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In Vitro Susceptibility of *Ehrlichia sennetsu* to Antibiotics

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Antibiotic efficacies were evaluated by Diff-Quik (Dade, Düdingen, Federal Republic of Germany) staining of *Ehrlichia sennetsu* in P388D$_1$ murine macrophages grown in 96-well microtiter plates. Sennetsu disease is generally cured with tetracyclines. In vivo, *E. sennetsu* is susceptible to doxycycline and is resistant to erythromycin, penicillin, and chloramphenicol. Our study confirmed, in vitro, the efficacy of doxycycline, which had an MIC of 0.125 μg/ml. *E. sennetsu* was found to be resistant to erythromycin, chloramphenicol, penicillin, gentamicin, and co-trimoxazole, while it was very susceptible to ciprofloxacin (MIC, 0.125 μg/ml) and rifampin (MIC, 0.5 μg/ml).

Since the recent description of human ehrlichiosis in the United States (2, 8), there has been increased interest in the genus *Ehrlichia*. The only *Ehrlichia* strain currently isolated from humans is *Ehrlichia sennetsu*, which is responsible for sennetsu disease. Sennetsu disease, also called infectious mononucleosis or glandular fever, has been described in Southeast Asia (3). The illness is characterized by fever, lymphadenopathy, asthenia, anorexia, and mononucleosis. The organism was first isolated from patients by Misao and Kobayashi (9) in 1955 by using *E. sennetsu*-infected mice. Since that time, the microorganism has been grown on HeLa cells (14), canine macrophages (5), human endothelial cells (6), and recently, murine macrophage P388D$_1$ cells (1). *E. sennetsu* is an obligate intracellular parasite and stains purple as morulae with Wright-Giemsa or Diff-Quik stain (12). The organism is round and often pleomorphic, and small intermediate and large forms of the organism have been described (12). *E. sennetsu* is taxonomically placed in the genus *Ehrlichia* of the family *Rickettsiaceae*. Sennetsu disease is cured by tetracyclines (3). In 1962, Kobayashi et al. (7) reported the in vivo efficacy of doxycycline by using *E. sennetsu*-infected mice. Erythromycin and chloramphenicol were tested and were not found to be effective (7). The purpose of the present study was to evaluate the susceptibility of *E. sennetsu* to antibiotics in a cell assay.

Penicillin (Diamant, Paris, France), ciprofloxacin (Bayer Pharma, Sens, France), erythromycin (Abbott, Rungis, France), co-trimoxazole (Roche, Neuilly-sur-Seine, France), doxycycline (Pfizer, Orsay, France), chloramphenicol (Msd-Chibret, Paris, France), gentamicin (Merck-Clevenot, Nogent-sur-Marne, France), and rifampin (Lepetit, Neuilly-sur-Seine, France) were tested. All antibiotics except erythromycin were dissolved in phosphate-buffered saline at 1 mg/ml. Erythromycin was dissolved at 1 mg/ml in 20% methanol–phosphate-buffered saline. Antibiotic solutions were prepared daily.

Immune serum was prepared in BALB/c mice that were inoculated intraperitoneally with *E. sennetsu* (4). The final titer was 1/200.

The *E. sennetsu* Miyayama strain (a gift from G. Dash, National Institutes of Health, Bethesda, Md.) was propagated in P388D$_1$ cells as described previously (1). The growth of the organism was verified with Diff-Quik stain (Dade, Düdingen, Federal Republic of Germany) and by immunofluorescence assay (IFA). A cytospin II system was used to carry out smears. Thus, 100 μl of infected supernatant was centrifuged on a slide. For IFA testing, the cells were fixed in cold acetone and dried.

