The Effect of Doxycycline Treatment on the Development of Protective Immunity in a Murine Model of Chlamydial Genital Infection

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Chlamydia trachomatis is a major cause of sexually transmitted disease (STD) worldwide. Antibiotics are effective in treating infection; however, reinfection is common. This observation has led to the conclusion that infection fails to elicit a protective antichlamydial immune response. It was postulated that high reinfection rates might be due to early eradication of organisms from genital tissue after antibiotic intervention, which could negatively influence the development of naturally acquired protective immunity. This hypothesis was tested by use of a murine model of female genital infection. The findings show that doxycycline intervention of infection, although very effective in eradicating chlamydiae from genital tissue and preventing upper genital tract disease, significantly inhibits the development of protective immunity. If antibiotic intervention of human chlamydial genital infection has a similar effect on protective immunity, it could have important implications in the understanding of immunity to infection and future public health efforts to control chlamydial STD.

Chlamydia trachomatis infections are the most common bacterial sexually transmitted diseases (STDs) in the United States [1], with an estimated 4 million cases of chlamydial genital infection occurring annually [2]. Infection of women constitutes a significant risk for the development of serious sequelae, including pelvic inflammatory disease, ectopic pregnancy, and reproductive disability [3–6]. The cost of treating these infections approaches 4 billion dollars annually, with 80% of these costs attributed to infection and disease of women [2]. Chlamydiae are sensitive to antibiotics, making antibiotic therapy a highly effective method for the treatment of chlamydial genital infection [7].

Evidence from epidemiological studies indicates that genital reinfection in women is common and plays an important role in the development of infection-related sequelae [8]. The high incidence of reinfection has led investigators to question whether there is natural protective immunity to chlamydial genital infection [9], or conversely, whether clinically inapparent long-term persistent infections develop in some women [10, 11]. The uncertainty about natural immunity to reinfection has had a significant impact on public health strategies to control chlamydial STD. For example, control of chlamydial STD by the development of an efficacious vaccine has received only a moderate level of enthusiasm and support because an important prerequisite in ascertaining vaccine feasibility is the ability of naturally occurring infections to provide protection against reinfection.

The murine model of C. trachomatis infection of the female genital tract [12–14] provides overwhelming evidence that protective immunity against chlamydial genital infection occurs. Intravaginally infected mice spontaneously resolve primary infection and exhibit significant resistance to reinfection. Compared with immunologically naïve mice, postinfected mice challenged intravaginally shed significantly fewer organisms for a much shorter infection period and exhibit only minimal and transient inflammatory changes in genital tissue [15–22]. Protective immunity correlates with the production of local IgA and IgG in vaginal washes and with a vigorous chlamydial-specific CD4+ Th1 cell–mediated immune response (CMI) [20–24]. Immunization capable of generating chlamydial-specific Th1 immunity is also highly protective [25].

Thus, an apparent paradox may exist between mice and humans in their ability to generate a protective immune response against chlamydial genital infection. This disparity might simply be the result of basic differences between the species in host response to infection. Conversely, it could also reflect the fact that infections of humans, unlike those of mice, are commonly treated with antibiotics, an intervention of natural infection that might impair the development of an adaptive protective immune response. Here, we have used the mouse model to test the hypothesis that antibiotic therapy might negatively affect...
the development of a protective immune response against chlamydial genital infection. Our findings show that doxycycline treatment of infected mice has a marked effect on the development of naturally acquired protective immunity. Thus, antimicrobial treatment early during the course of C. trachomatis urogenital infections may adversely affect the development of protective immunity in some women. These findings might have important implications in our understanding of immunity to C. trachomatis infections of humans that affect public health efforts to control chlamydial STD through the development of vaccines.

Materials and Methods

Chlamydia. The mouse pneumonitis strain of C. trachomatis (MoPn) was grown in HeLa 229 cells. Infectious elementary bodies (EBs) were purified by density gradient centrifugation and infection-forming units (IFUs) were determined as described elsewhere [26]. A single preparation of MoPn seed stock was used as the challenge inoculum for all of the experiments described.

Mice. Female C57BL/10 (H-2b) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used between 8 and 12 weeks of age. Animals were housed in microisolator caging under standard environmental conditions and were provided food and water ad libitum. The animal facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Chlamydial infection. Mice were given 2.5 mg of medroxyprogesterone acetate (Depo-Provera, Upjohn, Kalamazoo, MI) in 0.1 mL of saline subcutaneously 7 days prior to chlamydial cervicovaginal infection to synchronize estrus. Mice were infected by inoculation with 5 μL of MoPn (1500 IFUs, 100 ID50) in 10 mM phosphate, pH 7.2, containing 0.25 M sucrose and 5 mM l-glutamic acid into the vaginal vault. For rechallenge experiments, mice were treated with progesterone, as described above, and challenged intravaginally with 100 ID50 of MoPn 40 days after resolution of their primary infection.

