In Vitro Susceptibilities of Ehrlichia risticii to Eight Antibiotics

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Inhibition of the proliferation of Ehrlichia risticii cultured in murine macrophage P388D1 cells by eight antibiotics was evaluated by indirect fluorescent-antibody staining with an antiserum specific to E. risticii. There was a negative correlation between the percentage of infected cells and the log_10 of the concentrations of all antibiotics examined. The ranks of the antibiotics in the order of 50% inhibitory concentrations (on a microgram-per-milliliter basis) after 48 h of exposure were as follows: demeclocycline, doxycycline, and oxytetracycline < minocycline < rifampin < tetracycline < erythromycin and nalidixic acid. When the antibiotics were removed after 48 h of incubation, continuous inhibition of proliferation was evident at 72 h. At 96 h regrowth of the organisms occurred in most of the cultures. The rate of regrowth was the highest with nalidixic acid, followed by erythromycin, at all concentrations of the antibiotic tested. Regrowth was observed with less than 0.1 μg of minocycline per ml and less than 0.01 μg of oxytetracycline, tetracycline, and doxycycline per ml. With more than 0.01 μg of demeclocycline, however, the inhibition persisted for up to 72 h after removal of the antibiotic. These results indicate that demeclocycline was slightly more effective than doxycycline, oxytetracycline, and minocycline in eliminating E. risticii in macrophages in vitro, whereas tetracycline and rifampin were less effective. Nalidixic acid and erythromycin were ineffective.

Several assay systems have been developed to evaluate the susceptibilities of intracellular rickettsiae or chlamydia to antibiotics in vitro: examination of the cytopathic effect or plaque formation in cultured cells (1, 12, 16, 25, 26) and observation of the percentage of infected cells after staining with dyes (9, 26) or fluorescent antibody against the organism (9). These studies revealed that microorganisms belonging to the genus Rickettsia are susceptible to the tetracycline series of antibiotics (1, 12-14, 24-26), rifampin (16, 26), chloramphenicol (1, 12, 13, 16, 24, 26), and erythromycin (1, 12, 16, 26). On the other hand, penicillin (1, 12, 26) and streptomycin (1, 12) are not effective. It seems that to be effective, antibiotics need to be adequately hydrophobic to penetrate through the host cell membranes and to be retained in effective concentrations in the cytoplasm of the rickettsiae.

In this study, we used indirect fluorescent-antibody (IFA) staining to assay the inhibitory effects of eight antibiotics on the intracellular proliferation of E. risticii in vitro.

MATERIALS AND METHODS

Antibiotics. Tetracycline hydrochloride, oxytetracycline hydrochloride, doxycycline hydrochloride, and nalidixic acid, sodium salt, were obtained from Sigma Chemical Co. (St. Louis, Mo.). These antibiotics were dissolved at 1 mg/ml in phosphate-buffered saline (PBS), which contained 2 mM KH₂PO₄, 6 mM Na₂HPO₄, 2 mM KCl, and 136 mM NaCl (pH 5.5). Erythromycin (Sigma) was dissolved in dimethyl sulfoxide and diluted to 1 mg/ml in PBS (pH 7.2). Demeclocycline and minocycline (kindly provided by American Cyanamid Co., Pearl River, N.Y.) and rifampin (Sigma) were dissolved in dimethyl sulfoxide and diluted to 1 mg/ml in PBS (pH 5.5). Antibiotic solutions were prepared on the day of use. These antibiotic stock solutions were diluted more than 100 times in tissue culture medium immediately before use.

Sera. Immune serum was collected from a pony that had been injected with E. risticii-infected human histiocytic spinocytoma cell line P388D1 and was stored at −20°C. The immune serum was used at a 1:400 dilution in PBS (pH 7.2).

