In Vitro Activities of Ceftriaxone and Fusidic Acid against 13 Isolates of *Coxiella burnetii*, Determined Using the Shell Vial Assay

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The susceptibilities of 13 isolates of *Coxiella burnetii* to fusidic acid and ceftriaxone were determined by use of the recently described shell vial assay (D. Raoult, H. Torres, and M. Drancourt, Antimicrob. Agents Chemother., 35:2070–2077, 1991). At a concentration of 4 µg/ml, ceftriaxone was bacteriostatic for four isolates and slowed the multiplication of the other nine. Fusidic acid at a concentration of 2 µg/ml was bacteriostatic for six isolates and slowed the multiplication of three others. These results show that these compounds could be effective in the phagolysosome of *C. burnetii*-infected cells.

*Coxiella burnetii*, a strict intracellular bacterium, is the causative agent of Q fever. The acute form of the disease is usually a self-limiting acute febrile illness during which pneumonia or hepatitis may occur. The chronic form is, however, a severe disease in which endocarditis predominates (13, 18), but in some cases, vascular (19), aneurysm (13), or bony (16) infections may occur. For acute Q fever, bacteriostatic antibiotics are useful in helping a patient recover, but to cure a patient with chronic Q fever, a physician should use a bactericidal antibiotic regimen. To date, no antibiotic has been shown to be bactericidal against *C. burnetii* (17), a fact that may explain failures in treating Q fever patients. We recently developed a new technique for determining the antibiotic susceptibility of *C. burnetii* (20). In this model, an acute infection is achieved by inoculating suspensions of cell monolayers, and the bacteriostatic activity of the added antibiotics is evaluated after 6 days of incubation. This technique has been used to test the activities of 12 antibiotic compounds on three reference strains and 10 new human isolates of *C. burnetii*. The results revealed a heterogeneity of antibiotic susceptibility among the isolates implicated in Q fever endocarditis and led us to test other antibiotic compounds. Ceftriaxone is a broad-spectrum cephalosporin with a long half-life after parenteral administration. The broad-spectrum cephalosporins are very active against most of the gram-negative bacilli. Ceftriaxone has been successfully prescribed in some cases of Q fever (2). Fusidic acid is a steroid antibiotic, chemically related to cephalosporin P1. Although it is effective against some gram-negative bacteria (26), it is mainly active against gram-positive bacteria and is therefore most commonly used in the treatment of severe staphylococcal infections, including endocarditis (3, 6) and osteomyelitis (5, 11). This compound attains a high intracellular concentration (22) and is therefore a drug of interest against intracellular bacteria (7). Its half-life is 11.4 h. In this report, we describe the bacteriostatic effects of ceftriaxone and fusidic acid against 13 isolates of *C. burnetii*.

MATERIALS AND METHODS

*C. burnetii*. The *C. burnetii* isolates examined in this study were the same as those tested previously (20). The three reference strains were Nine Mile, obtained from O. Baca (University of New Mexico, Albuquerque), and Q212 and Priscilla, obtained from T. Hackstadt (Rocky Mountain Laboratories, Hamilton, Mont.). Nine Mile is the reference strain for acute infections, whereas Q212 and Priscilla (a goat strain) are reference strains for chronic infections (9). The 10 human isolates were obtained from eight cardiac valves, a vascular aneurysm, and a vascular prosthesis in patients suffering from chronic Q fever. These *C. burnetii* isolates were obtained by the inoculation of tissue homogenates onto human embryonic lung fibroblasts (HEL cells) in centrifugation shell vials as described previously (21). These isolates were named M (for Marseille), E (for endocarditis), AN (for aneurysm), P (for prosthesis), and I (for immunocompromised) and were numbered from 1 to 10.

In vitro cultivation of *C. burnetii*. The *C. burnetii* isolates were cultivated on HEL cells grown in antibiotic-free Eagle minimal essential medium supplemented with 1% glutamine and 10% fetal calf serum at 37°C in a 5% CO2 atmosphere. The cells were passaged until the degree of infection reached 90%. An inoculum was then prepared from each isolate.

Antibiotic preparation. Ceftriaxone sodium for injection (Roche, Neuilly-sur-Seine, France) and fusidic acid (sodium fusidate; Léo S.A., Montigny-le-Bretonneux, France) were used in this study. A stock solution of 1 mg/ml was prepared with sterile distilled water for each antibiotic. Stock solutions were sterilized via filtration (pore size, 0.22 µm) and stored at −20°C in approximately 4-ml aliquots.