Chlamydial cervicovaginal shedding. Chlamydial cervicovaginal shedding was assessed by quantifying the number of IFUs recovered from cervicovaginal swabs (Calgiswab type 1; Hardwood Products, Guilford, ME) taken at different times postinfection [18]. Monolayers of HeLa 229 cells grown in 96-well tissue culture plates were inoculated with cervicovaginal samples, and chlamydial inclusions were detected by indirect immunofluorescence staining by use of the chlamydial lipopolysaccharide genus-specific monoclonal antibody EVI-H1. Entire genital tracts were removed from mice at 40–60 days postinfection and scored for the presence of hydrosalpinx. Hydrosalpinx in this model accurately reflects infertility and is therefore thought to be a reasonable correlate and model for chlamydial salpingitis in women [14, 27].

Antibiotic treatment. Mice were treated daily with doxycycline-hyclate (Fujisawa USA, Deerfield, IL) by intraperitoneal injection of 0.1 mL of doxycycline (3 mg/mL) in distilled water (equivalent to 10 mg/kg body weight). Antibiotic treatment of different groups of mice was started at the time of infection (day 0) and at days 3, 7, and 10 postinfection. Mice were treated daily for a period of 14 consecutive days.

Figure 1. Kinetics of chlamydial clearance from genital tracts of female mice treated with doxycycline. Groups of 5–8 mice were infected intravaginally with chlamydiae followed by intraperitoneal administration of doxycycline for 14 consecutive days. Antibiotic treatment was initiated in individual groups of mice at 0, 3, 7, and 10 days postinfection. Mice were cultured at different time points postinfection, and numbers of infectious organisms shed from vaginas of mice were determined by calculating recoverable infection-forming units (IFUs) on monolayers of HeLa 229 cells. Numbers represent mean IFU ± SE.
Figure 2. Incidence of hydrosalpinx in female mice after chlamydial intravaginal infection and antibiotic intervention at different times postchallenge. Mice (4–6 per treatment group) were killed 40 days after clearance of primary infection and scored for presence of hydrosalpinx. Results shown are percentage of mice in each group exhibiting either unilateral or bilateral hydrosalpinx.

Figure 3. Serum antichlamydial IgG titers of infected doxycycline-treated mice. Sera of 3–5 mice in each experimental group were assayed individually by ELISA for IgG antibodies specific to formalin-killed mouse pneumonitis strain of Chlamydia trachomatis infectious elementary bodies. Results are expressed as mean antibody titer ± SD.
tion of infection at this time period postinfection did not significantly affect the generation of a serum IgG antibody response.

Chlamydial-specific IgA antibodies present in vaginal washes (figure 4) closely paralleled the serum IgG antibody responses (figure 3). The highest IgA responses were found in washes of untreated mice. The vaginal washes of mice treated at days 0 and 3 postinfection did not have detectable antichlamydial IgA antibodies. Chlamydial-specific IgA was present in the vaginal washes of mice treated at 7 and 10 days postinfection but at lower levels than untreated controls.

Antigen-specific cytokine responses of antibiotic-treated mice. CD4 T cells are known to play an important role in the development of protective immunity against murine chlamydial genital infection [18, 20, 21, 24]. To investigate whether antibiotic intervention affected the development of chlamydial-specific CD4 T cell responses, we assayed the secretion of IFN-γ and IL-10 from splenocytes of untreated and doxycycline-treated infected mice after culture with inactivated chlamydial organisms. CD4 T cells are classified as T helper (Th) 1 or 2, depending on the specific cytokine(s) they secrete after antigen stimulation [30]. CD4 Th1 cells produce IFN-γ, whereas Th2 cells produce IL-10. The concentrations of IFN-γ found in the culture supernatants of splenocytes of untreated and doxycycline-treated mice are shown in figure 5A. There was a clear reduction in the amount of IFN-γ produced by splenocytes of mice treated at days 0 and 3 postinfection compared with that of untreated control mice. IFN-γ production by splenocytes from both the day 7 and 10 postinfection treatment groups was not significantly different from that of untreated mice. Antigen-specific IL-10 production by cultured splenocytes was significantly less in the day 0 treatment group than that of controls. The production of IL-10 did not differ significantly between treated and untreated mice at later time periods. Thus, antibiotic intervention of acute chlamydial genital tract infection, particularly at early time periods postinfection, had a negative effect on the development of both chlamydial-specific antibody and CMI immune responses.

