kg intramuscularly every 3 days).

Specimen collection and testing. At 2-week intervals throughout the study, blood samples were collected for the measurement of antibodies to C. trachomatis and CHSP60, and cervical swabs were collected for the detection of C. trachomatis by culture and by ligase chain reaction (LCR). Sera were tested for IgG and IgM antibodies against C. trachomatis by the microimmunofluorescence (MIF) assay and for IgG antibodies to CHSP60 by an enzyme immunoassay, by use of recombinant CHSP60 as the antigen [17, 18]. CHSP60 antibody response was measured by absorbance at 450 nm. An absorbance value (optical density [OD]) of $\geq 0.2$ was considered to be a significant response on the basis of a previously determined mean absorbance $\pm 3$ SD from the mean, by use of sera from 50 humans seronegative for C. trachomatis [18]. The presence of C. trachomatis in the upper genital tract was detected by culture and/or by LCR, by sampling the fimbrial os at every inoculation, and at hysterectomy, 12–16 weeks after completion of treatment. During the hysterectomy, tissues from the fimbria, ampulla, uterus, and cervix were taken for routine light microscopy, immunocytochemistry, and in situ hybridization, as described elsewhere [16, 19].

Visual assessment. Disease progression was visually scored and recorded by videomicroscopy of the upper reproductive tract at each tubal inoculation, at laparoscopy 5 days after completion of treatment, and at hysterectomy [16]. The scoring index for gross pathological changes before and after treatment were as follows: gross adhesion score 0, normal, no genital tract damage; 1, dilatation (edema) of fallopian tubes, erythema; 2, dilatation plus mild adhesions, peritubal; 3, dilatation plus moderate adhesions, peritubal and adnexal; and 4, dilatation plus severe adhesions, peritubal, periaxial, and peritoneal.

Statistical analyses. MIF titers were converted to log2 units. Comparisons of medians were determined by the Wilcoxon rank sum test [20]. All other tests of significance were made by $x^2$ or 2-tailed Fisher’s exact tests with Yates’s correction where appropriate, depending on sample size.

Results

Prior to infection, all 40 monkeys were seronegative for C. trachomatis antibodies, as determined by the MIF and the CHSP60 assays. After the first inoculation, 10 monkeys were IgM antibody positive against C. trachomatis in the MIF assay, 4 were IgG positive, and none were CHSP60 antibody positive. After the second inoculation, a cumulative total of 15 (23%) monkeys were IgM positive, 36 (90%) were IgG positive in the MIF assay, and 8 (20%) were positive for CHSP60 antibody. At the end of 3 inoculations, although all monkeys had developed IgG antibodies against C. trachomatis and 18 (45%) had IgM antibodies by the MIF assay, only 23 (58%) developed...
IgG antibodies against CHSP60. Thirty-eight (95%) monkeys were cervical swab-positive, and 24 (60%) were fimbrial swab-positive (i.e., tubal-positive) by culture or by LCR just prior to the initiation of therapy.

Three patterns of CHSP60 antibody response were observed during the 22-week study period (table 1). Of the 40 monkeys, 17 (42.5%) did not mount a significant CHSP60 antibody response (OD < 0.2, nonresponders), 17 (42.5%) had a transient response (OD > 0.2) lasting < 8 weeks, and 6 (15%) had significant levels of CHSP60 IgG (OD > 0.2) that persisted until the end of the study. Prior to initiation of therapy, the mean MIF IgG titer and the mean of the maximum IgG titer attained by the monkeys in each of these 3 groups were not significantly different. Persistence of CHSP60 antibody response correlated with being culture- or LCR-positive in the fallopian tubes (P = .004) but not with being cervical swab positive (P = .83).

The gross adhesion scores were also not different among the 3 groups prior to or after treatment. There was no difference in the gross adhesion scores between the treated and untreated animals, as reported elsewhere [16]. Eight monkeys remained C. trachomatis LCR- or culture-positive posttreatment. A persistent CHSP60 response was correlated with being culture- or LCR-positive in the fallopian tube at hysterectomy (P = .02).