Potomac horse fever (PHF), also known as equine monocytic ehrlichiosis (8), equine ehrlichial colitis (14), and acute equine diarrhea syndrome (11), was first described in 1979 after an outbreak of acute systemic disease in Maryland in the vicinity of the Potomac River. Subsequently, the disease has been reported throughout North America. PHF is characterized by fever, anorexia, dehydration, leukopenia, diarrhea, colic, and in some cases, laminitis (11). The outcome of PHF varies: Approximately 70% of the horses recover and 30% of the horses die or are euthanized for humane reasons (11). In 1984, rickettsial organisms were identified by electron microscopy in the macrophages in the connective tissue of the large colon of an experimentally infected pony (18), and this agent was isolated in a monocye-macrophage cell line (3, 17) or in the canine primary monocyte culture (7) and subsequently proved to be the etiologic agent of PHF (7, 17).

The causative agent of PHF was toxonologically placed in the genus Ehrlichia of the family Rickettsiaceae based on morphological and serological studies, and was named Ehrlichia risticii (8). E. risticii, an obligate intracellular parasite, possesses all of the general phenotypic characteristics common to other members of the genus Ehrlichia (7, 17-19). The organism is host membrane bound (18). It is gram negative, and stains purple with Wright-Giemsa stain (7, 17, 18). The organism is round and often pleomorphic (17, 18). Small, intermediate, and large forms of the organism exist (18). Small electron-dense forms (0.2 to 0.4 μm in diameter), including those in the middle of binary fission, are found grouped in host membrane vacuoles, whereas most of the larger and lightly stained organisms (0.6 to 1.5 μm in diameter) are individually and tightly enclosed by the host membrane (18). The organism has fine strands of DNA and ribosomes and is surrounded by double membranes (18). The efficacy of antibiotics on the proliferation of ehrlichiae in vitro has not been reported previously.

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lymphoma U-937 cells (17). Serum samples were pooled, divided into fractions, and stored at −20°C until use.

E. risticii. E. risticii that was isolated from an experimentally infected horse and that was passaged more than 10 times through murine monocyte-macrophage P388D1 cells (American Type Culture Collection, Rockville, Md.) (15) was used. E. risticii was further propagated in P388D1 cells. The proportion of infected cells was assessed with a stain (Diff-Quik; American Scientific Products, Obetz, Ohio). Cultures infected more than 90% were used as a seed inoculum for assessing antibiotic susceptibilities.

Culture of E. risticii and antibiotic testing. Approximately 10⁶ P388D1 cells were propagated in each well of 12-well tissue culture plates (Flow Laboratories, Inc., McLean, Va.) containing 2 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) and 2 mM L-glutamine (GIBCO) at 35°C in a humidified atmosphere of 5% CO₂ and 95% air. After 2 to 3 days of incubation, when the cells were subconfluent, the culture medium was removed and 0.2 ml of tissue culture medium containing approximately 2 × 10⁹ E. risticii-infected P388D1 cells or uninfected P388D1 cells (control) was added to each well. The plates were incubated at 35°C as described above. After 4 h of incubation with periodic movement to evenly distribute the inoculum, 0.8 ml of culture medium was added to each well. After 24 h of incubation, 1 ml of culture medium containing various final concentrations of test antibiotics (10, 1, 0.1, 0.01, or 0 μg/ml) were applied to the wells (9 wells per dose). Uninfected P388D1 cells received the same amount of antibiotics. After 48, 72, and 96 h of incubation with the test antibiotics, the cells from groups of three wells were harvested with cell scrapers (Bellco Glass, Inc., Vineland, N.J.) and centrifuged, and pooled resuspended cells were used to determine the percentage of infected cells. Viable cells were enumerated by the trypan blue exclusion method.

Regrowth of the organisms subsequent to removal of the test antibiotics was examined as follows. After 48 h of incubation, the medium containing the antibiotics was removed and each well was washed with 1 ml of Hanks balanced salt solution (GIBCO) (pH 7.4). The dissociated cells that were present were centrifuged at 430 × g for 5 min, and the pellet was suspended in antibiotic-free RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The cell suspension was then pipetted back into the corresponding wells. The plates were subsequently reincubated for 72, 96, and 120 h before the percentage of infected cells was determined by IFA staining. All experiments were performed in triplicate.

