

Growth Cycle-Dependent Pharmacodynamics of Antichlamydial Drugs

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Chlamydiae are obligate intracellular pathogens that exhibit an extensive intracellular developmental cycle in vivo. Clinical treatment of chlamydial infection is typically initiated upon occurrence of symptomatology and is directed against an asynchronous population of different chlamydial developmental forms. Pharmacodynamics of antichlamydial drugs are predominantly characterized by MICs; however, in vitro determinations of MIC may not reflect differential susceptibilities of the developmental cycle. In this study, we correlated the antichlamydial effect of erythromycin, rifampin, doxycycline, and ciprofloxacin with the developmental stage of a fast-replicating and a slow-replicating chlamydial species. In addition, we describe the influence of concentration on killing. Extracellular elementary bodies and very-early-phase and late-phase chlamydiae were refractory to all tested antibiotics except rifampin, which was very effective against early-cycle chlamydiae. Rifampin was the most effective antibiotic overall, killed in a dose dependent matter, and exhibited moderate synergism with erythromycin. These considerations provide important information on chlamydial biology and antimicrobial susceptibility. A combinational therapy of rifampin and a macrolide should be considered in therapy-refractory infections.

Chlamydiae share a biphasic intracellular developmental cycle that is characterized by infectious and extracellularly viable elementary bodies (EBs) and the intracellular reticulate bodies (RBs). The latter replicate by binary fission within an intracytoplasmic parasitophorous vacuole called “inclusion.” The time kinetics of the developmental cycle are of great variance between different chlamydial species and range in vitro from as little as 36 h for *Chlamydia trachomatis* L2 to as much as 72 h for *C. pneumoniae*.

C. trachomatis is a major cause of ocular infections and sexually transmitted diseases resulting in serious sequelae such as trachoma, pelvic inflammatory disease, and tubal infertility (12, 14). *C. pneumoniae* causes upper respiratory tract infections, and more than 10% of community-acquired pneumonias and can be isolated from inflamed vascular tissue (3, 9, 13).

In order to prevent serious sequelae of chlamydial infections, successful antimicrobial treatment is of paramount importance, and treatment failures of chronic, long-lasting infections are of great concern (8, 12). Therapy-refractory organisms rarely exhibit increased MICs in in vitro models (7). These models usually determine antimicrobial activity against freshly inoculated cells (17). Clinical therapy, however, is typically directed against an established symptomatic infection. This fact requires the consideration of possible differences in antibiotic susceptibility related to the developmental cycle. Therefore, we determined the antichlamydial activity of clinically relevant antimicrobials at different developmental stages of the organism. We established time-kill curves at therapeutically relevant concentrations in order to allow for a more differentiated view of chlamydial susceptibility in comparison to those obtained by conventional MICs. These results may improve the understanding of chlamydial therapy and biology.

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MATERIALS AND METHODS

Cell culture. HeLa 229 cells were grown in 24-well cluster plates in Dulbecco's modified Eagle's medium (PAA Laboratories, Cölbe, Germany) supplemented with 10% fetal bovine serum (Gibco/Invitrogen, Karlsruhe, Germany). A total of 8×10^5 cells per well were infected with 4×10^5 inclusion-forming units (IFU) of *C. pneumoniae* strain AR-39 (ATCC 53592) or *C. trachomatis* serovar L2 (ATCC VR-902B) and centrifuged for 1 h at $550 \times g$ at 35°C and incubated at 35°C with 5% CO₂ in the presence of 1 µg/ml cycloheximide (Sigma, Deisenhofen, Germany). Calculation of time postinfection (p.i.) started after centrifugation.

Growth curves. Chlamydiae were harvested 1 h, 6 h, 12 h, 18 h, 24 h, 36 h, 48 h, 72 h, and 86 h postinfection and inoculated onto fresh monolayers. Chlamydiae were stained with an anti-lipopolysaccharide antibody, and inclusion-forming units were counted and given as a percentage of the inoculum.

