Steroids Alone or as Adjunctive Therapy with Doxycycline Fail To Improve Oviduct Damage in Mice Infected with Chlamydia muridarum

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In women, Chlamydia trachomatis can ascend from the cervix to the fallopian tubes, where an overly aggressive host inflammatory response can cause scarring that leads to chronic pelvic pain, infertility, or ectopic pregnancy. Although screening and treatment programs for women have resulted in reduced rates of sequelae, morbidities associated with oviduct scarring continue to occur. Since corticosteroids have anti-inflammatory and antifibrotic effects, we tested the ability of dexamethasone to inhibit inflammation and prevent oviduct scarring in mice genitally infected with Chlamydia muridarum. The administration of 1 or 2.5 mg/kg of body weight of dexamethasone on days 7 to 21 of infection resulted in reduced accumulation of inflammatory cells in the oviducts compared to that in controls. However, a concomitant increase in bacterial burden was observed, and chronic oviduct disease was not reduced. Adjunctive administration of a prolonged (21-day) or short (3-day) course of dexamethasone in combination with the antibiotic doxycycline also failed to reduce chronic oviduct pathology compared to antibiotic treatment alone. Steroids administered alone or adjunctively with antibiotics failed to prevent oviduct damage in this murine model of Chlamydia trachomatis infection.

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen in the world. Infected women are at risk for pelvic inflammatory disease (PID), which can lead to oviduct tissue scarring. Chronic oviduct damage may result in the morbidity of recurrent pelvic pain, infertility, and ectopic pregnancy. The study of chlamydial oviduct disease pathogenesis has long relied on macaque, guinea pig, and mouse animal models in which chlamydiala are inoculated directly into the oviduct bursa or at the level of the cervix. When the cervical route of inoculation is performed, bacteria ascend and infect the oviducts, where inflammation can lead to scar formation and fluid blockade resulting in hydrosalpinx development, similar to what is observed in women. Subcutaneous depot forms of progesterone are frequently used in mice to enhance initial infection rates by halting the estrus cycle (1–3). However, even in the absence of progesterone pretreatment, the majority of infected mice of multiple genetic backgrounds sustain severe oviduct disease after infection (4).

Studies in the murine model have demonstrated that soon after infection, mucosal epithelial cells and resident innate immune cells release inflammatory cytokines and chemokines. These molecules lead to an influx of neutrophils and monocytes that release tissue-damaging molecules such as matrix metalloproteinases and interleukin 1 (IL-1) (reviewed in Darville and Hiltke [5]). The intensity of neutrophil influx into the oviduct (pyosalpinx) correlates directly with eventual development of hydrosalpinx (6). Soon after neutrophil infiltration, recruited CD4+ T cells secreting gamma interferon (IFN-γ) promote resolution of infection and protection from oviduct damage (5, 7–9). CD8+ T cells also contribute to resolution of infection through their production of IFN-γ (10), but these cells can also promote oviduct damage through their production of tumor necrosis factor alpha (TNF-α) (11).

Doxycycline effectively kills Chlamydia in the murine model of chlamydial genital tract infection. In fact, early antibiotic treatment of mice can significantly reduce the rate of hydrosalpinx (9, 12), and multiple human studies have revealed that screening and treatment programs lead to reduced rates of PID and infertility (13–17). Antibiotic treatment early after cervical infection may completely prevent ascension of chlamydiae to the fallopian tubes or lead to a rapid resolution of oviduct infection. However, due to the asymptomatic nature of most chlamydial infections, they are often prolonged and many go untreated (18–20). Since an effective vaccine is not available, treatment regimens that inhibit tissue damage caused by injurious components of the host inflammatory response are urgently needed.

A multitude of clinical studies have established the beneficial effects of steroid therapy for inflammatory and infectious disorders (reviewed in Aberdein et al. [21]). Steroids are potent anti-inflammatory agents that work via a variety of mechanisms, including suppression of proinflammatory cytokines, increased transcription of anti-inflammatory proteins, and inhibition of neutrophil adhesion, chemotaxis, and phagocytosis (22, 23). They are also potent inhibitors of T cell growth, proliferation, and differentiation and can signal T cell apoptosis (23), which is poten-
tially problematic in regard to chlamydial infection, where CD4+ T cells play an essential role in resolution of infection (7, 11, 24, 25). In addition to use as a single-agent therapy, the adjunctive use of steroids with antibiotics has been demonstrated to improve outcomes in a variety of infectious disorders such as acute pyelonephritis (26) and tuberculous meningitis (27). Relevant to prevention of oviduct scarring, steroids have been shown to inhibit and even reverse fibrotic changes in a number of inflammatory diseases (28–31).