IFA assay for percentage infected cells. Pooled suspensions of E. risticii-infected P388D1 cells (10 μl) were pipetted into three wells of 12-well Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.)-coated slides (Carlson Scientific Inc., Peotone, Ill.) and air dried. Cells were fixed in acetone (Mallinckrodt, Inc., Paris, Ky.) at room temperature for 5 min. The slides were then dried and stored at 4°C.

Pony antiserum 10 μl to E. risticii diluted at 1:80 in PBS (pH 7.4) was pipetted into each well of the antigen-coated slides. After 30 min of incubation at 35°C, the slides were washed 3 times with PBS (pH 7.4) containing 0.002% Tween 20 (vol/vol) (Bio-Rad Laboratories, Richmond, Calif.). The cells were then incubated for 30 min at 35°C with 10 μl of fluorescein isothiocyanate-labeled anti-horse immunoglobulin G (IgG; United States Biochemical Corp., Cleveland, Ohio) diluted at 1:200 in PBS (pH 7.4) for 30 min. Afterward, the slides were washed with PBS as described above, counterstained for 3 min with 0.2% Evans blue (Sigma) diluted in PBS (pH 7.4), and finally washed with PBS (pH 7.4). Mounting fluid (90% glycerol in PBS) was immediately applied into each well following the removal of excess PBS. The slides were covered with cover slips (22 × 60 mm) (Fisher Scientific Co., Cincinnati, Ohio) and examined with a fluorescence microscope (Microphot-FX; Nikon). One hundred cells were counted in each well, and the mean and standard deviations of the percentage of infected cells of the three wells were determined.

The 50% inhibitory concentration (IC₅₀) of each antibiotic was determined from the dose-response curve after 48 h of exposure to the antibiotic.

Statistical analysis. One-way analysis of variance was used to determine whether any significant differences existed. The Duncan new multiple range test was used to determine significant differences among the doses of the eight antibiotics in terms of their effects on E. risticii growth, as reflected by the percentage of infected cells. Correlation coefficients of the percentage of infected cells and the concentration of antibiotics were calculated by correlation and simple linear regression. A value of P < 0.05 was considered to be statistically significant for all assays.

RESULTS

Inhibition of E. risticii growth by antibiotics. The effects of various antibiotics on E. risticii growth in vitro were evaluated by IFA staining by using anti-E. risticii serum and fluorescein isothiocyanate-labeled anti-horse IgG. No positive cytoplasmic fluorescence was demonstrable in E. risticii-infected P388D1 cells reacted with nonimmune horse serum or in uninfected P388D1 cells reacted with immune serum. In comparison with nonantibiotic-treated control cultures, all eight of the antibiotics tested significantly inhibited E. risticii proliferation after 48 h of exposure at the lowest concentration tested (0.01 μg/ml) (Fig. 1). A negative linear correlation between the logarithm of the concentration of antibiotics and the percentage of cells infected was observed (Fig. 1). The correlation coefficients (r) after 48 h of antibiotic treatment were 0.88, 0.88, 0.90, 0.91, 0.92, 0.93, 0.97, and 0.97 for demeclocycline, doxycycline, oxytetracycline, rifampin, nalidixic acid, tetracycline, minocycline, and erythromycin, respectively.

### Table 1. IC₅₀ of antibiotics on *E. risticii*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>IC₅₀ (µg/ml) at 48 h in:</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demeclocycline</td>
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<td>&lt;0.01</td>
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</tr>
<tr>
<td>Doxycycline</td>
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<td>0.01</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Oxytetracycline</td>
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<td>0.03</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Minocycline</td>
<td>0.45</td>
<td>&lt;0.01</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>Rifampin</td>
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<td>0.45</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
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<td>10.00</td>
<td>10.00</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* The average and standard deviation of three culture wells of the percentage of infected cells in experiments 1 to 3 without antibiotics were 47 ± 2.51 to 53 ± 2.51, respectively, at 0 h and 53 ± 2.51, respectively, at 48 h. The IC₅₀ was determined from the dose-response curve for each antibiotic which gave 50% inhibition of the percentage of infected cells of the control culture (without antibiotics) after 48 h of incubation.

