MODELS OF INFECTION

Factors influencing the induction of infertility in a mouse model of Chlamydia trachomatis ascending genital tract infection

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In women, infections due to Chlamydia trachomatis frequently result in long-term sequelae including chronic abdominal pain, ectopic pregnancy and infertility. In an attempt to characterise the pathogenesis of the infection, female C3H (H-2k) mice were inoculated intravaginally with different doses of C. trachomatis and then mated with proven male breeder mice. The inoculated mice developed a broad spectrum of clinical manifestations ranging from infertility to asymptomatic shedding. The dose inducing infertility in 50% of the mice was c. 10^5 inclusion-forming units of C. trachomatis. In another group of mice sampled at intervals after intravaginal inoculation, C. trachomatis was recovered from the upper genital tract starting at 24 h after infection. A higher percentage of animals infected during the luteal phase of the oestrous cycle had positive cultures from the middle and upper genital tract than when mice were inoculated during the follicular phase. These results indicate that rapid therapeutic intervention is required to avoid the sequelae resulting from C. trachomatis genital infection, and suggest that hormonal factors play a role in the pathogenesis of the disease.

Introduction

Chlamydia trachomatis is one of the most common human pathogens, second only to Trichomonas vaginalis as a causative agent of a sexually transmitted disease [1-3]. In women, cervicitis is the most prevalent clinical manifestation of chlamydial genital tract infection. The disease is frequently self-limited and the patient recovers without long-term sequelae [1-3]. However, in a number of cases, involvement of the upper genital tract may occur, leading in some individuals to long-term sequelae including chronic pain, ectopic pregnancy and infertility [1-3]. The pathogenesis of the infection, and particularly of the long-term sequelae, is poorly understood. Thus, there is significant interest in developing animal models that will facilitate the characterisation of the disease with the long-term goal of implementing preventive measures in the human population.

Several animal models are now available for the investigation of chlamydial genital tract infection [4-9]. For example, Barron et al. [4], used the C. trachomatis mouse pneumonitis (MoPn) biovar to demonstrate that intravaginal inoculation of white Swiss mice resulted in a genital tract infection that lasted for c. 4 weeks as determined by the isolation of the organism from vaginal swabs. Subsequently, Swenson et al. [5] and Swenson and Schachter [6] inoculated the upper genital tract of Swiss mice with the C. trachomatis MoPn biovar and were able to demonstrate that the animals developed salpingitis and long-term sequelae including infertility. Furthermore, Tuffrey et al. [7] showed that in mice pretreated with progesterone, intrauterine or intra-bursal inoculation with some of the human serovars of C. trachomatis resulted in infertility.

As an extension of the model described by Barron et al. [4], a recent study from this laboratory showed that intravaginal inoculation of three inbred strains of mice, BALB/c (H-2^k), C3H (H-2^k) and C57BL/6 (H-2^b), with the C. trachomatis MoPn biovar resulted in salpingitis and infertility [8]. Of the three strains of mice, C3H had the most severe disease and the highest rate of infertility. Thus, further investigation of the pathogenesis of C. trachomatis infection in this strain of mice was undertaken with the aim of gaining a better understanding of the factors involved in the long-term sequelae, in particular infertility.
Materials and methods

Organisms

The C. trachomatis MoPN biovar (strain Nigg II), obtained from the American Type Culture Collection (Rockville, MD, USA), was grown in HeLa-229 cells and elementary bodies were purified as described previously [9]. Stocks of the organism were frozen at −70°C in 0.2 M sucrose, 20 mM sodium phosphate (pH 7.2), 5 mM glutamic acid (SPG) [9].

Staging of the oestrous cycle

Vaginal smears were obtained by placing 100 μl of phosphate-buffered saline (PBS: 0.01 M sodium phosphate, pH 7.2; 0.15 M sodium chloride) in the vagina and then aspirating the fluid with a 250-μl micropipette. The vaginal aspirate was transferred to a glass slide; the cells were fixed with methanol and stained with Diff-Quik solution II (Baxter Healthcare, Miami, FL, USA). The criteria for staging were those proposed by Rugh [10].