Electron microscopy. For ultrastructural analysis, *Chlamydia*-infected cells were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Sigma, Deisenhofen, Germany) in 0.1 M cacodylate buffer, pH 7.2, for 1 h at room temperature. Cells were washed three times in cacodylate buffer and postfixed in 1% osmium tetroxide (Polysciences Inc., Eppelheim, Germany) for 1 h at 4°C. Samples were washed again, dehydrated in a graded ethanol series, and embedded in araldite (Fluka, Buchs, Switzerland). Ultrathin sections were cut on an Ultracut E (Reichert-Jung, Nußloch, Germany) and contrasted with uranyl acetate and lead citrate (UltraStainer Carlsberg System; LKB, Bromma, Sweden). The sections were examined using a Philips electron microscope, EM 400, at 60 kV.

MIC. The MIC was determined by adding twofold dilutions of the antibiotic and immunofluorescence staining after 72 h in *C. pneumoniae* and 36 h in *C. trachomatis*. The MIC was the concentration that reduced the IFU number by at least 1.5 logarithmic steps.

Time-kill curves. The growth cycle was subdivided in an early phase (starting at 0 h p.i.), mid phase (interval with midpoint at 18 h p.i. in *C. trachomatis* and 36 h p.i. in *C. pneumoniae*), and late phase (ending at 36 h p.i. in *C. trachomatis* and 72 h p.i. in *C. pneumoniae*). Time-kill curves were determined in these phases for ciprofloxacin, doxycycline, erythromycin (2 µg/ml each), and rifampin (8 µg/ml). Synergistic activities of all combinations in *C. trachomatis* were studied by simultaneous addition of the drugs. Antibiotic concentrations used were based upon reported mean serum levels (16). Additional concentrations (0.5 µg/ml, 2 µg/ml, or 8 µg/ml) were tested for the influence of concentration on killing. All antibiotics were purchased from Sigma, Deisenhofen, Germany, except ciprofloxacin, which was provided by Bayer, Leverkusen, Germany. Antibiotics were added for the indicated intervals and washed out three times with medium, and cells were cultured for a total of 36 h when infected with *C. trachomatis* and for 72 h when infected with *C. pneumoniae*. In order to detect reductions in IFU number of up to 3 logarithmic steps, chlamydiae were harvested, disrupted with glass beads, and inoculated onto new cell monolayers in two dilutions, resulting

