Induction of the Chlamydia muridarum Stress/Persistence Response Increases Azithromycin Treatment Failure in a Murine Model of Infection

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Viable but noninfectious (stressed/persistent) chlamydiae are more resistant to azithromycin (AZM) in culture than are organisms in the normal developmental cycle. Chlamydia muridarum-infected mice were exposed to amoxicillin to induce the organisms to enter the persistent/stressed state and subsequently treated with AZM. AZM treatment failure was observed in 22% of persistently infected mice, with an average of 321,667 inclusion-forming units (IFU) shed after AZM treatment. Productively infected mice had a 9% rate of AZM treatment failure and shed an average of 12,083 IFU. These data suggest that stressed chlamydiae are more resistant to frontline antimicrobial drugs in vivo.

Chlamydial species exhibit a unique biphasic developmental cycle, interchanging between the infectious elementary body (EB) and the replicative, noninfectious reticulate body (RB). Internalized EBs form vacuoles that fuse to form an inclusion, the membrane-bound structure in which EBs transform to RBs. After multiple rounds of division, RBs condense to form EBs, which are released to infect new host cells. In culture, exposure to environmental insults, including immunological stressors like gamma interferon (IFN-γ) and beta-lactam antibiotics such as amoxicillin (AMX), induces chlamydiae to reversibly detox from this normal developmental cycle (1, 2), entering a noninfectious, viable state (AMX), induces chlamydiae to reversibly detour from this normal interferon (IFN-γ) and beta-lactam antibiotics such as amoxicillin (AMX), induces chlamydiae to reversibly detox from this normal developmental cycle (1, 2), entering a noninfectious, viable state. In our model, AMX treatment decreased vaginal shedding of infectious chlamydiae by >99% without affecting viability. Shedding of infectious EBs resumed within 1 week after treatment cessation (4). These data demonstrate that AMX can be used to reversibly induce chlamydial persistence in vivo.

In culture, persistent chlamydiae are refractory to treatment with first-choice antibiotics. Persistent/stressed C. trachomatis strains within penicillin-exposed Hec1B cells are resistant to treatment with azithromycin (AZM) (5), and IFN-γ exposure makes C. trachomatis more resistant to doxycycline killing (6). Additionally, doses of AZM or ofloxacin up to 4× the MIC are ineffective against persistent C. pneumoniae infection in culture (7). Thus, many investigators have proposed that persistent chlamydiae may contribute to chronic disease by evading antibiotic treatment. While there is evidence that persistent infection occurs in vivo (8, 9), the absence of an experimentally tractable animal model of viable but noninfectious chlamydial infection has hampered direct testing of this prediction. This study will seek to determine if persistent organisms are resistant to treatment by the first-choice antibiotic, AZM, in our newly characterized mouse model (4). All animal experiments described in this manuscript were approved by the East Tennessee State University Committee on Animal Care.

To determine a dose of AZM adequate to eradicate chlamydial shedding, 6-week-old, progesterone-treated, female BALB/c mice were vaginally infected with 106 inclusion-forming units (IFU) of C. muridarum Weiss in 10 μL of 2SPG (sucrose phosphate glutamate buffer) or an equal volume of 2SPG. Mice were given AZM in either a single dose (16 mg/kg [body weight], 80 mg/kg, or 200 mg/kg) or lower repeated doses [20 mg/kg once daily for 3 days following a single 40 mg/kg loading dose [abbreviated 40/20] or 80 mg/kg three times over the course of 6 days [abbreviated 80(3)]] once daily by gavage beginning 6 days postinfection (dpi). Control mice received equal volumes of water each day, as appropriate. Mice were vaginally swabbed every third day, and swabs were used to determine infectious IFU shed, via subculture titer assay and antilipopolysaccharide staining as described previously (4). Both 16 mg/kg, which was selected based on its approximation of a typical human dose (10), and 80 mg/kg, which is a commonly published dosage used in mice (11), failed to eradicate productive infection (Fig. 1). The 200-mg/kg and 40/20 doses completely eradicated chlamydial shedding in the experimental period and were selected to determine AZM efficacy against persistent chlamydial infection in a mouse model.