Animal studies

C3H/HeN Sim (H-2b) female mice (7–8 weeks old) were obtained from Simonsen Laboratories (Gilroy, CA, USA), and kept in isolation cubicles at a constant temperature of 24°C with a cycle of 12 h of fluorescent light and 12 h of darkness.

To ascertain the dose needed to induce infertility, five groups of female mice, under Metofane (methoxyflurane; Mallinckrodt Veterinary Inc.; Mundelein, IL, USA) anaesthesia were inoculated intravaginally, with 10^1, 10^3, 10^5 or 10^7 inclusion-forming units (ifu) of C. trachomatis MoPN per mouse in 20 μl of SPG medium. As a control, mice were inoculated intravaginally with 20 μl of SPG medium alone. Vaginal cultures were taken at weekly intervals to ascertain shedding [9]. At 6 weeks after inoculation, a proven male-breeder mouse (Simonsen Laboratories) was placed in a cage with five female mice and the animals followed as described elsewhere [9]. Briefly, pregnancy was determined by measuring the weight gained and the mice that were considered pregnant were killed. After the first mating, female mice that were not pregnant were mated again with a male mouse that had successfully mated with another group of female mice and pregnancy was followed as above. The number of embryos was counted at the time the female mice were killed. Each group included 10 mice and the experiment was done twice.

To determine the kinetics of migration of C. trachomatis ifu in the genital tract, mice under Metofane anaesthesia were inoculated intravaginally, at 6 h into the diurnal cycle, with 3 × 10^7 ifu of C. trachomatis MoPN in 20 μl of SPG. Control mice were inoculated with 20 μl of SPG alone. Two mice were killed at 15 min, 1 h, 4 h, 7 h, 24 h, 36 h, 2 days, 3 days, 5 days, 6 days and 7 days after inoculation. The genital tract was divided into three portions: the upper section comprising the ovary and the oviduct, the middle corresponding to the body of the uterus, and the lower part including the cervix and upper region of the vagina. Each section of the tissue was suspended in 2 ml of SPG and minced with a tissue homogeniser (Tekmar Co, Cincinnati, OH, USA). Samples were inoculated on to McCoy cell monolayers and cultured as described previously [9]. A blind passage of the infected culture at 48 h was performed. All monolayers were stained with a hyperimmune rabbit polyclonal serum raised against C. trachomatis MoPN elementary bodies [8]. The limit of detection of this culture system was estimated to be 10^3 ifu of C. trachomatis/ml. In parallel, 14 other mice were similarly inoculated and, after 3, 5, 7, 14, 22 and 42 days, two or three mice were killed for histopathological analyses.

To further characterise the kinetics of migration, and to determine the effects of the oestrous cycle on the establishment of a C. trachomatis MoPN infection, vaginal smears were collected in the middle of the nocturnal light cycle and the mice were immediately inoculated intravaginally with 3 × 10^7 ifu of C. trachomatis MoPN; 100 mice were included in the experimental group and 20 mice were assigned to a control group inoculated with SPG medium alone. Half of the mice were killed at 36 h and the other half at 7 days after inoculation and samples from the genital tract were cultured as described above. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Immunoassays

The C. trachomatis MoPN specific antibody titre was measured by an indirect inclusion immunofluorescence assay (IFA) as described previously [11]. Briefly, C. trachomatis MoPN-infected McCoy cells were fixed with acetone, serial two-fold dilutions of the sera and incubated with serial two-fold dilutes of the sera. As a second antibody, a fluorescein-labelled goat anti-mouse polyclonal antibody diluted with PBS and containing Evan's blue 0.02 % w/v was added. The slides were observed with an epi-fluorescence microscope.