Among the 23 CHSP60 responders, 12 were tubal-positive and 11 were tubal-negative. Of the 12 tubal-positive monkeys, 6 were persistent CHSP60 responders, 4 were transient responders, and 2 did not respond. Seven of the 11 tubal-negative monkeys were transient responders, and 4 were nonresponders. Tubal positivity was correlated with a persistent CHSP60 response (P = .024, Fisher’s exact test; figure 1). The mean duration of CHSP60 positivity was 88.4 ± 41.6 days for the tubal-positive monkeys versus 33.6 ± 24.9 days for the tubal-negative monkeys (P = .002). The CHSP60 response in tubal-positive

Table 1. Correlates of duration of chlamydial heat-shock protein 60 (CHSP60) antibody response in an experimental model of chronic pelvic inflammatory disease.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>None (n = 17)</th>
<th>Transient (n = 17)</th>
<th>Persistent (n = 6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean MIF IgG</td>
<td>3.3 ± 0.8</td>
<td>3.6 ± 0.6</td>
<td>4.3 ± 1.0</td>
<td>.06</td>
</tr>
<tr>
<td>Maximum MIF IgG</td>
<td>6.2 ± 1.0</td>
<td>6.2 ± 0.8</td>
<td>7.0 ± 0.9</td>
<td>.18</td>
</tr>
<tr>
<td>Tubal CT*</td>
<td>16 (94%)</td>
<td>16 (94%)</td>
<td>6 (100%)</td>
<td>.83</td>
</tr>
<tr>
<td>Gross adhesion score</td>
<td>3.0 ± 0.8</td>
<td>2.7 ± 0.8</td>
<td>2.7 ± 0.8</td>
<td>.57</td>
</tr>
<tr>
<td>Posttreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubal CT*</td>
<td>1 (6%)</td>
<td>4 (24%)</td>
<td>3 (50%)</td>
<td>.02</td>
</tr>
<tr>
<td>Gross adhesion scoreb</td>
<td>2.8 ± 0.8</td>
<td>2.6 ± 1.1</td>
<td>3.0 ± 0.9</td>
<td>.71</td>
</tr>
</tbody>
</table>

NOTE: Nonresponder, CHSP60 antibody assay absorbance < 0.2; transient responder, CHSP60 antibody assay absorbance of < 0.2 for < 8 weeks; persistent responder, CHSP60 antibody assay absorbance of < 0.2 until end of study; MIF, microimmunofluorescence; CT, Chlamydia trachomatis.

* Positive for C. trachomatis by culture or by ligase chain reaction.

b Values represent mean ± SD of visual gross adhesion scores for monkeys in each group. Score of 0, normal; 1, edema in fallopian tubes, erythema; 2, edema plus mild peritubal adhesions; 3, edema with moderate adhesions, peritubal and adnexal; 4, edema plus severe adhesion, peritubal, periaxial, and peritoneal.

The CHSP60 response in tubal-positive monkeys persisted for 50.6 ± 26.1 days after the last culture/LCR-positive result, compared with 18.4 ± 16 days in tubal-negative monkeys (P = .005). Tubal positivity was also correlated with a CHSP60 antibody response more intense than that of tubal-negative monkeys. The mean absorbance for CHSP60, measured in OD units, was 0.15 ± 0.06 (median, 0.16; range 0.07–0.25) for tubal-negative monkeys, compared with 0.32 ± 0.21 (median, 0.26; range 0.13–0.84) for tubal-positive monkeys (P = .006).

Among the 23 CHSP60 responders, a more intense antibody response (OD > 0.5) was correlated with a more severe gross adhesion score at laparoscopy pretreatment (P = .04) but not at posttreatment (table 2). Although there were more tubal-positive monkeys with an OD of > 0.5, compared with the group with an OD of 0.2–0.5, that difference was not significant (P = .06).