*C. burnetii* inoculation titration test. Cells in 90% infected cultures were trypsinized and resuspended in culture medium, and the microorganisms were released from the host cells by strong homogenization (20); 0.5- or 1-ml fractions were stored at −80°C. Prior to the antibiotic exposure, the number of infective *C. burnetii* microorganisms per milliliter of infected-cell suspension was determined by the titration test described previously (20). In brief, dilutions (10−1 to 10−8) were inoculated onto confluent monolayers of HEL cells in centrifugation shell vials. After 6 days of incubation at 37°C in a 5% CO2 atmosphere, the infected cells were demonstrated by indirect immunofluorescence with a rabbit
antiserum to C. burnetii. The titer of the inoculum was calculated from the last dilution that resulted in at least one infected cell in a shell vial. The results obtained for each isolate varied but were all between 2.5 × 10^−4 and 5.0 × 10^−3 bacteria per ml. The inoculation titration test was also used to determine the dilution of the inoculum resulting in 30 to 50% infected cells after 6 days; this dilution was previously determined to be the most suitable dilution for antibiotic treatment experiments (20).

**Antibiotic susceptibility assay.** Antibiotics were used at the lower breakpoint in accordance with the French Committee of the Antibiogram (1). The antibiotic concentrations, expressed in micrograms per milliliter, were 4 for ceftriaxone and 2 for fusidic acid. These concentrations were obtained by diluting each antibiotic stock solution with the cell culture medium, the last 10-fold dilution being made in the shell vials after the inoculation step. We used the antibiotic challenge procedure described previously (20). For each C. burnetii isolate, confluent monolayers of HEL cells were inoculated with 0.4 ml of the infected-cell suspension dilution that resulted in 30 to 50% of the cells becoming infected after 6 days in the inoculum titration test. The shell vials were centrifuged at 30°C for 1 h at 700 × g. The supernatant was decanted, and 0.1 ml of the antibiotic stock solution and 0.9 ml of the cell culture medium were added to the shell vials. For each isolate, a reference shell vial of infected HEL cells without antibiotics (positive control) was prepared. Uninfected shell vials and slides fixed immediately after inoculation with each isolate were kept as negative controls. Three shell vials per challenge were used. Tests were repeated three times for each isolate. After 6 days at 37°C in a CO2 incubator, the number of HEL cells infected with C. burnetii was determined by indirect immunofluorescence.

**Indirect immunofluorescence for C. burnetii.** After fixation with methanol for 10 min, the HEL cells were rehydrated for 5 min with phosphate-buffered saline (PBS). The coverslips in the shell vials were incubated for 30 min at 37°C with 0.3 ml of anti-C. burnetii rabbit antiserum (titer, 1:2,000) diluted (1:200) in PBS and were washed three times (10 min each time). The cells were then incubated for 30 min at 37°C with 0.3 ml of fluorescein-conjugated goat anti-rabbit globulin (Bio-Mérieux, Charbonnières-les-Bains, France) diluted (1:300) in PBS with Evans blue diluted 1:400 as a counterstain. After each wash, shells in the vials were washed in water, and the shell vials were mounted in buffered glycerol on microscope slides and evaluated independently by both of us with a fluorescence microscope at a magnification of ×100.

**Test reading.** The C. burnetii organisms could be identified by their intense yellow-green fluorescence inside one or more vacuoles of infected cells. For each isolate, the effect of an antibiotic compound was assessed by comparison with infection in shell vials not treated with antibiotics (positive control). The results were scored as follows (20): resistant, infection with antibiotic treatment comparable to that of the positive control, meaning normal growth (30 to 50% infected cells); intermediate or increased susceptibility (fewer than 10% infected cells), meaning decreased growth; and susceptible, the absence of infected cells or the presence of isolated bacteria, meaning no growth. The percentage of infected cells was assessed at a magnification of ×100. For each isolate, the tests were repeated 2 months later with another inoculum and fresh antibiotic stock solutions.

**RESULTS**

Table 1 shows the efficacies of ceftriaxone and fusidic acid against the different isolates tested. Ceftriaxone at 4 μg/ml was bacteriostatic against four C. burnetii isolates, including the Nine Mile strain. The multiplication of the other nine isolates was slowed down. For all these isolates with intermediate susceptibility, round bacterial forms of C. burnetii were seen in the phagolysosomal vacuoles of the infected cells (Fig. 1), and the size of the infected vacuoles was reduced in comparison with that in the positive controls (Fig. 1). The activity of fusidic acid against C. burnetii was also shown to depend on the isolate. Fusidic acid was bacteriostatic for six isolates and reduced the growth of three; four were found to be resistant. No clear alteration of the C. burnetii morphology was observed with fusidic acid.

**DISCUSSION**

In vitro susceptibilities of C. burnetii to ceftriaxone and fusidic acid have not been tested before. In this study, we showed a heterogeneity of susceptibility to these two different compounds among three reference strains and 10 human isolates of C. burnetii implicated in chronic Q fever.