* ND, Not determined.

Antibiotics were ranked in the following order of efficacy in reducing the percentage of cells infected by *E. risticii* with regard to the IC₅₀ after 48 h of exposure: demeclocycline, doxycycline, and oxytetracycline < minocycline < rifampin < tetracycline < erythromycin and nalidixic acid (Table 1).

Although at 48 h rifampin and tetracycline only partially inhibited *E. risticii* growth even at 10 µg/ml, the continuous presence of these antibiotics showed further suppression of the percentage of infected cells at 72 and 96 h (Fig. 2a and b). The rest of the antibiotics did not show any significant differences in the percentage of infected cells with or without antibiotics (Fig. 3) at 72 and 96 h incubation.

![Graph](http://aac.asm.org/)

**FIG. 2.** Inhibition of growth of *E. risticii* in P388D1 cells by various concentrations of rifampin (a) and tetracycline (b).

After 48 h of exposure to 10 µg of any one of the eight antibiotics per ml, the viability of P388D1 cells was more than 80% by the trypan blue dye exclusion test. The presence of less than 0.01% dimethyl sulfoxide in the culture medium of some antibiotics had no effect on the morphology or growth characteristics of P388D1 cells or those of *E. risticii* in vitro under the same incubation conditions.

**Regrowth of *E. risticii* after removal of antibiotics.** To examine the effects of antibiotics on the viability of the organism, the antibiotics were removed from the cultures after 48 h, and the cultures were reincubated in antibiotic-free medium to permit the detection of resumption of infectivity of the surviving organisms. The percentage of infected cells remained suppressed at 24 h after removal of the drugs, and afterward the percentage of infected cells increased. The subsequent increase in the percentage of infected cells varied with the concentration and type of antibiotic. A rapid increase in the percentage of infected cells occurred at concentrations of 0.01 µg of tetracycline and rifampin per ml (Fig. 3e and f, respectively). With nalidixic acid, at all concentrations used, rapid regrowth occurred and the percentage of infected cells after 120 h of incubation was almost the same as that of untreated infected cells (Fig. 3h). Resumption of growth was also evident at all concentrations of erythromycin used (Fig. 3g).

A slight increase in the percentage of infected cells was observed after exposure to 0.01, 0.1, and 1 µg of minocycline per ml; 0.01 µg of doxycycline per ml; and 0.01 µg of oxytetracycline per ml (Fig. 3b, c, and d, respectively). No increases in the number of infected cells were observed at all concentrations of demeclocycline used (Fig. 3a).

**DISCUSSION**

The antimicrobial effects in vitro depend on the properties of the antibiotics, the properties of the ehrlichiae, the intracellular compartment occupied by the ehrlichiae, the properties of the host cell, and the infection cycle of the ehrlichiae.

Unlike members of the genus *Rickettsia*, which grow free in the cytoplasm of the host cell, ehrlichiae are always bound to the host membrane (17, 18). To be effective, antibiotics need to penetrate through plasma and vacuole membranes and, furthermore, the inner and outer membranes of ehrlichiae; and they must be retained in sufficient concentrations in active forms in the ehrlichial cytoplasm. All the antibiotics examined were hydrophobic enough to diffuse into the cell cytoplasm (2, 10). Demeclocycline was the most effective for *E. risticii* as for all the groups of brucellae (5), which are facultative intracellular (vaccular) bacteria.