Histopathological analyses

Tissues were fixed in buffered formalin and stained by standard techniques.

Statistical analysis

The two-tailed unpaired Student's t test and Fisher's exact test were employed for statistical analyses with the software programme Statview IV (Abacus, Berkeley, CA, USA).
Results

Dose of intravaginal inoculum of *C. trachomatis* MoPn required to induce infertility

Mice were inoculated intravaginally with inocula ranging from $10^7$ to $10^9$ ifu of *C. trachomatis* per mouse. Vaginal cultures and blood samples were collected at weekly intervals. All mice were mated at 6 weeks after inoculation. As shown in Table 1, all mice from the groups inoculated with $10^7$ and $10^8$ ifu of *C. trachomatis* were culture-positive at least once during the course of the experiment; 95% and 35% of the mice inoculated with $10^6$ and $10^4$ ifu of *C. trachomatis*, respectively, shed chlamydia during the period of observation. All mice ceased shedding by 35 days post-inoculation. All individual serum samples collected from these animals including those that were culture negative, showed anti-chlamydial antibodies starting at 2 weeks after inoculation. The geometric mean inclusion IFA antibody titres at day 42 after inoculation for the groups inoculated with $10^7$, $10^6$, $10^5$ and $10^4$ ifu of *C. trachomatis* were 3377, 1940, 3377 and 970, respectively. Vaginal cultures and antibody titres were always negative in control mice.

As shown in Table 1, of the mice inoculated with $10^7$, $10^6$ and $10^5$ ifu, 80%, 70% and 45%, respectively, became infertile in contrast with 10% in the control group (p<0.05). The percentage of animals that hydrosalpinx was also higher in the mice inoculated with the three high doses of *C. trachomatis* than in the control group. The mean number of embryos per mouse was 0.8, 1.7 and 3.5 for the groups inoculated with $10^7$ and $10^6$ ifu of *C. trachomatis*, respectively, whereas in the control group the mean number of embryos was 7.4 (p<0.01). When the mean number of embryos in both uterine horns per pregnant mouse was calculated, there was a statistically significant difference only for the groups inoculated with $10^7$ and $10^6$ ifu of *C. trachomatis* when compared with the control group.

However, when the mean numbers of embryos in the right or left uterine horn in the fertile mice were compared there was no difference between the animals infected with *C. trachomatis* and the control group inoculated with SPG alone. No statistically significant differences in the number of embryos, number of infertile mice or number of mice with hydrosalpinx, were found between the group inoculated with $10^6$ ifu of *C. trachomatis* and the control group.

<table>
<thead>
<tr>
<th>Dose of <em>C. trachomatis</em> MoPn ifu</th>
<th>Mean number of embryos/mouse (SD) in group</th>
<th>Number of mice that shed antibodies (%)</th>
<th>Number of mice with hydrosalpinx (%)</th>
<th>Number of infertile mice (%)</th>
<th>Mean number of embryos/mouse (SD) of pregnant mouse</th>
<th>Isolation of <em>C. trachomatis</em> from different regions of the genital tract*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^7</td>
<td>0.8 (1.6)</td>
<td>15 (75)</td>
<td>16 (80)</td>
<td>3.8 (1.3)</td>
<td>3.6 (1.5)</td>
<td>Lower</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 (100)</td>
<td>1 (50)</td>
<td>28 (56)</td>
<td>4.0 (0)</td>
<td>Middle</td>
</tr>
<tr>
<td>10^6</td>
<td>1.7 (3.1)</td>
<td>8 (40)</td>
<td>9 (45)</td>
<td>5.6 (3.0)</td>
<td>3.3 (1.0)</td>
<td>Upper</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 (100)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>3.5 (2.4)</td>
<td></td>
</tr>
<tr>
<td>10^5</td>
<td>3.5 (3.6)</td>
<td>3 (15)</td>
<td>3 (15)</td>
<td>6.4 (2.3)</td>
<td>4.7 (1.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 (95)</td>
<td>2 (10)</td>
<td>8.1 (2.2)</td>
<td>4.5 (1.6)</td>
<td></td>
</tr>
<tr>
<td>10^4</td>
<td>6.8 (3.6)</td>
<td>3 (15)</td>
<td>2 (10)</td>
<td>8.0 (2.2)</td>
<td>0.0 (1.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 (75)</td>
<td>2 (10)</td>
<td>4.0 (2.2)</td>
<td>4.2 (1.5)</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05 by the Fisher’s exact test and ^p<0.05 by the Student’s t test, compared with the value for the control mice.

