Effects of inoculating dose on the kinetics of Chlamydia muridarum genital infection in female mice

Alison J Carey, Kelly A Cunningham, Louise M Hafner, Peter Timms and Kenneth W Beagley

Chlamydia trachomatis infections have been implicated in problems such as pelvic inflammatory disease and infertility in females. Although there are some studies examining the kinetics of ascending infection, there is limited information on the kinetics of pathology development and cellular infiltrate into the reproductive tissues in relation to the effects of inoculating dose, and a better understanding of these is needed. The murine model of female genital tract Chlamydia muridarum infection is frequently used as a model of human C. trachomatis reproductive tract infection. To investigate the kinetics of ascending genital infection and associated pathology development, female BALB/c mice were intravaginally infected with C. muridarum at doses ranging from 5×10^2 to 2.6×10^6 inclusion forming units. We found that the inoculating dose affects the course of infection and the ascension of bacteria, with the highest dose ascending rapidly to the oviducts. By comparison, the lowest dose resulted in the greatest bacterial load in the lower reproductive tract. Interestingly, we found that the dose did not significantly affect inflammatory cell infiltrate in the various regions. Overall, this data show the effects of infectious dose on the kinetics of ascending chlamydial infection and associated inflammatory infiltration in BALB/c mice.

Immunology and Cell Biology (2009) 87, 337–343; doi:10.1038/icb.2009.3; published online 10 February 2009

Keywords: Chlamydia infection; female reproductive tract; inflammation

Chlamydia trachomatis is the most common sexually transmitted disease worldwide, causing a large socioeconomic burden on health-care systems. The World Health Organization (WHO) estimates that 92 million new chlamydial infections are detected each year. It is estimated that up to 70% of infections in females and 50% in males are asymptomatic, causing sequelae such as pelvic inflammatory disease (PID) and epididymitis, respectively. The rise in genital chlamydial infections has coincided with the rise not only in PID but also in ectopic pregnancy, tubal infertility and salpingitis. The health-care cost associated with infections is estimated to be between US$2–10 billion each year, with PID alone estimated to cost US$5.5 billion annually.

There has been a significant amount of research into the development of an efficacious vaccine in animal models (reviewed in Hafner's et al. work). However, there is little consistency in the infectious challenge dose used, with the doses ranging from 1.5×10^5 to 1×10^7 inclusion-forming units (i.f.u.). Rank et al. estimated the transmission dose of Chlamydia caviae in guinea pigs to be 10^2 i.f.u., after examining the levels and progression of infection in female guinea pigs acquired through mating experiments. The variation in inoculating dose is a problem, as it is unclear how the infectious dose will alter the disease outcomes and the response of the animal.

There is a basic understanding of the cellular pathogenesis of Chlamydia (reviewed in Stephens), with limited information about the kinetics of ascending infection and the associated pathology development. A chlamydial infection induces an influx of inflammatory cells, including neutrophils, T cells, B cells and macrophages, which are stimulated by the production of proinflammatory cytokines and chemokines. Studies show that even low levels of infection induce a profound immune response. Ex vivo studies using human fallopian tube tissues have indicated that interleukin-1 (IL-1) is the initial proinflammatory cytokine activated by a chlamydial infection and confirm that this cytokine is involved in tissue destruction. Acute and chronic/persistent infections can promote foci of inflammatory responses along with promoting tissue remodeling, cellular proliferation and healing that, if persist, lead to scarring. Although there is a role for an adaptive immune response in chlamydial disease, it is secondary to the secretion of proinflammatory cytokines and chemokines from infected non-immune cells.

However, the exact time frame of chlamydial ascension along the female reproductive tract and the level of infection required to induce this response are not known. Knowledge of the kinetics of infection is essential to aid in the development of a vaccine, and would show how the challenge dose affects the final outcomes of the disease. A study by Maxion et al. has shown that the infectious dose modulates the innate immune response and that an increased level of infection correlates with a decrease in oviduct sequelae. In a murine model, the effects of infection can vary depending not only on the inoculating

School of Life Sciences and Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland, Australia

Correspondence: Professor KW Beagley, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Ave, Kelvin Grove, Brisbane, Queensland 4059, Australia.

E-mail: k2.beagley@qut.edu.au

Received 9 October 2008; revised 8 December 2008; accepted 12 December 2008; published online 10 February 2009
dose and the serovar of Chlamydia but also on the age of the animal, the mouse strain used and the hormone levels present.

As there is limited information on the effects of inoculating dose on the kinetics of C. muridarum genital infection and its associated pathology development, this study aimed to examine these in a murine model.