In contrast to the susceptibility of *Rickettsia prowazekii* (26) and *Rickettsia rickettsii* to erythromycin (12), this antibiotic was not as effective as the tetracycline series of antibiotics against *E. risticii*. Similar findings have been reported for *Coxiella burnetii* (13, 20). This result may be related to the fact that *E. risticii* and *C. burnetii* are in membrane vacuoles, while *R. prowazekii* are *R. rickettsii* are not. Erythromycin may be inactivated in the vacuoles, since it is sensitive to acid pH (2). Although nalidixic acid has been reported to inhibit the division of the reticular body of chlamydia (22), another membrane-bound obligate intracellular bacterium, and to partially inhibit *R. rickettsii* (12), this antibiotic only weakly inhibited ehrlichial growth.

The antibiotics tested differed in their mechanisms of action. Most of the antibiotics used inhibited protein synthesis by a variety of mechanisms. Tetracycline, minocycline,
FIG. 3. Inhibition of growth of *E. risticii* in P388D1 cells by various concentrations of antibiotics and subsequent recovery on removal of the drug 48 h after antibiotic treatment. (a) Demeclocycline; (b) minocycline; (c) doxycycline; (d) oxytetracycline; (e) tetracycline; (f) rifampin; (g) erythromycin; (h) nalidixic acid.
demeclocycline, doxycycline, and oxytetracycline work by reversibly binding to 30S ribosomal subunits, thereby inhibiting binding of aminoacyl tRNA to those ribosomes (6). Erythromycin works by binding to 50S ribosomal subunits (2). Rifampin inhibits RNA synthesis by a DNA-dependent RNA polymerase (23). Nalidixic acid alters the DNA structure by inhibiting DNA gyrase (21). Ribosomas and DNA strands have been identified in *E. risticii* by electron microscopy (18). Our findings suggest that *E. risticii* synthesizes proteins by mechanisms similar to those of other bacteria and rickettsiae and that *E. risticii* possesses a similar synthetic apparatus.

*E. risticii* can be demonstrated by Giemsa or Diff-Quik staining (17, 18). However, in evaluating the antimicrobial activity against *E. risticii* in culture cells, we chose IFA staining because it was more specific, reliable, and distinctly visible, while the organism was difficult to distinguish from other cellular structures such as cytoplasmic granules with the Giemsa stain (17). When compared with biological assays, the ability to test for the viability of organisms was limited by these staining procedures, since in both procedures the loss of antigenicity or stainability may occur sometime after death or inactivation of the organism.

Some antibiotics were active only when present in the medium and showed little residual effects after they were removed. For example, in the case of rifampin, the percentage of infected cells decreased progressively with time to zero, however, while the organism resumed infection of new cells 24 h after removal of the drug. With nalidixic acid, after removal of the antibiotic, the organism resumed infection at a higher rate than did untreated infected cultures, and the percentage of infected cells was as high as that in the untreated cultures at 72 h after removal of the antibiotic. The increased percentage of infected cells on removal of certain concentrations of these drugs indicates that the drugs at those concentrations are rickettsiastic rather than rickettsioidal.

By transmission electron microscopy, we found that chloramphenicol is slightly more effective than tetracycline in inhibiting the growth of *E. risticii* in P388D1 cells in vitro. The inhibitory effect of chloramphenicol was, however, not further examined by the experimental procedure described here, since the 1-h half-life of chloramphenicol in horses is too short for clinical use (4).

Although the exact mechanism of the decrease of the percentage of infected cells after treatment with antibiotics is unknown, it is not due to the death of infected cells and the concomitant increase of uninfected cells but is due to the specific elimination of *E. risticii* from the cytoplasm of infected macrophages. Results of our preliminary ultrastructural study have revealed the active involvement of lysosomes of the infected macrophages in this process.

In conclusion, the in vitro susceptibility of *E. risticii* to the seven antibiotics tested was dose dependent. *E. risticii* was highly susceptible to demeclocycline, doxycycline, oxytetracycline, and minocycline; less susceptible to tetracycline and rifampin, and relatively unsusceptible to erythromycin and nalidixic acid on a microgram-per-milliliter basis.

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LITERATURE CITED


