Bactericidal Activities of Antibiotics against Intracellular Francisella tularensis

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MICs of many antibiotics for Francisella tularensis are low in axenic medium, whereas only aminoglycosides, tetracyclines, and fluoroquinolones are useful in treating tularemia in cell culture. Only these antibiotics, rifampin, and telithromycin were bactericidal against intracellular F. tularensis. These results correlate better with clinical data than MIC data do.

Francisella tularensis bv. tularensis and F. tularensis bv. palaearctica are responsible for tularemia (17, 25). The geographical distribution of the former biovar has long been considered restricted to North America, but this species has recently been isolated from fleas and mites parasitizing small mammals in Slovakia (15). This biovar is considered more virulent in humans and animals than F. tularensis bv. palaearctica, which is found essentially in Europe and Asia and to a lesser extent in North America. Antibiotic therapy of tularemia has been empirically determined. Only streptomycin treatment allows a 100% cure rate (7, 12). Because this antibiotic is potentially toxic and may not be widely available nowadays, alternatives have been proposed. Gentamicin has been used successfully (5, 20) but has a similar toxicity potential. Tetracyclines are considered a useful alternative, although relapses are observed in about 10% of cases (9, 24, 28). More recently, fluoroquinolones have been useful in a limited number of patients (26, 29, 31). Other antibiotics, including beta-lactams, macrolides, and chloramphenicol, are considered less effective or ineffective (4, 7, 9, 24). Only a few in vitro studies have determined the antibiotic susceptibilities of F. tularensis. Baker et al. (2) determined MICs for 15 F. tularensis strains, and they showed that the expanded-spectrum cephalosporins (cefotaxime, ceftriaxone, and ceftazidime), aminoglycosides, tetracycline, chloramphenicol, erythromycin, and rifampin were all bacteriostatic. Similar results were reported by Markowitz et al. (19). Thus, in vitro susceptibility data do not correlate well with the clinical experience in treating human tularemia. Because F. tularensis is a facultative intracellular bacterium (10, 11, 14), we hypothesized that determination of the activity of antibiotics against the intracellular form of the bacterium would yield more accurate predictions of the clinical efficacy of antibiotics.

F. tularensis strain. We used an F. tularensis bv. palaearctica strain recently isolated in our laboratory from a patient suffering from typical ulceroglandular tularemia after receiving a tick bite in Mulhouse, northern France (13).

MIC and MBC determination. MICs were determined using a modified Mueller-Hinton broth and a microplate assay, as previously described by Baker et al. (2). An F. tularensis inoculum (~10^7 CFU/ml) was prepared from a chocolate agar-grown culture (BioMérieux, Lyon, France) and dispensed into the wells of a 96-well microtiter plate (Poly Labo, Paris, France). Antibiotics were tested at twofold serial concentrations (0.125 to 256 µg/ml) in triplicate. F. tularensis displayed exponential growth in modified minimum essential medium (MEM), with a 2.2-log increase in CFU counts after 24 h of incubation (37°C, 5% CO2). MICs were determined after a 24-h incubation of antibiotic-containing cultures, compared to drug-free controls. Minimal bactericidal concentrations (MBCs) were determined at the same time, by placing onto chocolate agar 10-fold serial dilutions of bacterial suspensions from wells with no visible growth; the minimal antibiotic concentration allowing a 3-log or greater reduction of the primary bacterial inoculum titer was considered the MBC.

Among beta-lactams tested, only ceftriaxone (an expanded-spectrum cephalosporin) displayed a bacteriostatic activity (Table 1); none displayed a bactericidal activity. Aminoglycosides (i.e., streptomycin, gentamicin, and amikacin), thiamphenicol, macrolides (i.e., erythromycin, clarithromycin, and telithromycin), rifampin, fluoroquinolones (i.e., ofloxacin, pefloxacin, and ciprofloxacin), and doxycycline displayed both a bacteriostatic and a bactericidal activity against our F. tularensis strain, although only the MBCs of rifampin, telithromycin, and fluoroquinolones were in the range of concentrations achievable in human sera. Cotrimoxazole was poorly effective, but the addition of vitamins in the Mueller-Hinton broth may have impaired the activity of both sulfamethoxazole and trimethoprime as previously mentioned by Baker et al. (2). These results are concordant with MICs previously reported by Baker et al. for 15 F. tularensis strains isolated in the United States (2).