RESULTS

The infectious dose affects the course of vaginal shedding in mice

To monitor the course and degree of infection in the mice, vaginal swabs were collected every 3 days and were cultured on McCoy cells; the cutoff for a productive infection was set at 300 i.f.u. The mid (5 × 10^4 i.f.u.) and high (2.6 × 10^6 i.f.u.) dose infections caused an initial infection three-fold greater (P < 0.001) than the low dose, which decreased rapidly by day 9 post infection (p.i.) (Figure 1). The animals that received these two doses reached the cutoff level by day 35 p.i. In contrast, the low dose (5 × 10^2 i.f.u.) infection group shed significantly greater (P < 0.05) levels of Chlamydia 9 days p.i. On day 35 p.i., this group was still infected, secreting 18-fold more i.f.u. than the mid and high dose animals, but was not significantly different. By day 42 p.i., the low dose group reached the cutoff level.

The infectious dose affects the ascension of Chlamydia in the murine female reproductive tract

Although swab collection and analysis allows the level of bacterial shedding in the lower genital tract to be determined, the removal and culture of tissues allows the level of viable bacteria present within the submucosal layers of all regions of the genital tract to be examined. Culture of the cervico-vaginal region revealed that although the difference between the mid and high inoculating dose is quite large, the level of infection that occurred in the tissues was not significantly different (Figure 2a). In both groups, infection was not detected in the cervico-vaginal tissues from day 21 p.i. However, the low dose group had a significantly higher initial infection (day 6 p.i.) in this region than the mid (P < 0.01) and high dose (P < 0.05) groups.

In the uterine horn tissues (Figure 2b), the low dose group had a similar course of infection as the mid and high doses, with the exception of a peak in infection at day 9 (P < 0.001), which was not observed with the other doses. The chlamydial burden within the uterine horns for all three doses was much lower than that observed in the cervico-vaginal region and oviducts.

The oviduct tissues had the greatest chlamydial burden of any of the regions. The high dose had a 7.5- to 13-fold greater infection (P < 0.001) than the mid and low doses, respectively, on day 6 p.i. (Figure 2c). The mid and low dose groups had a similar degree of

---

**Figure 1** Course of infection determined by vaginal swabs after vaginal inoculation with varying doses of *Chlamydia muridarum*. Vaginal swabs were collected on days 6, 9, 12, 15, 21, 35 and 42 after vaginal infection to determine levels of viable organisms by McCoy cell culture. Data are mean ± s.e. of mean for 10 mice, from two separate experiments, with a productive infection classified as > 300 i.f.u. Two-way ANOVA was performed with Bonferroni’s post-test. *P < 0.05* (low dose compared with high dose); †P < 0.001 (low dose compared with mid-dose).

**Figure 2** Kinetics of infection in (a) cervico-vaginal, (b) uterine horn and (c) oviduct tissue homogenate culture after vaginal inoculation with varying doses of *Chlamydia muridarum*. Tissues collected at various time points were homogenized, equal amounts cultured on McCoy cell monolayers and i.f.u./mg of tissue was determined. Data are mean ± s.e. for 10 mice. Two-way ANOVA with Bonferroni’s post-test was performed. *P < 0.05*; †P < 0.01; and ‡P < 0.001.
infection. From day 9 p.i., all three groups had the same pattern of infection. Interestingly, the chlamydial burden within the oviducts was higher than that seen in the uterine horn and cervico-vaginal tissues for all three doses during the early stages of infection.

Overall, the infectious dose affects the degree of ascending infection in the murine female reproductive tract (FRT), with rapid ascension to the oviducts observed in mice that received the high dose, despite the low dose causing greater infections in the lower regions of the reproductive tract.

The infectious dose affects the development of pyosalpinx but not hydrosalpinx
Pyosalpinx is defined as the oviduct containing pus and is often a result of acute salpingitis. Hydrosalpinx is defined as dilatation of the oviducts and luminal filling with a clear serous fluid and is used as an indicator of infertility. The animals that received the low dose did not appear to develop gross visual pathology as severe as the two higher doses by 42 days p.i., despite similar infection levels (Figure 3a). This group was therefore extended, with animals examined at days 49 and 70 p.i., and showed that hydrosalpinx developed to the same degree as seen with the two higher doses, but at later time points. The low dose group had very low levels of pyosalpinx until day 15 p.i. In contrast, the mice that received the mid dose of infection developed pyosalpinx by day 6 p.i., with it being most severe at 9 days p.i. (Figure 3b). By day 35 p.i., hydrosalpinx was present and quite severe, and continued to day 42 p.i. when the experiment was terminated. Similarly, the mice that received the high dose of infection also developed pyosalpinx, but the severity peaked at day 12 p.i. By day 35 p.i., hydrosalpinx had developed in these mice (Figure 3c).