Effect of the oestrous cycle on the establishment of a *C. trachomatis* genital infection

Vaginal smears were collected c. 5–6 h into the nocturnal light cycle. The mice were then immediately inoculated intravaginally with $3 \times 10^7$ ifu of *C. trachomatis* and killed at 36 h and 7 days after inoculation. Animals were divided into two groups, those inoculated...
during pro-oestrus and oestrus, also called follicular phase, the only stage at which mice mate, and those inoculated at metoestrus and dioestrus, also termed the luteal phase. As shown in Table 3, C. trachomatis was recovered from the middle and upper genital tract from a lower percentage of mice inoculated during the follicular phase than from the groups inoculated during the luteal phase. For example, at 7 days after inoculation, cultures were positive from the middle and upper genital tract in 73.6% and 63.1%, respectively, of the mice inoculated during the follicular phase, whereas C. trachomatis was recovered from 96.7% and 93.5% of the mice inoculated during the luteal phase (p < 0.05).

**Histopathological analyses**

Sections from the lower, middle and upper genital tract were obtained from mice inoculated with 3 x 10^7 ifu of C. trachomatis, and examined for histopathological changes. By 7 days after inoculation a mild acute inflammatory reaction, mostly perivascular, consisting of polymorphonuclear leucocytes was observed infiltrating the endometrium, and the mucosal and muscular layers of the fallopian tubes. By 14 days after inoculation, the polymorphonuclear infiltrate was very extensive, particularly in the upper genital tract, occupying the lumen and penetrating through all the layers of the oviduct with abscess formation (Fig. 1). The acute inflammatory infiltrate started to subside by the third week after inoculation and was progressively replaced by a mononuclear infiltrate with a significant number of plasma cells. By 42 days after inoculation, on gross examination, most of the animals had a unilateral or a bilateral hydrosalpinx. On histological examination, the tubes with hydrosalpinx had a dilated lumen and flattening of the mucosal layer. The specimens from the control mice remained within normal limits during the course of observation.

**Discussion**

C. trachomatis genital tract infections represent one of the most common preventable causes of infertility in women [1-3]. As a significant number of C. trachomatis genital infections in women are clinically silent, the ultimate goal should be to develop a vaccine to prevent the infection or at least the sequelae of the disease [12]. However, in the meantime a better understanding of the pathogenesis of the disease is required in order to implement more effective therapeutic measures.

In an attempt to ascertain the factors that are important in the pathogenesis of infertility, this study used a mouse model of intravaginal inoculation with C. trachomatis that closely resembles an ascending genital tract infection in women [12]. In the C3H mice there was a dose-response to C. trachomatis and the higher the inoculum the higher the percentage of animals that became infertile. The infertility could have been due, among other causes, to a complete obstruction of the oviduct, or to an alteration of the implantation site. However, it was observed, that in mice that became pregnant after inoculation with a high dose of C. trachomatis the total number of embryos in both uterine horns was lower than in the control group. Nevertheless, the average number of embryos in each gravid uterine horn in mice inoculated with C. trachomatis was the same as that in the control group. These results indicate that, in the oviducts that were patent, a normal pregnancy occurred and that the infertility observed was most likely due to an obstruction of the oviduct. This supports the findings by Tuffrey et al. [7], based on a model of intrabursal challenge, that suggested that infertility following chlamydial salpingitis was caused by a transportation failure of the egg to the oviduct.