In this study, we initially sought to determine if administration of dexamethasone alone during Chlamydia muridarum genital infection might protect the oviduct from scar formation. We examined the effects of dexamethasone treatment on infection course, inflammatory response, and chronic oviduct tissue pathology. In addition, we sought to determine whether the administration of steroids as adjunctive therapy with the antibiotic doxycycline would result in preservation of oviduct structure and reduction of hydrosalpinx after murine genital tract infection.

**MATERIALS AND METHODS**

**Strains, cell lines, and culture conditions.** Plaque-purified *Chlamydia muridarum* strain Nigg was propagated and titrated in L929 cells as previously described (32). Bacteria were titrated as inclusion-forming units (IFU) using fluorescently tagged anti-chlamydial lipopolysaccharide monoclonal antibody (Bio-Rad, Hercules, CA) (33).

**Mice.** Eight-week-old female C3H/HeOuJ or C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the animal facilities at the research center of Children’s Hospital of Pittsburgh. These two murine strains were chosen based on previous data that revealed enhanced chronic oviduct disease risk in C3H/HeOuJ mice but increased early neutrophil infiltration in C57BL/6 mice (33). Mice were given food and water *ad libitum* in an environmentally controlled room with a 12-hour light/dark cycle. All animal experiments were performed according to the institutional policies for animal health and were approved by the University Institutional Animal Care and Use Committee.

**Chlamydia infection and monitoring.** Five days prior to infection, mice were treated subcutaneously with 2.5 mg of medroxyprogesterone (Depo-Provera; Upjohn, Kalamazoo, MI) to induce a stage of anestrus in order to facilitate successful infection (3). Anesthetized mice were intra-vaginally inoculated with 1 × 106 IFU of *C. muridarum* in 30 μl of sucrose-sodium phosphate-glutamic acid (SPG) buffer (250 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid [pH 7.2]). Mice were monitored for cervicovaginal shedding via endocervical swabs (34), and mice were euthanized on day 42 and their oviduct histopathology was compared to that of mice receiving doxycycline or PBS alone.

**Evaluation of oviduct immune cells by flow cytometry.** Cell surface proteins were stained with peridinin-chlorophyll protein (PerCP)-Cy5.5 anti-mouse CD45 (clone 30–F11), V450 anti-mouse CD3 (clone 500A2), phycoerythrin (PE)-Cy7 anti-mouse CD8 (clone 53-6.7), and fluorescein isothiocyanate (FITC) anti-mouse CD11b (clone M1/70) (all from BD Biosciences) and allophycocyanin (APC) anti-mouse CD4 (clone RM4-5) and PE-anti-mouse Ly6G (clone RB6-8C5) (from eBioscience). Data were acquired using an LSR II analyzer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Fifty to 100,000 cells were collected for each data point. The absolute number of leukocytes per oviduct was determined by the following calculation: number of cell marker-specific cells per oviduct = (number of gated cells positive for the cell marker of interest as determined by flow cytometry) × (number of cells in the oviduct as determined by hemocytometer count/number of cells analyzed by flow cytometry).

**Evaluation of MMP-9 in oviduct homogenates.** An aliquot of each oviduct homogenate was analyzed for matrix metalloproteinase-9 (MMP-9) using an enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Minneapolis, MN). Each sample was run in duplicate.

**Histopathology.** At the time of euthanasia, gross pathology, including presence of hydrosalpinx formation, was noted, and the entire genital tract was removed and fixed in 10% formalin. The specimens were then encased in a paraffin block and sectioned into 4-μm slices for placement on a slide. They were stained with hematoxylin and eosin and were evaluated by a pathologist blinded to the experimental design. Oviducts were individually scored for erosion and dilatation using a four-tiered, semi-quantitative scoring system as previously described (33, 38). Briefly, scoring was performed for erosion (0, normal; 1, minimal presence of parameter; 2, scattered aggregates of parameter; 3, numerous aggregates; and 4, confluent of parameter) and for dilatation (0, normal; 1, minimal luminal dilatation with no loss of plicae; 2, dilatation twice the normal diameter with loss/flattening of some plicae; 3, dilatation with complete flattening of mucosa; and 4, marked dilatation with rupture of the wall).