Overall, it was found that the low dose group had the least severe pyosalpinx development, and all three groups developed very similar degrees of hydrosalpinx, although the low dose group was at a later time point.

The infectious dose does not significantly alter the level of inflammatory cell infiltration throughout the course of infection
The histopathological changes in the three regions of the reproductive tracts were measured using hematoxylin and eosin stain, and the levels of acute (neutrophils) and chronic (lymphocytes) inflammation were measured.

Acute inflammation. An increased presence of neutrophils is indicative of an acute infection at the site, and they are important in this model as they are required for the clearance of chlamydial infections. The greatest levels of infiltrate were seen during the acute phase of infection in all three groups in all the regions (Figure 4). Surprisingly, within the cervico-vaginal region, there was no significant difference in the level of neutrophils observed between any of the groups or in comparison with the control (Figure 4a). In the uterine horn tissues, all three groups had significantly greater levels of neutrophil infiltrate than the controls on day 6 p.i. ($P < 0.001$, $P < 0.05$, $P < 0.01$, respectively), but decreased to very low levels by day 15 p.i. in all three groups (Figure 4b). On day 9 p.i., the low dose group had significantly greater infiltrate than the mid and high dose groups ($P < 0.05$ and $P < 0.001$, respectively), correlating with the greater chlamydial burden seen in the uterine tissues at this time point. Interestingly, the numbers of neutrophils present in the uterine horn tissues during the very early stages of infection were much greater than those seen in the cervico-vaginal regions. In the oviducts, the level of neutrophils was greatest on days 9–15 p.i., after which, they decreased, coinciding with the clearance of Chlamydia from the oviduct tissues (Figure 4c). Neutrophil presence in the mid and high dose groups at these time points correlates with the presence of pyosalpinx (Figure 3).

Chronic inflammation. The presence of lymphocytes within the tissues can represent chronic inflammation, and in these experiments,
all three regions of the reproductive tract had elevated levels of lymphocyte infiltration compared with the negative and progesterone controls (Figure 5). Specifically, in the mice that received the mid dose, the levels of infiltrate in the cervico-vaginal region were significantly greater than controls on days 9 and 12 p.i. ($P < 0.01$ and $P < 0.05$, respectively; Figure 5a). Within the uterine horn tissues, both the low and mid dose groups showed a trend of increased levels of lymphocyte infiltration compared with both control groups at numerous time points (Figure 5b), but overall, it was the mid dose that induced the greatest lymphocyte infiltrate present at all time points until day 35 p.i. In the oviducts (Figure 5c), the overall level of infiltrate was much lower than that observed in the cervix/vagina and uterine horns. All lymphocyte infiltrate in the oviduct had subsided by day 35 p.i., the time at which hydrosalpinx was observed. There was an overall trend, with the mid dose having the greatest levels of lymphocytic infiltrate in all three regions of the reproductive tract during the acute stages of infection (days 6–21 p.i.).

The levels of neutrophils were only elevated for a short period of time that coincided with the peak infection levels within the tissues. In contrast, even after the infection had cleared from the tissues, the level of lymphocyte infiltration remained elevated. Overall,
the level of inflammatory cell infiltrate was not significantly affected by the levels of infection seen in the three regions of the reproductive tract.

**DISCUSSION**

In this study, we have shown the kinetics of ascending genital tract chlamydial infection and the associated inflammation kinetics in female BALB/c mice. We have shown that the infectious dose of *C. muridarum* alters both the rate and the level of clearance and ascension in the female reproductive tract and the development of gross pathology. However, the overall level of inflammatory cell infiltrate was not significantly affected by the infectious dose administered, highlighting that inoculum that is 5000-fold less than the highest used here is sufficient to cause inflammatory infiltrate to a similar, if not greater degree.