To test the hypothesis that persistent chlamydiae are more resistant to AZM in vivo, mice were infected as described before and exposed to 2 mg/kg AMX in sterile water twice daily by gavage from 5 to 11 dpi to induce the chlamydial persistence/stress response in vivo (4). For controls, gavage was similarly performed with sterile water. Mice were treated with 200 mg/kg AZM as described before, beginning 6 dpi. Vaginal swabs were collected and processed for enumeration of infectious organisms (4). Experiments were repeated in triplicate with 8 to 16 mice per group (Fig. 2). Treatment failure, defined as any animal resuming shed-
ding of infectious EBs after AZM treatment, was observed in 22% of AMX-stressed, persistently infected animals (Table 1). Notably, the 200-mg/kg AZM dose was more effective at eliminating shedding from productively infected mice, with only 9% treatment failure observed in these animals. Additionally, persistently infected mice that failed AZM treatment shed an average of 321,667 IFU/mouse, while productively infected animals that failed AZM treatment shed only 12,083 IFU/mouse, corresponding to a difference that was significant \( (P = 0.0448; \text{Fig. 2D}) \). As expected, mock-infected animals did not shed infectious chlamydiae (not shown). Additionally, shedding was undetectable immediately after AMX stress but resumed thereafter in all mice receiving AMX alone (persistently infected controls), as previously observed (4). Taken together, these data suggest that persistent/stressed chlamydiae are more resistant to AZM \( \text{in vivo} \), as reported in cell culture (5).

We also assessed treatment failure in AZM 40/20 dose-exposed mice. All 40/20 dose-treated, chlamydia-infected mice that underwent gavage with water (productively infected controls) or AMX ceased shedding following AZM treatment and did not resume shedding at any time up to 21 dpi. Thus, unlike the single 200-mg/kg AZM dose, multiple AZM doses (40 mg/kg

**FIG 1** Shedding of infectious chlamydiae observed in mice treated with various standard AZM treatment doses at 6 dpi. Azithromycin was given either as a single dose of 16, 80, or 200 mg/kg or lower repeated doses [20 mg/kg once daily following a single 40 mg/kg loading dose [abbreviated 40/20] or 80 mg/kg three times over the course of 6 days [abbreviated 80(3)]]. Shedding data from untreated, infected control animals are indicated as “No Tx.” The key indicates the day postinfection each sample was collected.

**FIG 2** Intensity and duration of shedding in chlamydia-infected mice treated with the AMX stressor alone, AZM alone, or AMX and AZM. Shedding curves from untreated, infected control animals are indicated as “No Tx.” Shedding curves from triplicate experiments with 8 mice (A), 16 mice (B), and 12 mice (C) per group are shown. The x axes show days postinfection, while the y axes show average IFU per mouse. The inset in each graph shows the shedding observed 9 to 21 dpi (A) or 9 to 27 dpi (B and C). (D) Average IFU per mouse exhibiting treatment failure at any time from day 15 to day 21 postinfection are shown. Student’s unpaired \( t \) test was used to compare average post-AZM shedding per mouse observed in the AZM-alone group versus that in the AMX/AZM group, following removal of outliers by Grubbs’ test \( (P \leq 0.05 \text{ was considered significant}) \).
followed by three 20-mg/kg doses) eliminated shedding of viable chlamydiae after persistence/stress induction. Therefore, the intrinsic AZM resistance of persistent/stressed chlamydiae can be overcome by increasing the AZM treatment duration.

Treatment failure is a significant, ongoing problem in the context of human chlamydial infections (12, 13). For example, C. trachomatis positivity is reported in 10 to 15% of women after following recommended treatment regimens (14) and 23% of male patients are positive for chlamydial infection 4 weeks after AZM therapy (15). Furthermore, recurrent infections with the same serovar occur in patients that have received antimicrobial therapy (16). While these data are consistent with either failure of treatment for ongoing infection or reinfection by a positive sexual partner, there is currently no evidence of human clinical chlamydial isolates displaying stable homotypic resistance to antimicrobials in vivo (13, 14, 17). Because persistent/stressed chlamydial forms (ABs) have been observed in samples from treated patients (18) and persistent chlamydiae have increased antimicrobial resistance in culture (5–7), it has been proposed that persistent/stressed chlamydiae may contribute to treatment failure in vivo (5, 6, 13). Our observations that (i) the percentage of AZM treatment failures is increased when persistence is induced with AMX and (ii) shedding of infectious EBs is higher in persistently infected mice that fail AZM therapy support this hypothesis, particularly given that reinfection cannot occur in our model system.

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