Reinfection and the development of protective immunity in antibiotic-treated mice. These findings clearly showed that antibiotic intervention has a negative effect on the ability of mice to generate a chlamydial-specific immune response. It was therefore important to ascertain whether this negative effect on the development of immunity could be correlated with the level of resistance to chlamydial reinfection. Protective immunity was studied by rechallenging mice intravaginally 40 days after resolution of their primary infections. Secondary reinfection kinetics was monitored by culture at different time periods postchallenge. The results clearly showed that antibiotic-treated mice were much more susceptible to reinfection than untreated control mice (figure 6). Intravaginally challenged, untreated mice exhibited infections characterized by the recovery of few organisms (≤10) over the entire infection period. In contrast, mice treated with doxycycline at day 0 and 3 postinfection were highly susceptible to reinfection. Infections were characterized by the shedding of high numbers of infectious organisms (4–4.5 log10), with infections lasting for 3–4 weeks. Shedding and clearance kinetics closely mimic those of immunologically naïve animals (figure 1, untreated). That result was not particularly surprising for the day 0 treatment group, because they never became infected, nor did they produce detectable antichlamydial immune responses. However, the susceptibility of mice treated at 3 days postinfection was surprising, because these animals exhibited a vigorous infection of the genital tract accompanied by the shedding of large amounts of organism and antigen (figure 1). Moreover, a significant difference in the development of adaptive protective immunity was also observed in those groups of mice whose genital infections were treated as late as 7 and 10 days postinfection. Mice in those treatment groups shed significantly more chlamydiae and exhibited infections of longer duration than untreated control mice, despite the fact that only marginal differences were observed in their immune response to infection (figures 3, 4, and 5). These results show that antibiotic intervention of chlamydial genital infection in mice has a marked negative impact on the host’s ability to generate a protective antichlamydial immune response. A similar but less dramatic inhibition in resistance to reinfection was also observed in mice whose infections were eradicated in the midpoint of the natural infection period. These results suggest that optimum protective immunity depends on the natural pro-
Discussion

In this report, we used a murine model of chlamydial infection of the female genital tract to investigate the effect of antibiotic intervention on infection and on the development of adaptive protective immunity. Our results show that although early intervention with doxycycline is highly effective in eradicating chlamydial genital infection and preventing upper genital tract disease, it has a marked negative influence on the development of adaptive protective immunity. Our results show a significant impairment in the ability of antibiotic-treated mice to generate chlamydial-specific antibody and CMI responses. Furthermore, the marked differences that were found among antibiotic-treated and untreated mice in their ability to resolve secondary chlamydial genital infection is potentially of great importance in human infection. Profound differences in the clearance kinetics of chlamydiae from the genital tracts of rechallenged mice were observed in all treatment groups compared with untreated controls. The most significant effect on the development of protective immunity was observed in mice treated with antibiotic at day 3 after primary infection. Despite the production of a marked acute infection by those mice for a period of 4–5 days, levels of protective immunity were no greater than that of uninfected (day 0 treatment group) or naïve control mice. Perhaps most intriguing and relevant finding of this study is that mice treated with doxycycline as late as 7 and 10 days postinfection were significantly less immune than untreated control animals. At those postinfection time periods, the bulk of chlamydial organisms had been cleared from the genital tract, and the infection had progressed to the point of its natural resolution. Thus, maximal protective immunity against chlamydial infection of the female mouse genital tract requires that acute infection be allowed to proceed into the late stages of the natural infection course. Our studies provide new insight into the effect of antibiotic treatment on the development of protective antichlamydial immunity and provide an impetus to readdress current thinking about whether natural chlamydial genital infection in humans generates a protective immune response [11]. Antibiotic treatment early in the course of infection is beneficial, as it likely eradicates chlamydiae and prevents the development of severe disease, but, as shown in this study, it may also negatively affect the development of protective immunity. Understanding the effect of antibiotic treatment on the development of natural immunity to human chlamydial genital infection is particularly relevant when considering future chlamydial vaccine endeavors. For example, if protective immunity against human chlamydial genital infection occurs naturally, then the successful development of an efficacious vaccine is probably feasible.

It is difficult to address this question in humans because of ethical concerns. However, Parks et al. [31] have reported spontaneous chlamydial clearance in women who delay seeking antibiotic treatment, which led them to conclude that host immune responses may be mediating chlamydial clearance. Our findings in mice provide experimental support for that conclusion. Furthermore, our results and those of other investigators [29]...
clearly show the beneficial effect of antibiotic intervention on the eradication of chlamydial infection and the prevention of upper genital tract disease. Nevertheless, it is paradoxical that such an effective intervention approach may in fact result in a cohort of women who fail to develop adequate levels of antichlamydial protective immunity. If our animal model accurately reflects what occurs in humans, then women who are treated early during the course of infection will be at risk of reinfection or recurrent chlamydial infection, a situation that is known to exist in our society today. Thus, the observations reported here could have important public health implications in future efforts to control chlamydial STD.

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