**Statistics.** Statistical comparisons between groups for lower genital tract bacterial burden were made by two-factor repeated-measures analysis of variance (ANOVA) with a Bonferroni posttest. Comparisons between groups for oviduct bacterial burden, flow cytometry data, and
MMP-9 levels were made with one-way ANOVA. A Kruskal Wallis test was used to determine significant differences in pathology scores. Analysis of the data was performed on GraphPad Prism, and differences were considered significant at $P$ values of $<0.05$.

RESULTS

Histopathology of oviducts harvested on day 21 of infection reveals preservation of the oviduct structure with steroid treatment. In our initial experiment (Fig. 1, experiment 1), mice ($n = 5$ per group) receiving 0.05, 0.5, 1, or 2.5 mg/kg per day of dexamethasone or PBS were euthanized at day 21 of infection, and their oviducts were harvested for histopathology. Microscopic examination revealed relative preservation of the oviduct structure in mice receiving 1 mg/kg per day of dexamethasone (Fig. 2B) or 2.5 mg/kg per day of dexamethasone (Fig. 2C) compared to a control group receiving PBS (Fig. 2A) or mice receiving 0.05 and 0.5 mg/kg (data not shown), which suggested a potential beneficial effect of steroid treatment and prompted further investigation.

Leukocyte numbers are reduced in the oviducts of infected mice receiving dexamethasone but MMP-9 is similar and bacterial burden is increased compared to controls. We hypothesized that treatment with dexamethasone would lead to a decrease in the influx of inflammatory cells into the oviduct and a subsequent reduction in chronic pathology. Oviducts were harvested from steroid-treated C57BL/6 and C3H/HeOuJ mice on day 11 or 14 of infection, respectively (Fig. 1, experiments 2 and 3). These are days on which we have noted that neutrophil numbers peak in the oviducts of the respective mouse strains after C. muridarum infection (9, 39). We detected a 6-fold reduction in the numbers of neutrophils (Fig. 3A) and a non-statistically significant reduction in the numbers of macrophages (Fig. 3B) in the oviducts of C3H/HeOuJ mice treated with 1 or 2.5 mg/kg of dexamethasone compared to infected controls. Steroid treatment also resulted in decreases in the numbers of CD4$^+$ (Fig. 3C) and CD8$^+$ T cells (Fig. 3D) consistent with the dual effect of steroids to inhibit the proliferation of T cells as well as chemotaxis and adhesion of phagocytes. Similar results were observed in C57BL/6 mice (data not shown). Despite a reduction in neutrophil influx, steroid treatment had no effect on MMP-9 levels. Ramsey et al. demonstrated that neutrophils were a major source of MMP-9 production (40); thus, persistently elevated MMP-9 levels suggest that neutrophil release of proteases was not inhibited by steroid administration.

**FIG 1** Treatment diagram for six independent experiments using dexamethasone (Dex) with or without the addition of doxycycline (Doxy). Experiments 1, 3, 4, and 5 included 5 C3H/HeOuJ mice per group, experiment 2 included 5 C57BL/6 mice per group, and experiment 6 included 15 C57BL/6 mice per group.
(Fig. 3E) or that uninhibited production of MMPs by oviduct stromal cells contributed to oviduct damage (41). Additionally, increased chlamydial 16S rRNA detected on day 14 in mice receiving dexamethasone suggests that steroid treatment had a detrimental effect on host defense mechanisms (Fig. 3F). Although an overall dose-response effect was not observed with the higher dose of 2.5 mg/kg, there was one animal in this group with minimal response to steroids that skewed the results toward the control.

Dexamethasone treatment failed to reduce chronic oviduct pathology and led to an increase in lower genital tract bacterial burden. Lower genital tract bacterial burden was monitored through day 42 in groups of C57BL/6 and C3H/HeOuJ mice.
treated with dexamethasone on days 7 to 21 (Fig. 1, experiments 2 and 3). A divergence in chlamydial burden was observed beginning on day 10 of infection, with statistically significant increases occurring on days 14 to 21 in C57BL/6 mice receiving 1 or 2.5 mg/kg per day of dexamethasone compared to controls (Fig. 4A). The fact that increased bacterial burden occurred during the time of steroid administration suggested again that steroids were acting to inhibit immune mechanisms important for control of infection. Histologic evaluation of oviducts harvested on day 42 revealed no decrease in mucosal epithelial erosion (Fig. 4B) or dilatation (Fig. 4C). Results in C3H/HeOuJ mice were similar (data not shown). Oviducts from mock-infected C57BL/6 and C3H/HeOuJ mice exhibited no erosion and minimal to no dilatation (median dilatation and erosion scores, 0 in both murine strains; maximum dilatation score, 1 in both strains; maximum erosion score, 0 in both strains). These findings prompted a trial of high-dose dexamethasone in combination with the antibiotic doxycycline in an effort to suppress inflammation while effectively eliminating the inflammatory bacterial trigger.