The pattern of chlamydial shedding from the cervico-vaginal region was similar to that reported earlier.Interestingly, there were only two time points where the levels of shed bacteria significantly differed from each other. However, examination of the chlamydial burden within the tissues of the three regions of the mouse FRT highlighted differences between the groups, with the low dose able to ascend to infect the uterine horns to a greater degree than the mid and high dose. In contrast, it was the high dose that ascended to the oviducts to the greatest degree. This difference within the oviducts may have been because the lower reproductive tract was overgrown with *Chlamydia* and anicullar spread has allowed *Chlamydia* to not be bound to epithelial cells to migrate to the areas where there were uninfected cells, including the oviducts. This overloading of the epithelium with *Chlamydia* has been suggested earlier by Kelly et al. The infectious dose modulates the innate immune response in relation to the infection, with the level of chlamydial burden being directly related to the development of oviduct pathology. Maxion et al. reported that there were differences in oviduct dilatation and trends in the ability of increasing doses to cause a greater infiltration of both adaptive and innate immune cells such as polymorphonuclear cells. This was not seen in our study, with the high dose causing the greatest infection in the oviducts and no significant differences in the levels of infiltrating cells. This may be linked to different variants of *C. muridarum* being used. There are two naturally occurring isolates of *C. muridarum*, Weiss and Nigg II. Recently, it has been found that although these two variants are identical in their patterns of infection, they differ in their virulence. Maxion et al. do not state which strain of *C. muridarum* has been used, but based on their obtained ID$_{50}$ (2.5 × 10$^5$ f.u.) and all of the animals in this study becoming infected at 5 × 10$^5$ f.u., using the same strain of mice, this suggests that different variants may have been used by Maxion et al. and our group and, therefore, could explain the differences found.

The chlamydial burden seen in the uterine horns was much lower than that observed in the oviducts. Recruitment of CD45$^+$ major histocompatibility complex class II (MHC II) cells limit the level of infection in uterine tissues early in infection, suggesting that an early MHC II response may have limited the level of infection within the uterine tissues in this case. The cervico-vaginal regions had a greater overall chlamydial burden than that of the reproductive tract and uterine tissues and a more prolonged infection. This may be because the immune system in that region of the reproductive tract may be dampened due to its continual contact with natural flora and other potential pathogens, allowing the bacteria to initially infect epithelial cells to a greater degree.

We have also shown that the development of gross pathology is not necessarily dose-dependent, with the low dose developing a similar level of hydrosalpinx as the mid and high dose groups but at a later time point. Pyosalpinx is defined as the presence of polymorphonuclear cells, or pus, within the oviduct, and in these experiments was found to occur during the acute infection stages. Hydrosalpinx occurs when the oviducts are occluded and clear serous fluid accumulates causing oviduct dilatation. Many have used the presence of hydrosalpinx as a marker for infertility in the mouse model, with Shah et al. demonstrating that oviduct occlusion directly correlates with infertility in mice. The presence of hydrosalpingeal fluid in women undergoing *in vitro* fertilization has been linked to decreased implantation rates. The exact reasons behind this are unclear, but it is believed that the fluid contains cytokines such as IL-2, that are involved in the development of pathology, and it is this pathology development that decreases the rate of successful pregnancy outcomes. Here we found that the mid dose had the greatest overall level of hydrosalpinx development, possibly related to greater levels of chronic pathology (lymphocytes) and also a more prolonged presence of neutrophils. Although the development of pyosalpinx and hydrosalpinx has been examined in both C57BL/6 and C3H/HeN mice, this is the first time, to our knowledge, that the kinetics of their development has been examined in the BALB/c model, at multiple time points and at varying infectious doses.

Importantly, we have shown that the infectious dose administered did not significantly affect the overall level of inflammatory cell infiltration. We have shown that neutrophils are present in the early stages of infection in all three regions of the mouse reproductive tract. Treatment of animals with granulocyte-depleting monoclonal antibodies has shown that neutrophils play a critical role in the clearance of early-stage chlamydial infections from the reproductive tract, but too intense a neutrophil response may promote pathology development. Here we have seen that the mid dose had a more prolonged elevation of neutrophil infiltration in the oviducts and the greatest level of hydrosalpinx development at the earlier time points. It is believed that the actual chlamydial infection is not the cause of the inflammation or pathology development, but rather the host immune response. This is supported by the findings from fallopian tube samples of hysterectomy patients, where infection levels were disproportional to the severity of tissue destruction. Upon infection, an epithelial cell secretes various proinflammatory cytokines, and it is believed that this cytokine secretion triggers a cascade of events that leads to the development of chronic pathology, scarring and tubal infertility.

With the transmission rates of *C. trachomatis* on the rise, 50–60% of infections being asymptomatic and potential sequelae such as PID, there is a need to understand the mechanisms of ascending infection leading to pathology development and to develop ways of preventing the damage. This study highlights that a low-level inoculum can cause a similar level of damage as one more than 5000 times greater, suggesting that using a high inoculum to establish infection is unnecessary and, in fact, may result in an underestimation of the effectiveness of experimental vaccines. Importantly, we have shown the kinetics of not only ascending genital tract infection in mice but also the development of infection-related inflammation and pathology in relation to varying infectious doses.