For antibiotic assays, we used 96-well microtiter plates (3596; Costar Cambridge, England). Approximately 10^4 50% *E. sennetsu*-infected P388D$_1$ cells were inserted into each well. After 1 h of incubation, the supernatant was removed and replaced with a medium containing minimum essential medium, 10% fetal bovine serum, and 5% L-glutamine, with or without antibiotics (positive control). Doxycycline, rifampin, and ciprofloxacin were tested at final concentrations of 2, 1, 0.5, 0.25, and 0.125 μg/ml; co-trimoxazole and chloramphenicol were tested at 4, 2, and 1 μg/ml; and erythromycin was tested at 4, 2, 1, 0.5, and 0.25 μg/ml. Penicillin was used at 1,000 μg/ml, and gentamicin was used at 100 μg/ml. Two experiments were done. The plates were incubated in the same medium in a CO_2 incubator at 37°C, and in order to study the regrowth of the organism, the medium was removed on day 5 and replaced with antibiotic-free medium. The percentage of infected cells was evaluated on days 1, 2, 3, 5, 6, 7, and 8 in both experiments. For the evaluation of the percentage of infection, the cells were harvested, centrifuged on a slide by using the cytospin II system, dried, stained with Diff-Quik (Dade), mounted, and observed on a light microscope (Nikon).

For comparison between Diff-Quik staining and IFA for evaluation of the percentage of infected cells, five samples were stained by IFA and with Diff-Quik. The percentage of infected cells was not found to be different by the two staining methods. Because Diff-Quik was easier to use than IFA, we used Diff-Quik to evaluate the percentage of infected cells.

The MIC was defined as the lowest concentration of antibiotic that was able to reduce the percentage of infected cells to 10% by day 5 of the experiment. At the concentrations used in this study, doxycycline and ciprofloxacin appeared to be the most effective. The MIC of doxycycline was 0.125 μg/ml, but 10% of the cells remained infected on day 5 at all concentrations tested (Fig. 1). Ciprofloxacin appeared to be the most effective of the antibiotics tested (MIC, 0.125 μg/ml). However, at all concentrations tested, all the cells were cured of *E. sennetsu* infection by day 3 (Fig. 1). Rifampin was effective at concentrations higher than 0.5 μg/ml. Penicillin and gentamicin were not found to

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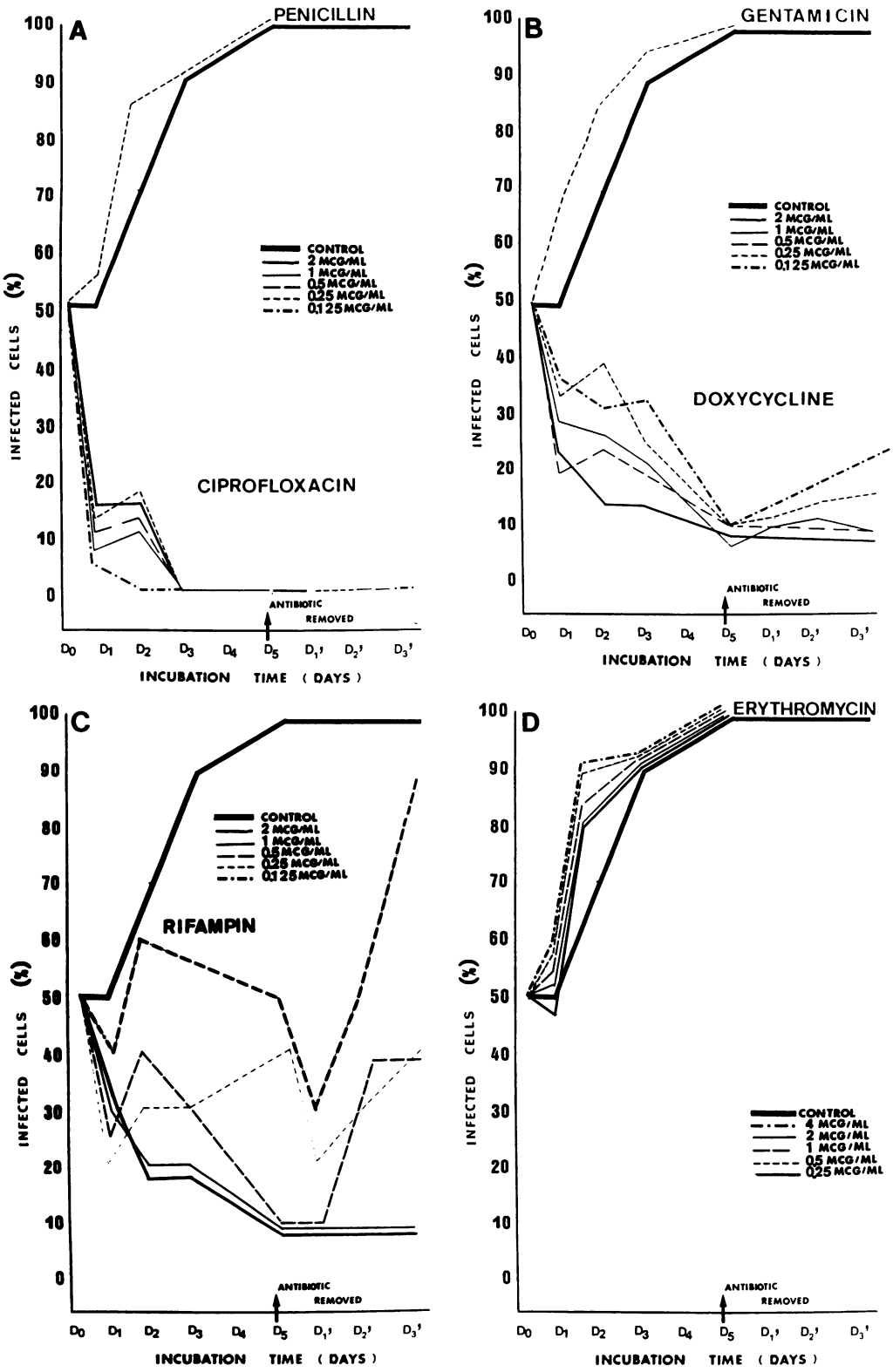