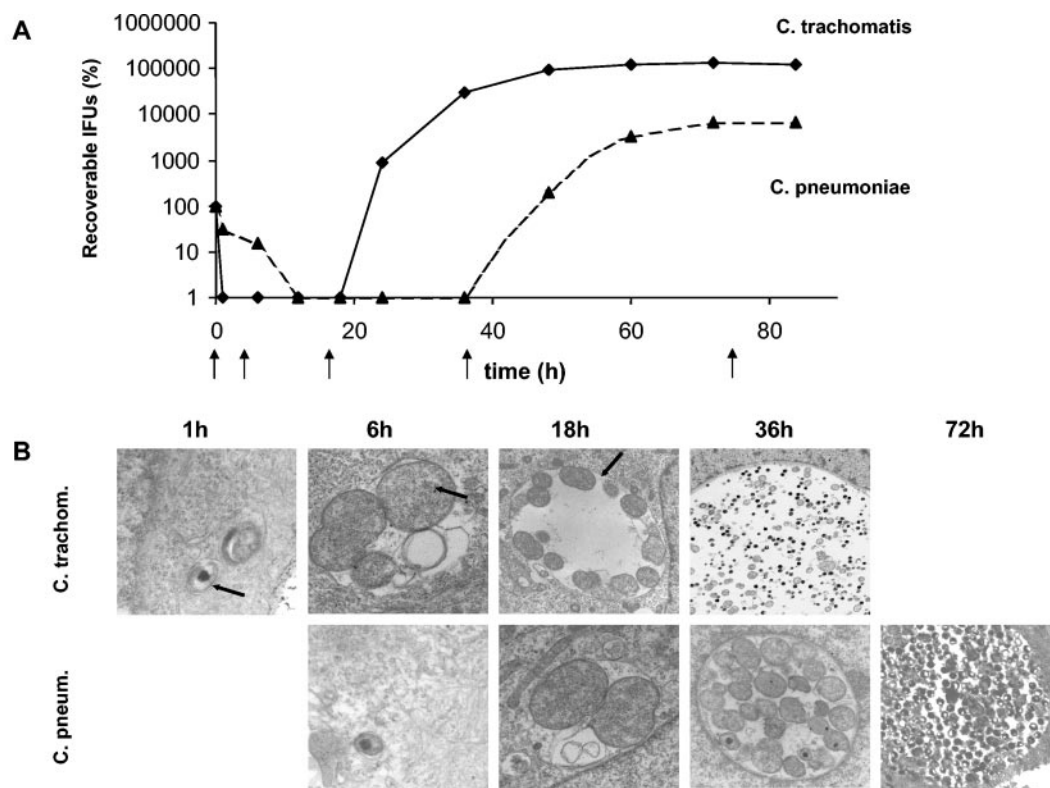


FIG. 1. Growth cycle of fast-replicating *C. trachomatis* serovar L2 and slow-replicating *C. pneumoniae* as reflected by recoverable IFUs (A) and electron microscopy (B). The early phase is characterized by the transition of EBs (black arrow at 1 h in panel B) into RBs (black arrow at 6 h in panel B) and the loss of infectivity (indicated by reduced recoverable IFUs in panel A). This phase is protracted in *C. pneumoniae* for more than 6 hours. The mid phase (18 h in *C. trachomatis* and 36 h in *C. pneumoniae*) is dominated by replicating RBs within the growing chlamydial inclusion (black arrow at 18 h in panel B). RBs recondense into EBs during the late phase and regain infectivity (indicated by increased recoverable IFUs in panel A). The growth cycle lasts 36 h in *C. trachomatis* and 72 h in *C. pneumoniae*.

in about 10 and >1,000 IFUs per high-power field in the antibiotic-free control. Infected cells were fixed with methanol after 24 h in *C. trachomatis* or 72 h in *C. pneumoniae* and stained with a genus-specific anti-lipoplysaccharide antibody, and inclusion-forming units were counted. The average of four replicates was calculated and expressed as a percentage of the untreated control; the variability of the replicates was below $\pm 33\%$ of the arithmetic mean in all experiments. Susceptibility of elementary bodies was tested by adding the antibiotic to purified elementary bodies with a titer of 1,000 IFUs in a total volume of 10 μ l for the indicated time periods. In order to reduce the drug concentrations below the MIC for cultivation, elementary bodies were diluted in 1 ml medium, and the medium was replaced after centrifugation. IFUs were counted after 24 h (*C. trachomatis*) or 72 h (*C. pneumoniae*).

RESULTS

Chlamydial growth cycle. Fig. 1 reflects the differences in the growth cycle between the fast-replicating *C. trachomatis* L2 and the slow-replicating organism *C. pneumoniae*. Recoverable IFUs (Fig. 1A) dropped soon after infection, indicating transition of EBs into noninfectious RBs and the beginning of replication (Fig. 1B). An increase of recoverable IFUs was associated with recondensation of RBs into EBs and started 24 h p.i. in *C. trachomatis* and 48 h p.i. in *C. pneumoniae*. The total length of the developmental cycle was about 36 h in *C. trachomatis* and 72 h in *C. pneumoniae*. The infectious burst of *C. trachomatis* exceeded that of *C. pneumoniae* by 50-fold. In summary, the chlamydial growth cycle can be morphologically divided into three phases. The early phase, starting at 0 h p.i.,

is characterized by elementary bodies and the transition of EBs into RBs. Replicating RBs dominate the mid phase (around 18 h p.i. in *C. trachomatis* and 36 h p.i. in *C. pneumoniae*), and the recondensation of RBs into EBs reflects the late phase (ending at 36 h p.i. in *C. trachomatis* and 72 h p.i. in *C. pneumoniae*).