Intracellular antibiotic susceptibility testing. F. tularensis was grown in P388D1 murine macrophage-like cells (ATCC CCL46). Confluent cell monolayers grown in shell vials (Bibby Sterilin, Stone, Staffordshire, England) were seeded with 1 ml of a 10^5-CFU/ml F. tularensis inoculum, in MEM supplemented with 10% fetal calf serum (Poly Labo) and 2 mM glutamine (Poly Labo), and immediately centrifuged (1 h, 3,500 × g, room temperature). In order to remove nonphagocytized bacteria, cell supernatants were replaced by MEM containing 10 µg of gentamicin per ml, and cultures were incubated at 37°C for 4 h. We previously determined that such a protocol allowed a 4-log reduction of an F. tularensis inoculum. Because penetration of eukaryotic cells by gentamicin is slow, detectable only after 24 to 48 h of antibiotic exposure (33, 34), this protocol would allow eradication of nonphagocytized bacteria without significant killing of intracellular F. tularensis. Gentamicin-containing supernatants were then discarded and...
replaced by MEM supplemented with 4% fetal calf serum and 2 mM glutamine, which does not support extracellular growth of *F. tularensis*. Antibiotics were added to culture media at a single concentration in the range of levels achievable in the serum of humans (Fig. 1). As for ceftriaxone and gentamicin, approximately peak and trough concentrations in serum were determined by thermic shocks (2180 and 180°C), harvesting cell monolayers by scraping, and inducing cell lysis by lytic agents. Dilutions of cell lysates were plated (1 h at room temperature) in 10-mm-diameter coverslips in shell vials. After a 48-h incubation, CFU counts were determined. A bactericidal activity corresponding to the primary inoculum dose, as determined by using the Student t test and a 95% confidence limit. The lack of cell toxicity of both the experimental procedure and the antibiotics used was verified by (i) examination of cell monolayers under an inverted microscope before each inoculum titration and (ii) repeating the experimental procedure and, using trypan blue dye exclusion test, determining cell viability, which was considered acceptable if fewer than 2% of cells were stained (Sigma, Paris, France).

*F. tularensis* multiplied within P388D1 macrophage-like cells, with a 0.65-log increase in CFU after a 24-h incubation of cultures, whereas stagnation in CFU counts was observed after 48 h of incubation (Fig. 1), and incubation prolonged to 72 h resulted in the complete destruction of cell monolayers, preventing the determination of CFU counts in controls at that time. No cell toxicity was observed with antibiotics at the concentrations tested. Penicillin G, amoxicillin, ceftriaxone, thiamphenicol, and erythromycin failed to display any significant activity against intracellular *F. tularensis* compared to drug-free controls after 24- and 48-h incubations (Fig. 1), whereas incubation prolonged to 72 h resulted in the destruction of cell monolayers, which can be considered evidence of lack of intracellular bacteriostatic activity as well. In contrast, infected cells remained viable for the 3-day period of the experiments in cultures containing antibiotics with significant intracellular bactericidal activity, including the aminoglycosides streptomycin, gentamicin, and amikacin, but also telithromycin, doxycycline, rifampin, and the fluoroquinolones ofloxacin and ciprofloxacin (Fig. 1).

**Location of *F. tularensis* within P388D1 cells.** The location of *F. tularensis* within P388D1 cells was assessed both by a transmission electron microscopy technique and by confocal microscopy analysis. P388D1 cells were infected with *F. tularensis* using the previously described procedure, including the use of gentamicin to remove nonphagocytized bacteria. Infected cell cultures were then incubated at 37°C in drug-free supplemented MEM for 48 h. In the electron microscopy assessment, infected P388D1 cells were harvested by trypsinization and pelleted by centrifugation. Cell pellets were fixed (1 h at room temperature) in cacodylate buffer (0.1 M, pH 7.2) containing 2.5% glutaraldehyde and then incubated overnight at 4°C in fresh cacodylate buffer. They were incubated (1 h at room temperature) in 1% osmium tetroxide, dehydrated through increasing concentrations (25 to 100%) of ethanol, and then embedded in Epon 812. Thin sections were cut out and post-stained with a saturated solution of methanol-uranyl acetate and lead citrate in water before examination on a JEOL JEM 1200 EX electron microscope. In the confocal microscopy assessment, *F. tularensis*-infected P388D1 cells were grown on 10-mm-diameter coverslips in shell vials. After a 48-h incubation of cultures, *F. tularensis* was stained by indirect immunofluorescence, using a locally prepared rabbit anti-*F. tularensis* serum (1:200) and a goat anti-rabbit immunoglobulin-fluorescein conjugate (1:200) (BioMérieux, Marcy L’Etoile, France). Cells were stained by using Evan’s blue dye (BioMérieux). Fluorescence was analyzed with a laser scanning confocal fluorescence microscope (LEICA DMIRBE) equipped with a 100× oil immersion lens. The intracellular location of *F. tularensis*, in infected P388D1 cell cultures, was confirmed both by electron microscopy and by confocal microscopy analysis. An electron micrograph of *F. tularensis*-infected P388D1 cells showed that after 48 h of incubation bacteria were localized within vacuoles. These vacuoles were of variable size and contained one to several bacteria. Likewise, confocal microscopy revealed the presence of several fluorescent intracellular bacteria within each infected cell examined.