FIG. 1. Inhibition of growth of *E. sennetsu* in P388D$_1$ cells by various concentrations of antibiotics and subsequent recovery after removal of the drug on day 5 after antibiotic treatment.

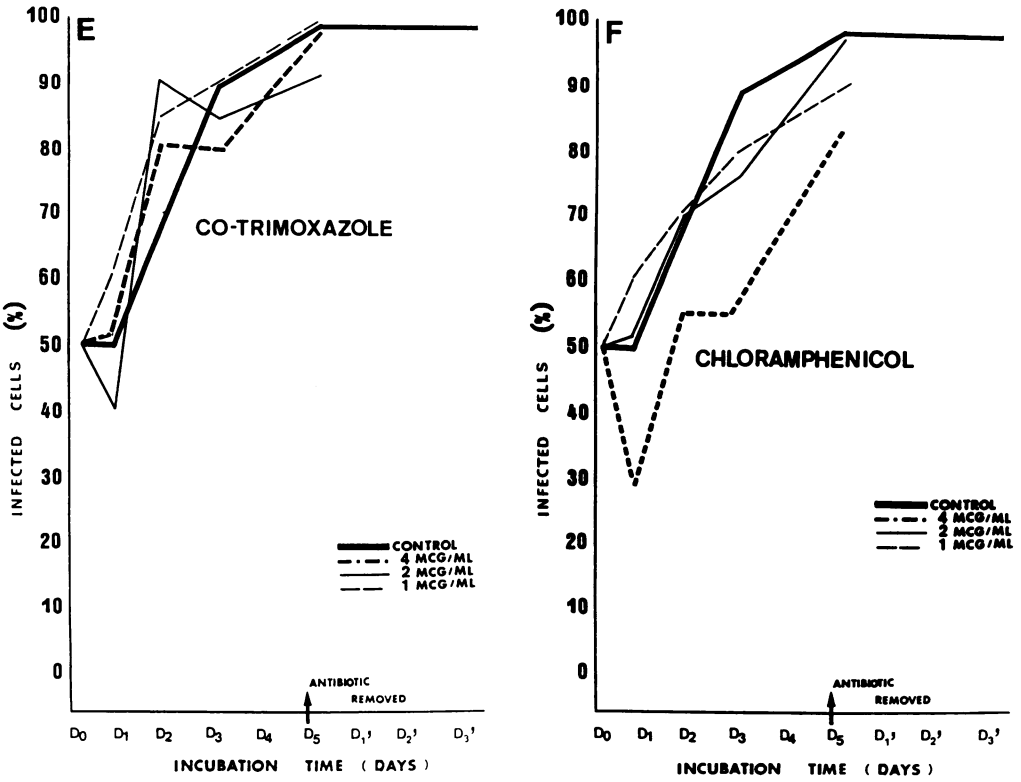

FIG. 1—*Continued*

be effective at any of the concentrations tested, with the percentage of infected cells being equal to that of the control. Erythromycin was also found to be ineffective against *E. sennetsu* at any of the concentrations tested, and the percentage of infected cells was similar to that of the control (Fig. 1). *E. sennetsu* appeared to be resistant to co-trimoxazole as well as to chloramphenicol. For cells treated with co-trimoxazole and chloramphenicol, the percentages of infected cells were similar to those of the control (Fig. 1).