The pattern of CHSP60 antibody response was not different in the 4 treatment groups (P = .47; table 3). Of interest, al-
Pretreatment tubal CT, though 5 of 10 monkeys in the doxycycline only group were CHSP60 responders, none of them were persistent responders. In contrast, 2 of 5 responders in the doxycycline with triamcinolone group were persistent responders, as were 2 of 7 responders in the doxycycline with ibuprofen group and 2 of 7 in the placebo group. Antimicrobial therapy was initiated at week 6 of the study. Because of the small number of monkeys in each treatment group and the wide variation in the intensity of CHSP60 antibody responses prior to the initiation of treatment, it was not possible to determine whether antimicrobial treatment had an effect on CHSP60 antibody response.

Discussion

These results demonstrate heterogeneity in CHSP60 antibody responsiveness among experimentally infected primates. There were 3 types of CHSP60 antibody response among the monkeys: nonresponders, transient responders, and persistent responders. Similarly, heterogeneity in disease outcome after genital chlamydial infection has been observed in humans, since only a subset of infected women developed long-term sequelae of salpingitis, ectopic pregnancy, or tubal infertility [21]. These adverse outcomes have been strongly correlated with antibody responses to CHSP60. In studies of trachomatis scarring in trachoma endemic communities in the Gambia, CHSP60 antibody response was positively correlated with HLA class II allele DRB1*0701 and negatively correlated with DQB1*0301 and DQB1*0501 [22, 23]. However, since none of these alleles were directly correlated with evidence of disease, susceptibility to disease due to the genetic regulation of host immune response may be complex. Genetic restriction in immune responses to CHSP60 may partially explain the heterogeneity in disease outcome after genital chlamydial infection. All the animals in this study had extensive upper genital tract damage, as reported elsewhere [16], but only 58% of animals mounted a CHSP60 antibody response. Thus, other host or microbial determinants probably contribute to the development of immunopathology after genital chlamydial infection, at least in this model system.

This animal model provides an unique opportunity to determine whether CHSP60 response can be used as a marker of persistent chlamydial infection in the upper genital tract. Such an assay may be useful in identifying women at risk of developing an immunopathologic response leading to tubal occlusion [24]. Our results show that a prolonged or persistent CHSP60 antibody response is correlated with the presence of C. trachomatis in the fallopian tubes. Compared with the MIF response, the CHSP60 antibody response appeared to be a more specific marker for chlamydial tubal infection.

Animals with detectable C. trachomatis in their fallopian tubes were more likely to have an intense CHSP60 response that persisted longer. Among the CHSP60 responders, a more intense CHSP60 response was correlated with a more severe gross pathology before treatment. Similar findings were previously reported in human studies. In a prospective study of 157 women with a clinical diagnosis of PID, 73 women had laparoscopically confirmed salpingitis and were assessed for perihepatitis [25]. An OD of >0.5 in CHSP60 serum antibody response was detected in 12 (67%) of 18 women with laparoscopically confirmed perihepatitis, compared with 12 (28%) of 43 in the salpingitis only group (P = .005). The mean CHSP OD was also significantly higher in the perihepatitis group (P = .02). Perihepatitis was correlated with moderate to severe pelvic adhesions but not with peritoneal pus, suggesting a more intense inflammatory response in these patients than in women with salpingitis alone. In a study of human PID, the highest CHSP60 titers were found in 80% of women who had occluded tubes [2]. The strong association of CHSP60 antibody and tubal occlusion was independent of MIF IgG antibody, as was also shown in this study. Thus, an intense CHSP60 response can be an important marker to predict adverse outcome leading to tubal occlusion or pelvic adhesions. The similarity of disease outcome observed in this animal model to that in human studies makes this a useful model for further research on the role of CHSP60 in the pathogenesis of chlamydial disease, which may yield clues on strategies to prevent chlamydia-associated tubal infertility.