MIC testing. MICs for the fast- and slow-replicating chlamydial isolates differed not more than one dilutional step (Table 1). Rifampin was the most active drug, with a MIC three to five dilutional steps lower than that of erythromycin and doxycycline and six to seven dilutional steps lower than ciprofloxacin. Comparing MIC to antimicrobial concentrations in the human serum (approximately 8 μ g/ml for rifampin and 2 μ g/ml for ciprofloxacin, doxycycline, and erythromycin), serum levels were 1,600-to 3,200-fold higher than the MIC of rifampin,

TABLE 1. MICs of antibiotics against fast-replicating *C. trachomatis* and slow-replicating *C. pneumoniae*

Drug	MIC (μ g/ml)	
	<i>C. trachomatis</i>	<i>C. pneumoniae</i>
Rifampin	0.0025	0.005
Doxycycline	0.05	0.05
Erythromycin	0.1	0.05
Ciprofloxacin	0.25	0.5

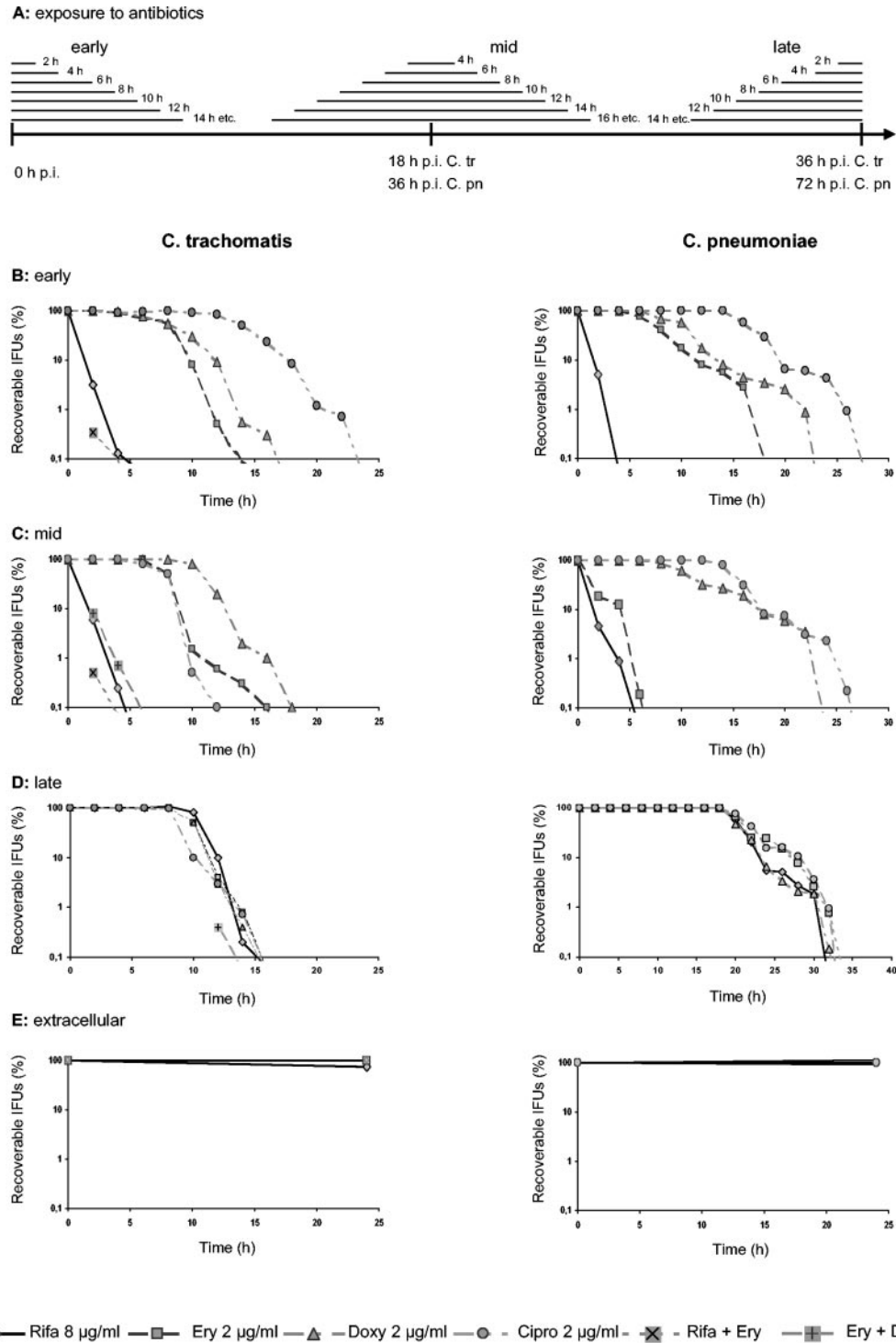


FIG. 2. Time-kill curves of *C. trachomatis* and *C. pneumoniae* at different developmental stages. Diagram A depicts schematically the exposure to antibiotics during early, mid, and late phases of the cycle. Exposure to early-phase chlamydiae (B) started at 0 h p.i. and lasted for the time periods indicated on the x axis. Cell culture proceeded until 36 h p.i. in *C. trachomatis* and 72 h p.i. in *C. pneumoniae*. Surviving chlamydiae were recultured, and recoverable IFUs are shown on the y axis as a percentage of the antibiotic-free control. Mid-cycle chlamydiae (C) were exposed for a time period that was centered at 18 h p.i. for *C. trachomatis* and 36 h p.i. for *C. pneumoniae*. Exposure to late-cycle chlamydiae (D) started at different time points and ended uniformly at 36 h p.i. for *C. trachomatis* and 72 h p.i. for *C. pneumoniae*. The x axis of the diagram showing extracellular chlamydiae (E) indicates the time of exposure to antibiotics before bacteria were cultured. Antimicrobial combinations were tested in *C. trachomatis* and displayed when synergistic activities were observed. C. tr, *C. trachomatis*; C. pn, *C. pneumoniae*; Rifa, rifampin; Ery, erythromycin; Doxy, doxycycline; Cipro, ciprofloxacin.