**Dexamethasone administration in conjunction with doxycycline does not reduce chronic oviduct disease.** In an adjunctive steroid treatment protocol (Fig. 1, experiment 4, and Table 1), C3H/HeOuJ mice were given higher doses of dexamethasone in an effort to further suppress oviduct inflammation. Groups of mice (n = 5 per group) received a 14-day course of 2.5, 5, or 10 mg/kg per day of dexamethasone and 5 days of doxycycline therapy during a 7-day steroid taper. A control group received doxycycline alone for 5 days. Histopathologic evaluation of oviducts obtained at day 42 showed no improvement in oviduct dilatation scores in mice receiving dexamethasone as an adjunct to doxycycline compared to those mice receiving doxycycline alone (Table 1, experiment 4). As observed in experiments 2 and 3 (Fig. 4), an increase in lower genital tract bacterial burden was observed during the time of high-dose steroid treatment (Fig. 1A; see also Fig. S1 in the supplemental material).

These findings led to a further refinement of our experimental treatment protocol to a shortened, 3-day burst of dexamethasone on days 11 to 13 (C57BL/6) or 14 to 16 of infection (C3H/HeOuJ) in combination with doxycycline for 5 days (Fig. 1, experiments 5 and 6 and Table 1). Treatment days were chosen based on our prior determination of peak days of oviduct neutrophil influx in C57BL/6 (39) and C3H/HeOuJ mice (9). Although no increase in bacterial burden was observed during short-course steroid treatment, and a rapid resolution of infection occurred with doxycycline therapy (Fig. 1B; see also Fig. S1 in the supplemental material), no improvements in oviduct dilatation scores were observed on day 42 postinfection (Table 1, experiments 5 and 6). All groups that received doxycycline alone exhibited lower dilatation scores than control groups given PBS and those given dexamethasone together with doxycycline, but these differences were not statistically significant (Table 1).

**DISCUSSION**

An overly robust host inflammatory response to *Chlamydia* causes damage to the delicate structures of the oviduct (5, 42). Steroids given alone or as an adjunct to antibiotics are potent anti-inflammatory agents that have been shown to reduce pathology and disease outcomes.

### TABLE 1

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Treatment protocol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Disease outcome (median oviduct dilatation score)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>Dexamethasone (mg/kg) (days of treatment)</td>
<td>Doxycycline (12 mg/kg)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4&lt;sup&gt;b&lt;/sup&gt; (5 C3H/HeOuJ mice per group)</td>
<td>0</td>
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<td></td>
<td>2.5 (3–2)</td>
<td>+</td>
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<td></td>
<td>5 (3–24)</td>
<td>+</td>
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<tr>
<td></td>
<td>10 (3–24)</td>
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<tr>
<td>5&lt;sup&gt;b&lt;/sup&gt; (5 C3H/HeOuJ mice per group)</td>
<td>0</td>
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<td></td>
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<td>2.5 (14–16)</td>
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<tr>
<td>6&lt;sup&gt;b&lt;/sup&gt; (15 C57BL/6 mice per group)</td>
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<td>2.5 (11–13)</td>
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<sup>a</sup> Control mice received PBS in place of indicated steroid or antibiotic treatments.

<sup>b</sup> Oviducts were harvested on days 35 to 42 of infection, and pathology scores within each experiment were compared by Kruskal-Wallis test; some were significantly different (0, normal; 1, minimal luminal dilatation with no loss of plicae; 2, dilatation twice the normal diameter with loss/flattening of some plicae; 3, dilatation with complete flattening of mucosa; and 4, marked dilatation with rupture of the wall).

<sup>c</sup> +, received doxycycline; –, did not receive doxycycline.