Another observation worth pursuing is that obtained with the mice inoculated with 10^4 ifu of C. trachomatis. In this group of mice the fertility rate, cases of hydrosalpinx and number of embryos were not significantly different from those of the control group. However, most probably all these animals had a chlamydial infection – as shown by the presence of vaginal shedding in 35% of the mice and by the detection of chlamydial antibodies in all of them. It would be interesting to determine if multiple intravaginal inoculations with 10^4 ifu leads to infertility. In a monkey model of chlamydial genital tract infection, multiple vaginal inoculations resulted in more severe pathological changes, although the long-term sequelae were not evaluated [13]. Thus, the dose-response results in this murine model suggest that, as most probably occurs in women, different inocula lead to

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**Table 3. Effect of the oestrous cycle on the recovery of C. trachomatis from the lower, middle and upper genital tract**

<table>
<thead>
<tr>
<th>Oestrous phase at time of inoculation</th>
<th>Number of culture-positive mice (%)</th>
<th>36 h after inoculation</th>
<th>7 days after inoculation</th>
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<tbody>
<tr>
<td></td>
<td>n Lower Middle Upper</td>
<td>n Lower Middle Upper</td>
<td></td>
</tr>
<tr>
<td>Follicular phase (pro-oestrous + oestrous)</td>
<td>6 4 (66.6) 1 (16.6) 0 (0)</td>
<td>19 18 (94.7) 14 (73.6) 12 (63.1)</td>
<td></td>
</tr>
<tr>
<td>Luteal phase (metoestrus + dioestrous)</td>
<td>44 39 (88.6) 32 (72.7)* 28 (63.6)*</td>
<td>31 31 (100) 30 (96.7)* 29 (93.5)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n, Number of mice tested.</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>*p &lt; 0.05 by the Fisher's exact test, compared with the mice in the follicular phase.</td>
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</table>
different degrees of disease, depending upon the environmental and genetic factors of the individual. This is in contrast to the guinea-pig model, where a dose-response was not observed [14].

It is important to evaluate the time taken for *C. trachomatis* MoPn ifu to migrate from the vagina to the upper genital tract, as it may determine the urgency required for the implementation of appro-

Fig. 1. Histopathological sections of the genital tract of mice 14 days after inoculation with *C. trachomatis* (a, b) compared with a control section (c). a, note the heavy acute inflammatory infiltrate permeating all the layers and the lumen of the oviduct and resulting in abscess formation in the ovarian bursa (×45). b, the lumen of the oviduct is filled with neutrophils and necrotic exudate (×680). c, normal appearance of the genital tract (×45). Arrows, oviduct; arrow-heads, ovary.
priate therapy and thus affect the immediate and long-term sequelae of the infection. In the mouse model, the organism could be recovered from the lower genital tract from the time of inoculation until the end of the experiment 7 days later. This suggests that the original inoculum remains viable at least until a new round of infectious elementary bodies is generated every 36–72 h as a result of the infection. Infectious particles were detected in the upper genital tract within 24 h of inoculation, indicating that it takes only a short time for the organism to migrate to the upper genital tract. As the limit of detection of this culture system is c. 10^5 ifu/ml, it is probable that viable C. trachomatis can break the cervical mucus barrier and migrate to the oviduct in <24 h. This finding is consistent with those of other studies performed in women indicating that particulate material placed in the vagina can be recovered from the peritoneal cavity within 30 min [15]. In mice (but not in women), the oviduct is not open to the peritoneal cavity and thus it is possible that an increased resistance may limit the migration of the organisms upward in the genital tract.