After removal of the antibiotics, no regrowth was observed with doxycycline, ciprofloxacin, or rifampin (Fig. 1). For ciprofloxacin, regrowth was not observed at any of the concentrations tested. For doxycycline, regrowth was observed only for concentrations of 0.25 and 0.125 μg/ml. No regrowth was observed if rifampin was used at concentrations greater than 1 μg/ml.

Antibiotic susceptibility studies on obligate intracellular parasites such as rickettsiae require cell models. Such an evaluation is necessary before an antibiotic can be used in vivo. Doxycycline and ciprofloxacin appeared to be the most effective antibiotics used in this study, as determined by their MICs. This is not surprising. Tetracyclines have been reported to be very effective against members of the family *Rickettsiaceae*, with MICs ranging from 0.06 to 0.125 μg/ml by microtiter plate assays for *Rickettsia conorii*, *Rickettsia rickettsii*, and *Rickettsia prowazekii* (10). These same data have been reported with *Coxiella burnetii* in embryonated eggs (13). More recently, doxycycline has been reported to be effective against *Ehrlichia risticii*, a species that causes pericarditis in horses (11). Oxytetracycline has been reported to deprive *E. risticii* of its ability to inhibit phagolysosomal fusion (15). Ciprofloxacin has also been reported to be effective against a diverse array of intracellular organisms, including members of the family *Rickettsiaceae* (10). Our data suggest that rifampin might be effective against human ehrlichiosis, but its MIC is higher than those of doxycycline and ciprofloxacin. However, the MIC obtained in our study with *E. sennetsu* is close to that found with other members of the family *Rickettsiaceae*, including *R. conorii* and *R. rickettsii* (10). A lack of regrowth was noted in our study for cultures treated with rifampin, doxycycline, and ciprofloxacin. This was dose dependent and required concentrations greater than 0.5 μg/ml for doxycycline and greater than 1 μg/ml for rifampin. *E. sennetsu* was resistant to erythromycin, chloramphenicol, co-trimoxazole, penicillin, and gentamicin. The susceptibilities of rickettsial organisms to erythromycin have been reported to be very heterogeneous. *R. rickettsii* and *R. conorii* have been reported to be resistant, with MICs ranging from 4 to 8 μg/ml, while *R. prowazekii* has been reported to be very susceptible, with an MIC of 0.008 μg/ml (10).

E. risticii has been reported to be resistant to erythromycin (11). The most surprising result of our study was the discrepancy found between our results and those reported in the literature concerning the susceptibilities of members of the family *Rickettsiaceae* to chloramphenicol. In our study, *E. sennetsu* appeared to be resistant to chloramphenicol, while this antibiotic has been shown to be effective against *R. conorii*, *R. rickettsii*, *R. prowazekii*, *Rickettsia typhii* (10), *C. burnetii* (13), and *E. risticii* (11). As was reported for other members of the family *Rickettsiaceae*, penicillin, co-trimoxazole, and gentamicin are not effective against *E. sennetsu* at currently achievable concentrations in humans. The in vitro results observed in our study were in accord with those described in vivo by Kobayashi et al. (7) with infected mice. Infected mice treated with erythromycin or chloramphenicol died on day 10, while those treated with tetracycline survived. Moreover, human ehrlichiosis has been reported to be

easily cured by treatment with the tetracyclines (3). These facts are in accord with our findings and lead us to think that the tetracyclines should be proposed as first-choice therapy in humans with erhlichiosis. More clinical studies are necessary to evaluate the efficacies of the quinolones against *E. sennetsu*.

In conclusion, in vitro susceptibility studies of *E. sennetsu*, as evaluated in P388D_1 cells, showed that it is susceptible to doxycycline, ciprofloxacin, and rifampin and is resistant to penicillin, gentamicin, co-trimoxazole, erythromycin, and chloramphenicol.

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