<sup>d</sup> Mice treated with dexamethasone in experiment 4 received a 7-day steroid taper.
improve outcomes in a variety of inflammatory disorders (21, 26, 27, 29, 31, 43). Therefore, we sought to determine whether the administration of steroids to mice with chlamydial genital tract infections would decrease oviduct inflammation and reduce chronic oviduct pathology.

We determined that although dexamethasone administered in doses of 1 and 2.5 mg/kg per day led to statistically significant decreases in the numbers of neutrophils infiltrating the oviducts during a time of peak inflammation, subsequent pathological evaluation of tissues from mice treated similarly revealed no reduction in chronic oviduct damage compared to controls. Additionally, while early necrosis and cellular infiltrates are reduced in the oviducts of mice given dexamethasone, it appears that there is a minimum threshold of damaging inflammation that is surpassed despite treatment with steroids, such that no improvement in chronic pathology is observed. The detection of equivalent levels of tissue-damaging MMP-9 in the oviducts of mice treated with these doses indicated that the anti-inflammatory effects on neutrophils were insufficient to be protective or that MMP-9 production continued in alternative cell types such as the oviduct stromal cells. These same doses led to decreased infiltration of protective CD4+ T cells and an increase in oviduct and lower genital tract bacterial burden. Although polymorphonuclear neutrophils (PMNs) appear to be most important in driving tissue damage, they also contribute to bacterial killing, and inhibition of neutrophil influx may also contribute to enhanced bacterial burden in steroid-treated mice. Higher doses of dexamethasone given for 3 weeks combined with doxycycline during a 7-day steroid taper also failed to improve chronic oviduct pathology. Both 2- and 3-week courses of dexamethasone led to increased lower genital tract bacterial burden, indicating that prolonged steroid treatment had a derogatory effect on host defense mechanisms, although we cannot rule out a direct effect of dexamethasone on epithelial host cells, leading to enhanced bacterial growth. Unfortunately, the provision of a short course of dexamethasone combined with doxycycline treatment also failed to improve outcomes.

While Maichuk demonstrated improved therapeutic efficacy in patients treated for paratrachoma with antibiotics plus steroids compared to antibiotics alone (44), our data are more consistent with the negative findings of Patton et al., who demonstrated that when steroids were added to doxycycline treatment in female macaques whose fallopian tubes were directly inoculated with C. trachomatis, the addition of steroids led to no improvement in oviduct pathology and persistent oviduct infection (45). Prior studies revealed that steroid treatment led to increased C. trachomatis lung burden after intranasal inoculation of mice (46) and failed to prevent infertility in mice inoculated directly into their ovarian bursa with C. muridarum (47).

Huang and colleagues demonstrated that antibiotics and adjuvantic steroids led to reductions in both the occurrence and severity of renal scarring in pediatric patients with pylonephritis (26). We were unable to replicate these findings in the murine model of chlamydial pelvic inflammatory disease. The tubal anatomy of the oviduct may enhance the risk for permanent damage from inflammation, as scarring at any site along the tube will lead to proximal or distal obstruction and damage to contiguous sites while the kidney contains multiple adjacent renal pyramids that may compensate for the loss of others.

Extrapolation of our findings to women is limited greatly by the relative virulence of C. muridarum in mice compared to C. trachomatis infection in woman. Chlamydia muridarum universally ascends to the oviducts of mice, and the majority of infected mice of multiple inbred strains exhibit high rates of hydrosalpinx and infertility (4, 33). A minority of women infected with C. trachomatis develop complications (48, 49), but it is this high-risk group who would most benefit from a drug with anti-inflammatory effects. Our murine data suggest that the T cell inhibitory effects of steroids can be detrimental during chlamydial infection, and drugs with more discriminating anti-inflammatory activities are needed. Improved understanding of specific immune mechanisms responsible for oviduct scarring should allow for development and testing of agents that inhibit these processes selectively. In the interim, creating an effective antichlamydial vaccine remains of paramount importance to avoid the long-term sequelae of Chlamydia pelvic inflammatory disease.

ACKNOWLEDGMENTS

This work was supported by the NIH-NIAID via grants R01 AI05624 and U19 AI084024 (to T.D.) and by a grant from the Magee Women’s Research Institute Clinical Trainee Research Award (to T.E.C.).

We thank Alison Logar of the Rangos Research Center at Children’s Hospital of Pittsburgh for her assistance with flow cytometry.

REFERENCES