On the other hand, Rank et al. [16] performed a similar experiment by inoculating guinea-pigs intravaginally with the C. psittaci agent of guinea-pig inclusion conjunctivitis (GPIC). As in the present study, they were able to recover the organism from the cervix immediately after inoculation. However, <10% of their specimens from the uterine cavity were positive at 3 days after inoculation and at 5 days after inoculation only c. 15% of the fallopian tube specimens were positive. These conflicting findings may represent a true biological difference between the animal models, or they may be due to the different methodologies used. The biology of the infection in the guinea-pig system may be different, as there is no evidence to indicate that inoculation with C. psittaci results in salpingitis, unless the animals are pretreated with oestradiol or an immunosuppressive agent. Furthermore, there are no data to indicate that long-term sequelae, including infertility, occur in this model [14, 16].

The oestrous cycle and sex hormones appear to play a significant role in the pathogenesis of chlamydial infection. For example, Ito et al. [17] found that mice inoculated intravaginally during dioestrus with the human C. trachomatis H serovar developed cervicitis in 50% of the cases, while those inoculated during oestrus did not become infected. Unfortunately, the infection was apparently only local and thus the effects of oestrous cycle on the upper genital tract infection could not be addressed [17]. In the present study with the C. trachomatis MoPn biovar in a murine model, the role of the oestrous cycle on the progression of the infection into the upper genital tract was investigated. During the follicular phase of the cycle mice were less prone to develop an upper genital infection than during the luteal phase, a result in agreement with that reported by Ito et al. [17]. On the other hand, in the guinea-pig model, Rank et al. [16] observed that animals inoculated on day 11 after ovulation, a time corresponding to the late follicular phase of the cycle, had more severe inflammation and fibrosis of the mesosalpinx than animals inoculated 6 and 16 days after ovulation. These authors indicated that because it takes 6 days for C. psittaci to reach the upper genital tract in their model, by that time the oestradiol levels will be at their peak and this may explain the increased histopathological findings.

Other in-vivo studies have attempted to assess the effects of different sex hormones on the pathogenesis of genital tract infections. For example, studies characterising the cervical mucus during the menstrual cycle have shown that, in the follicular phase, the mucus is abundant and watery with a parallel arrangement of the glycoproteins, whereas during the luteal phase the water content is low and the glycoproteins are distributed in an interlacing network [18]. If the cervical mucus plays a role in the pathogenesis of pelvic inflammatory disease, this would suggest that C. trachomatis may more easily migrate through this barrier to the upper genital tract during the follicular phase than during the luteal phase. However, acute salpingitis has been found to start more often during or shortly after menstrual bleeding than in the luteal phase [19]. The apparent protective effects of progesterone against ascending infections have been explained by the luteal-type cervical mucus as well as by the inactive endometrium induced by this hormone. In support of these findings, Barron et al. [20] reported that guinea-pigs infected with C. psittaci GPIC and treated with oestrogen-dominant oral contraceptives had more cases of hydrosalpinx than the controls, while Pasley et al. [21] found no effect of progesterone on the pathogenesis of the infection. However, Tuffrey et al. [7] have shown that the human serovars of C. trachomatis can induce a more severe genital infection in mice pretreated with progesterone. The reason for this effect could be the local action of progesterone in the genital tract, e.g., by increasing the susceptibility of the epithelium to an infection with C. trachomatis, or by the generalised immunosuppressive effect of this hormone [22]. In this respect women using combined oral contraceptives have been found to have more cervical cultures positive for C. trachomatis than non-users [23]. However, women infected with C. trachomatis while using these contraceptives do not develop pelvic inflammatory disease as often as non-users, although the fertility prognosis appears to be similar in both groups [1, 24, 25].

In conclusion, the factors involved in the pathogenesis of C. trachomatis infection are fairly complex and not clearly understood. As shown here, the rapid migration of C. trachomatis to the upper genital tract indicates that therapeutic measures have to be implemented as soon as possible to prevent or minimise long-term
References


C. TRACHOMATIS INFECTION AND INFERTILITY 605