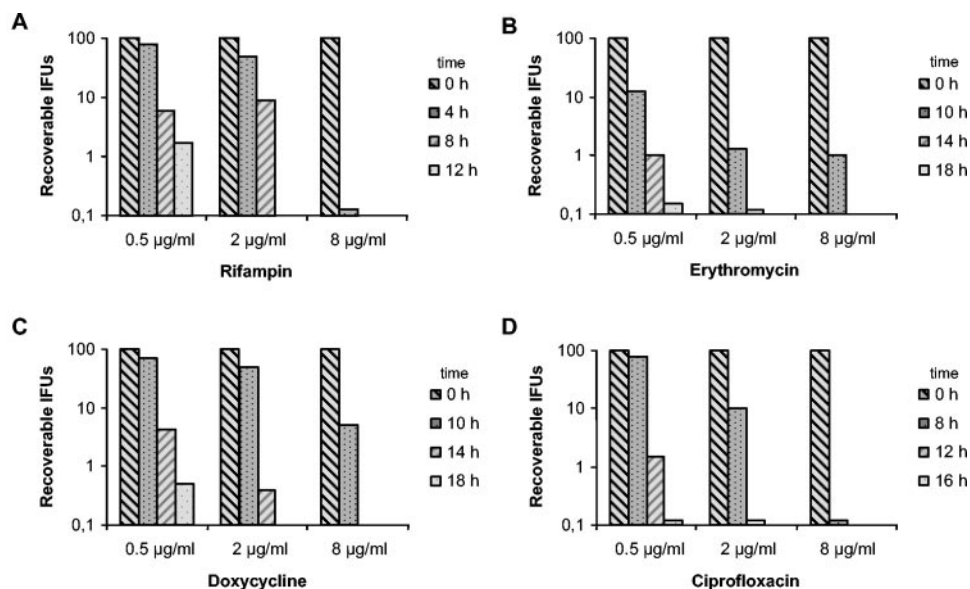


FIG. 3. Influence of concentration on killing of *C. trachomatis*. Rifampin, erythromycin, and doxycycline were applied during early phase (starting at 0 h p.i.) for the indicated time periods. Ciprofloxacin was studied during mid phase for a time period centered at 18 h p.i. Recoverable IFUs were determined after recultivation. All antibiotics showed concentration-dependent killing.

40-fold higher than that of doxycycline, 20- to 40-fold higher than that of erythromycin, and 4- to 8-fold higher than that of ciprofloxacin.

Time-kill curves. Antibiotic treatment of EBs had little effect on infectivity. Only rifampin showed a slight reduction of 30% within 24 h (Fig. 2). During early phase, rifampin showed the fastest killing (99.9% within 4 h) for both *C. trachomatis* and *C. pneumoniae*, followed by doxycycline and erythromycin (99.9% within 14 h to 16 h for *C. trachomatis* and 18 h to 23 h for *C. pneumoniae*). Ciprofloxacin had no effect during the first 15 h of the cycle; 23 h in *C. trachomatis* and 28 h in *C. pneumoniae* were required for a 99.9% reduction. Synergism was studied in *C. trachomatis*, and the combination of rifampin and erythromycin proved moderately synergistic on time-killing.

Mid-phase chlamydiae were highly susceptible to all antibiotics. Rifampin decreased recoverable IFUs by 99.9% within a 4- to 6-hour interval (16 h p.i. to 20 h p.i. for *C. trachomatis* and 33 h p.i. to 39 h p.i. for *C. pneumoniae*). Ciprofloxacin, erythromycin, and doxycycline required 12 h, 16 h, and 18 h, respectively, for killing of *C. trachomatis* and 24 h, 6 h, and 26 h, respectively, for killing of *C. pneumoniae*. Strong synergistic activity was noted for the combination of erythromycin plus doxycycline and moderate synergistic activity was observed for rifampin plus erythromycin in *C. trachomatis*.

Late-phase chlamydiae are refractory to antibiotics when applied later than 28 h p.i. in *C. trachomatis* and 54 h p.i. in *C. pneumoniae*. A 99.9% reduction was achieved by all antibiotics when drugs were added before 22 h p.i. in *C. trachomatis* and 40 h p.i. in *C. pneumoniae*. The combination of erythromycin plus doxycycline proved a moderate synergistic activity in *C. trachomatis*.

Concentration-dependent killing. The influence of increasing antimicrobial concentrations on killing was studied in *C. trachomatis* (Fig. 3). Rifampin, erythromycin, and doxycycline showed a strong effect of concentrations between 0.5 µg/

ml and 8 µg/ml on time-killing during early phase (starting at 0 h p.i.). The effect of ciprofloxacin was less pronounced (data not shown). Therefore, ciprofloxacin was studied at mid phase (centering at 18 h p.i.), and chlamydiae were highly susceptible to increasing concentrations.