The origin of continued antigenic stimulation to sustain the antibody response to CHSP60 is undefined but presumably results from continued ongoing infection in the fallopian tubes. Evidence of C. trachomatis DNA and antigen have been found in the fallopian tubes of women with postinfectious tubal infertility [26]. MIF IgA responses in serum is also significantly correlated with the finding of CHSP60 IgG response in women with confirmed PID but not among women with acute chlamydial infection [2]. Because serum IgA has a short half-life, the data suggest that these women may be harboring a persistent chlamydial infection. Thus, the CHSP60 antibody response may be a marker of persistent chlamydial infection in the fallopian tubes, as was shown in this model.

Neither gross adhesion scores nor histologic pathology in these monkeys were affected by the treatment regimens, as reported elsewhere [16]. A study in mice showed that antiinflammatory agents also had little effect on chlamydial salpingitis

### Table 2. Correlates of intensity of chlamydial heat-shock protein 60 (CHSP60) antibody response in chronic pelvic inflammatory disease.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CHSP60 absorbance</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2-0.5</td>
<td>&gt;0.5</td>
<td>(n = 11)</td>
<td>(n = 12)</td>
<td>(n = 23)</td>
</tr>
<tr>
<td>Pretreatment cervical CT*+ (%)</td>
<td>9 (82)</td>
<td>10 (92)</td>
<td>.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment tubal CT*+ (%)</td>
<td>3 (27)</td>
<td>8 (67)</td>
<td>.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posttreatment tubal CT*+ (%)</td>
<td>2 (18)</td>
<td>5 (42)</td>
<td>.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gross adhesion score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>2.3 ± 0.7</td>
<td>3.0 ± 0.7</td>
<td>.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posttreatment</td>
<td>2.4 ± 1.3</td>
<td>3.0 ± 0.8</td>
<td>.28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Positive for Chlamydial trachomatis (CT) by culture or by ligase chain reaction.
tubal tissue was found in 1 of 10 doxycycline-treated monkeys in this study, compared with 6 of 10 placebo-treated monkeys and with 7 of 10 of each of the 2 groups of monkeys treated with doxycycline and anti-inflammatory drugs. Of interest, none of the monkeys in the doxycycline group were persistent CHSP60 responders. In contrast, 4 of 4 monkeys with persistent CHSP60 response were in the group treated with doxycycline and anti-inflammatory drugs, and all had evidence of persisting chlamydial antigen or DNA. It is possible that the anti-inflammatory drugs may have affected the efficacy of doxycycline or the immune system of the host, or both, in eradicating the organism [16].

We had anticipated that if persistent chlamydial infection, characterized by antibody response to CHSP60, was the cause of immunopathology in acute chlamydial PID, then antimicrobial treatment that eradicates the infection should be effective in modulating the antibody response and the subsequent development of tubal pathology. On the other hand, if CHSP60 itself is the cause of inflammation in the absence of infection, possibly because of an autoimmune response to the human HSP60, then the effect of treatment on CHSP60 antibody response and immunopathology would be limited. In the present study, the wide variation in the intensity of the CHSP60 antibody response prior to treatment and the small number of monkeys in each treatment group made it impossible to draw any conclusions on the effect of antimicrobial treatment on CHSP60 response. However, as shown in table 2, a more intense CHSP60 antibody response was correlated with a more severe gross adhesion score pretreatment, and this correlation was not observed after treatment, suggesting a possible relationship among treatment, CHSP60 response, and tissue damage, although the precise nature of the relationship remains unclear.

In conclusion, the results of this study suggest that the CHSP60 antibody response may be useful as a marker of persistent infection in the fallopian tubes and therefore may be useful to identify women at risk of the development of tubal pathology. In such women, it may be possible to initiate intervention, such as more aggressive antimicrobial treatment, before tubal occlusion occurs. The efficacy of antimicrobial regimens presently recommended for the treatment of human chlamydial PID is largely based on the resolution of symptoms. Whether the infection or organism is actually eradicated from the fallopian tubes, hence preserving fertility, is uncertain. Hence, further studies using this animal model to explore the relationship among antimicrobial treatment, CHSP60 antibody response, and the prevention of tissue damage in chronic chlamydial PID are warranted.

Acknowledgment

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References