**METHODS**

*Chlamydia strain*

*Chlamydia muridarum* (Weiss strain; ATCC VR-123, Manassas, VA, USA), formerly the mouse pneumonitis biovar of *C. trachomatis* (MoPn), was grown by inoculation of McCoy cell monolayers in Dulbecco’s minimal essential
medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin sulfate, 2 μg/ml gentamicin and 20 μg/ml HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Elementary bodies were purified using a discontinuous Renografin gradient as described earlier.29

Mice
Female BALB/c mice, 6–8 weeks of age, were obtained from The Animal Resource Centre (Perth, Australia) and housed in an accredited laboratory animal care facility under specific pathogen-free conditions. Animals received food and water ad libitum. All procedures were approved by the Queensland University of Technology Animal Research Ethics Committee.

Infection
Mice were given 2.5 mg of modroxyprogesterone acetate (Depo-Provera, Pfizer, West Ryde, NSW, Australia) subcutaneously 7 days before infection. The mice were anesthetized intraperitoneally using ketamine (Parnell Laboratory, Sydney, NSW, Australia) and xylazine hydrochloride (Bayer, Pymble, NSW, Australia) and infected intravaginally with 20 μl of sucrose-phosphate-glutamate (SPG) containing one of the three infectious doses, 5×10¹⁴ i.f.u. (low), 5×10¹⁵ i.f.u. (mid) or 2.6×10¹⁶ i.f.u. (high). Mice were then sacrificed on days 6, 9, 12, 15, 21, 35 and 42 p.i. The low dose group also had animals sacrificed on days 49 and 70 p.i. to examine the extended pathology development.

Detection of C. muridarum infection
Infection was monitored by collecting cervico-vaginal swabs (Copan, Murrieta, CA, USA) on the days mentioned above. Swabs were placed in tubes containing 500 μl SPG and glass beads and were stored at −80 °C. To monitor the infection, individual wells of McCoy cell monolayers in 48-well plates were inoculated with 10 μl of swab specimen and media. The plates were incubated for 4 h at 37 °C, after which the swab solution was removed and replaced with fresh supplemented media containing 1 μg/ml cycloheximide. The wells were incubated for a further 24–30 h and then fixed with methanol. The inclusions were visualized by staining with rabbit anti-C. trachomatis antibody (Pierce/Progen, Richlands, NSW, Australia) and Immunopure ABC/DAB Staining Kit (Pierce/Progen), as described elsewhere.21 An animal was classed as having a productive infection if there were >300 i.f.u. per swab.

Assessment of ascending infection
To monitor the progress of the infection along the reproductive tract, the cervico-vaginal region, uterine horns and oviducts representing the upper reproductive tract were removed upon sacrifice, placed in 100% SPG and stored at −80 °C. To determine the chlamydial burden within the tissues, individual cervico-vaginal and uterine horns and pooled oviducts were weighed and homogenized, and 10–25 μl of homogenate was placed onto individual wells of McCoy cell monolayers and cultured and stained as above. Inclusions were visualized by staining with rabbit anti-C. trachomatis antibody (Pierce/Progen, Richlands, NSW, Australia) and Immunopure ABC/DAB Staining Kit (Pierce/Progen), as described elsewhere.21 An animal was classed as having a productive infection if there were >300 i.f.u. per swab.

Assessment of gross pathology
Upon sacrifice, the reproductive tracts were examined in situ for macroscopic changes. The presence of prostatiplex and hydrosalpinx was recorded and the level of fluid retention was also scored, with those having small amounts of fluid present in the oviducts given a score of 1, those with moderate amounts a score of 2 and those with large amounts of fluid given a score of 3.

Histopathology assessment
Tissues were removed at the time of sacrifice, fixed in 10% formaldehyde and embedded in paraffin wax. Sections (5 μm) were cut, dewaxed and rehydrated through graded ethanol solutions to phosphate-buffered saline. Hematoxylin and eosin staining was performed. Regions of the reproductive tract, cervix/vagina, uterine horn and oviducts were counted separately to each other. Ten random fields (×1000 magnification) of each were counted ensuring to include both epithelium and sub-mucosa, with the observer blinded to the time point and dose being examined.

Statistics
All data are presented as mean ± s.e. All statistics were performed using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA, USA). Significant differences in the swab clearance data, tissue i.f.u. and inflammatory cell infiltration were determined using a two-way analysis of variance with Bonferroni’s post-test with significance set for P<0.05. All experiments contained five mice and were repeated twice.

ACKNOWLEDGEMENTS
This work was supported by the Australian National Health and Medical Research Council (Australia, Grant no. 351113). AJC is supported by the National Health and Medical Research Council Postgraduate Training Scholarship (no. 497260).