Results obtained in our cell model correlate well with current clinical experience in treating human tularemia and explain for the first time some previously mentioned discrepancies. First, the observation that aminoglycosides display high in vitro activity against extracellular but also intracellular *F. tularensis* is compatible with their usefulness in treating tularemic patients (7, 20). These results confirm early observations by Nutter and Myrvik (23) that streptomycin but not penicillin G could inhibit growth of *F. tularensis* within rabbit alveolar macrophages in vitro. Also, our results are compatible with current knowledge of the pharmacokinetic properties of this class of antibiotics. Thus, activity of aminoglycosides against intracellular *F. tularensis* increased progressively with prolonged incubation time, reaching a maximum after 72 h of incubation of cultures, which correlates well with the mechanism of pinocytosis by which aminoglycosides progressively concentrate within eukaryotic cells, reaching significant intracellular levels only after 48 to 72 h of antibiotic-cell contact (21, 33, 34). Although expanded-spectrum cephalosporins display high in vitro activity against *F. tularensis*, Cross et al. (4, 5) reported a 100% failure rate in eight tularemia patients treated with ceftriaxone. Ceftriaxone was bacteriostatic against *F. tularensis* in axenic medium. However, although ceftriaxone has been shown to penetrate within phagocytic cells (18), the lack of bacteri-

### Table 1. MICs and MBCs for clinical strains of *F. tularensis* bv. *palearctica*, as determined by Baker’s method (2)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
<th>MBC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>2</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Amikacin</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Thiamphenicol</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Télihemycin</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>8</td>
<td>256</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Pefloxacin</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Cotrimoxazone*</td>
<td>16/80</td>
<td>256/1280</td>
</tr>
</tbody>
</table>

* MICs and MBCs to trimethoprim and sulfamethoxazole compounds, respectively (cotrimoxazole is the combination of trimethoprim and sulfamethoxazole).
cidal activity against both extracellular and intracellular *F. tularensis* may well explain why this compound cannot cure *F. tularensis*-infected patients (4, 7). The same reasoning may apply to thiamphenicol and erythromycin, compounds for which relapse rates in tularemic patients are high (4, 7) and which lack any bactericidal activity against *F. tularensis* despite their intracellular accumulation (21). Rifampin penetrates well within eukaryotic cells and displays a significant activity against intracellular *F. tularensis*. However, this compound is not considered a safe alternative to treat tularemia because of frequent relapses upon antibiotic withdrawal (7). In vitro selection of rifampin-resistant mutants has been previously reported for *F. tularensis* (3) as well as for many other bacteria (1, 8, 16, 22, 32) and may well explain the poor clinical efficacy of this compound when used alone. Doxycycline can inhibit *F. tularensis* growth in broth and displays a bactericidal activity in
P38RD1 cells, which correlates well with the usefulness of tetracycline compounds in patients with tularemia. However, the frequent occurrence of clinical relapses (7, 9) when a tetracycline is administered may indicate that in vivo activity is merely bacteriostatic. As for the fluoroquinolones, a high activity against \textit{F. tularensis} was shown both in axenic medium and in cells, which fits well with current clinical experience in using these drugs to treat tularemia patients (29, 31). Newer macrolide compounds potentially represent a safe alternative in populations for which tetracyclines and fluoroquinolones are contra-indicated, including pregnant women and children. Interestingly, the ketolide telithromycin displays a bactericidal activity against \textit{F. tularensis} in axenic medium and in our cell system. Telithromycin has been shown to reach high intracellular concentrations in polymorphonuclear neutrophils (35) and is highly active in vitro against other intracellular pathogens, including \textit{Chlamydia} (27) and \textit{Legionella} (6, 30). Its activity against intracellular \textit{F. tularensis} warrants clinical trials to establish its clinical usefulness in treating patients with tularemia.

REFERENCES