To date, no beta-lactam antibiotic has been shown to be effective against C. burnetii in vitro or in vivo. The data obtained in embryonated eggs, a model of acute infection, showed that C. burnetii was resistant to penicillin G and to cephalothin, a narrow-spectrum cephalosporin (15,25). In our assay system, all C. burnetii isolates were resistant to amoxicillin (20). In vivo, penicillin alone or in combination with aminoglycosides has failed to cure C. burnetii endocarditis (18). We found that the new broad-spectrum cephalosporin ceftriaxone was bacteriostatic against four isolates of C. burnetii and reduced the growth of the other nine. Other intracellular bacteria have also been shown to be susceptible to ceftriaxone. Brucella melitensis, a facultative intracellular bacterium that can multiply in mononuclear phagocytes, is more susceptible to ceftriaxone than to the narrow-spectrum cephalosporins and ampicillin (4, 14). Ceftriaxone has also been found to be active against Legionella spp. (23), a facultative intracellular parasite. Ceftriaxone and the new cephalosporins have been shown to have poor activity against Chlamydia trachomatis, an obligate intracellular parasite growing in the phagosomes of infected cells. The MIC of ceftriaxone against C. trachomatis varied between 16 and 32 μg/ml, whereas the MIC of cefotaxime was ≥128 μg/ml (10). With 8 to 32 μg of ceftriaxone per ml in the cell

**TABLE 1.** Susceptibilities of 13 C. burnetii isolates to antibiotics

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<tr>
<th>Isolate</th>
<th>Fusidic acid</th>
<th>Ceftriaxone</th>
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<tr>
<td>Nine Mile</td>
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*a* Susceptible (no growth); I, intermediate (decreased growth); R, resistant (normal growth).
culture medium, aberrant chlamydial inclusions were formed rather than chlamydial inclusions disappearing (27).

Our results showed that the MIC of ceftriaxone against C. burnetii was <4 µg/ml for about one-third of the studied isolates, so we can assert that C. burnetii is more susceptible to ceftriaxone than C. trachomatis, the other pathogen tested in cell culture systems. Ceftriaxone shows satisfactory penetration into cells (12). The altered forms of C. burnetii that we observed with ceftriaxone for isolates of intermediate susceptibility may have been the result of an alteration in the synthesis of the bacterial cell wall without a complete inhibition of growth.

Fusidic acid is highly active against Staphylococcus epidermidis and Staphylococcus aureus, including penicillinase-producing strains and strains with multiple antibiotic resistance (11). Fusidic acid is also very active against corynebacteria but shows poor activity against streptococci (8). The susceptibility of certain gram-negative bacteria to fusidic acid was first documented for members of the genus Neisseria (8). More recently, it was shown that Legionella spp. were susceptible in vitro to fusidic acid (28). A recent report shows that fusidic acid is active against Mycobacterium leprae (7). The inhibition of metabolic activity in M. leprae, as measured by a radiorespirometric analysis, was clearly dose dependent. The authors obtained an approximately 50% reduction in activity in both extracellular and intracellular M. leprae with fusidic acid concentrations of 1.25 to 2.5 µg/liter. This result is consistent with the effective intracellular penetration of this drug (intracellular concentrations are 40 to 100% extracellular concentrations [22]).

Like C. burnetii, M. leprae is an obligate intracellular parasite (29). In our work, a fusidic acid concentration of 2 µg/ml inhibited the growth of 6 of 13 isolates of C. burnetii tested. We can parallel this result with the susceptibility of certain gram-negative bacilli, including Legionella spp., to fusidic acid and with the activity of fusidic acid against intracellular M. leprae.

Fusidic acid shows effective intracellular penetration related to its lipophilic nature. Repeated oral or intravenous administrations of fusidic acid (three 500-mg doses per day) result in a maximum concentration in serum of >100 mg/liter (24). Fusidic acid has a long half-life (about 10 h) (24), is highly protein bound, and is mainly excreted by the liver (22). Serum and tissue fusidic acid concentrations are well above the MIC for most susceptible bacterial strains. Fusidic acid has a hydrophobic manner of penetration through the bacterial cell wall. The presence of the charge of lipopolysaccharide at the surface of the outer membrane of gram-negative bacilli generally prevents the penetration of hydrophobic antibiotics, such as erythromycin and fusidic acid. The mode of action of fusidic acid is to inhibit bacterial protein synthesis.

Our experiments showed a heterogeneity of susceptibility to ceftriaxone and fusidic acid among the C. burnetii isolates tested. In our previous report on C. burnetii antibiotic susceptibility, the quinolone ciprofloxacin, used at a concentration of 1 µg/ml, distinguished between susceptible and intermediate isolates. Four isolates and the Nine Mile strain were susceptible to ciprofloxacin (20). Of these, Nine Mile, MP 7 and ME 8 are susceptible to both ceftriaxone and...
fusidic acid, and ME 1 and ME 3 are susceptible to fusidic acid and intermediate in susceptibility to ceftriaxone. Many endocarditis-associated isolates show decreased susceptibility to several antibiotics, despite the different modes of action of these drugs. These resistant isolates may present a reduction in the permeability of the bacterial cell wall, affecting the penetration of many antibiotics. This hypothesis was proposed by Yeaman et al. (30), who showed a general diminution of the antibiotic susceptibility of the Priscilla strain in comparison with that of the Nine Mile strain. Quantitative or qualitative modifications of lipopolysaccharide, as described by Hackstadt (9), may explain the variation in antibiotic susceptibility among the C. burnetii isolates.

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REFERENCES