DISCUSSION

Treatment failures of chlamydial infections are still a matter of concern, despite the in vitro sensibility of the organisms by MIC testing (8, 12). Persistent developmental forms have been described in vitro and associated with antimicrobial refractoriness (1, 6, 10). Our model instead focuses on the active developmental cycle and studies differential susceptibility of its various developmental forms. This issue requires consideration, as clinical treatment is usually directed against a fully established infection and a mixture of multiple developmental forms. In the present in vitro model, we divided the cycle into three phases and the extracellular EBs and described the pharmacodynamic characteristics of the antimicrobials used most often by time-killing at various serum concentrations. Since chlamydiae differ in the length of the growth cycle, we compared a fast-replicating and a slow-replicating species.

We have demonstrated a reduced antimicrobial susceptibility in the extracellular phase and the very late and early states of the cycle. These phases can be correlated to the presence of elementary bodies that have been shown to have a reduced metabolism (2). One remarkable exception is the immediate activity of rifampin against early stages of both *C. trachomatis* and *C. pneumoniae*. Especially the latter one was killed before the replicative phase commenced. Microarray analyses have identified the immediate-early genes of *C. trachomatis* (2), of which none was described to interact with rifampin. The RNA polymerases as a major target of rifampin may be present in early chlamydiae as a preformed protein, but the superior

activity of rifampin in comparison to other drugs requires further studies. In mid phase, differences between the antibiotics were more distinct. By comparing the two species, killing was faster for ciprofloxacin in *C. trachomatis* and for erythromycin in *C. pneumoniae*. Rifampin was again most effective. In late phase, all tested antibiotics act with the same kinetic. The time p.i. before drugs have to be applied in order to achieve sufficient killing corresponds to 60% of the cycle in fast- and slow-replicating species. Recondensation into EBs was associated with refractoriness to treatment. As antibiotics are classically divided into concentration-dependent and concentration-independent agents, we tested the influence of three physiologically relevant doses on killing of *C. trachomatis*. To our knowledge, this is the first study that reports the increased susceptibility of chlamydiae to higher concentrations of antimicrobials and justifies the application of the highest tolerable dose.

The pharmacodynamics of antichlamydial antibiotics are most often characterized by MICs. They correspond to the drug concentration at the site of infection that is supposed to be exceeded in order to mediate activity. In addition, MIC is a useful tool to monitor the development of resistance. As resistance is not a matter of serious concern in chlamydiae and serum as well as intracellular concentrations of all tested antibiotics exceed MICs, comparison of MICs in the case of chlamydiae is of little value for the comparison of antibiotics. Additionally, MIC determination often neglects the fact that the in vivo treatment is directed against an established infection (17). Our model of time-killing resembled the in vivo situation more closely. Physiologically relevant doses of drugs were used, and different stages of the cycle were distinguished. Drug interactions like synergism could be easily monitored. The results provided a rationale for improved antimicrobial treatment and gave information on the biology of the pathogen during the cycle. The model did not account for developmental forms resembling persistent infections, as they could be induced in vitro under various conditions (1, 6, 10). Killing was detected over 3 logarithmic steps; regrowth at lower levels that has been reported in some studied was not monitored (4).

Chlamydial infections are usually treated with macrolides or tetracyclines. Nevertheless, therapeutic failures and reinfections often occur, resulting in serious sequelae like pelvic inflammatory disease or tubal infertility (7, 8, 12). Our in vitro study demonstrated the superior activity of rifampin over other often-used antibiotics. In addition, rifampin is well tolerated and cheap. The drawback of rifampin is the fast occurrence of resistance by a point mutation in the *rpoB* gene (5). Failure due to resistance has been described in *C. trachomatis* (15) but interestingly not in *C. pneumoniae*. A combination of rifampin with other drugs has been proven successful in preventing resistance (11). Macrolides seem to be a good combinational partner as they not only are the second most active antimicrobial class but also show synergistic activity with rifampin. The combination of azithromycin and rifampin was already shown

to be effective in an animal model of *C. pneumoniae* mouse pneumonitis (18).

In summary, our model of chlamydial time-killing provided further evidence for rifampin as a highly active antichlamydial drug. Clinical studies are needed to demonstrate its clinical effectiveness in therapy-refractory infections.

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