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In Vitro Antibiotic Susceptibility of the Newly Recognized Agent of Ehrlichiosis in Humans, *Ehrlichia chaffeensis*

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Ehrlichiosis in humans, a rickettsial disease recently discovered in the United States, is generally treated successfully with tetracyclines; however, treatment with these agents is usually avoided with children and pregnant women. The in vitro susceptibility of *Ehrlichia chaffeensis*, the agent of human ehrlichiosis in the United States, was assessed by a quantitative evaluation of infected DH82 cells cultivated in 96-well microtiter plates in the presence of different concentrations of selected antibiotics. Extracellular MICs and MBCs were evaluated after 72 h of exposure to the antibiotics. Doxycycline and rifampin were found to exert rapidly bactericidal effects, with MBCs in the extracellular culture medium of less than 0.5 and 0.125 μg/ml, respectively. *E. chaffeensis* was resistant to chloramphenicol, ciprofloxacin, erythromycin, co-trimoxazole, penicillin, and gentamicin, which had MICs greater than 16, 4, 8, 4, 40, and 32 μg/ml, respectively. These observations are consistent with the finding that human ehrlichiosis appears to respond to tetracycline therapy, which has been the therapy of first choice. Further clinical investigations are necessary to evaluate the role of rifampin in the treatment of human ehrlichiosis, especially in children.

Ehrlichiae are strictly intracellular gram-negative bacteria which parasitize the leukocytes of humans and animals. The first ehrlichial disease was reported in 1935 by Donatien and Lestoquard, who observed the agent of canine ehrlichiosis, namely, *Ehrlichia canis*, in Tunisia (10). Thirty years later, Misao and Kobayashi (19) and Fukuda et al. (15) reported the isolation of a rickettsial agent, later named *E. sennetsu*, from humans with an infectious mononucleosis-like illness in Japan. This agent has been cultivated on numerous cell lines and is highly susceptible to doxycycline, ciprofloxacin, and rifampin in vitro (5). Despite the fact that physicians are aware of possible ehrlichiosis infection as an alternative diagnosis to infectious mononucleosis, no human patients with sennetsu ehrlichiosis have been reported in Japan since 1970 (27). Recently, two new ehrlichial diseases, Potomac horse fever and human ehrlichiosis, were discovered in the United States (16, 18). These discoveries created a new interest in ehrlichial diseases among veterinary and medical investigators. Human ehrlichiosis in the United States was first described by Maeda et al. in 1987 (18). Since that time, more than 200 cases have been reported in the literature (9). It was first suggested on the basis of serologic data that human ehrlichiosis might be caused by *E. canis*. In fact, the causative agent, *E. chaffeensis*, has been recently isolated and cultivated from human blood on a continuous cell line (9). Infected patients usually present with fever, myalgia, arthralgia, headache, weight loss, nausea, vomiting, and occasionally meningitis or pneumonia (13, 14). Laboratory data include elevated levels of aspartate aminotransferase and alanine aminotransferase and absolute lymphopenia. Most patients recover with or without antibiotic therapy, but in a previous study some cases of human ehrlichiosis led to death (12). Patients are usually treated successfully with doxycycline (8). However, doxycycline is not widely accepted for the treatment of children and is contraindicated for the treatment of pregnant woman. In previous studies, some children were treated and apparently cured with chloramphenicol, but some adults got worse or died despite this therapy (12, 18). The aim of this study was to test the in vitro susceptibility of the human agent of ehrlichiosis in America to various antibiotics.

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MATERIALS AND METHODS

Ehrlichia cultivation. *E. chaffeensis* was obtained from Jacqueline Dawson (Centers for Disease Control, Atlanta, Ga.) and was inoculated into a DH82 canine malignant histiocytic cell line (29). Briefly, 1 ml of infected DH82 cells frozen at −80°C in minimal essential medium was inoculated into a 25-cm^2 flask containing a confluent culture of DH82 cells. After 2 h of gentle agitation, 5 ml of fresh minimal essential medium containing 12.5% fetal calf serum and 2 mM L-glutamine was added to the flask. The medium was changed twice per week. Since DH82 is a slowly growing cell line, the cells were not subcultivated for 1 to 2 months, until 50% of cells were infected. Continuous propagation was achieved by passaging 3 ml of supernatant from a 100% infected culture onto an uninfected monolayer.

Antibiotics. 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 evaluated. All antibiotics were intravenous formulations and were prepared daily. The concentrations of antibiotics used in our experiments were chosen according to the resistance and sensitivity levels of each antibiotic, as determined by the National Committee for Clinical Laboratory Standards (NCCLS) (25). For each antibiotic, the highest dilution tested corresponded to the resistant MIC breakpoint determined by the NCCLS. Decreasing serial dilutions of antibiotic were also tested such that concentrations corresponding to the susceptible MIC breakpoint of the antibiotic as stated by the NCCLS were included. Penicillin and gentamicin were tested only at 40 and 32 μg/ml, respectively, concentrations which corre-

spond to their resistant MIC breakpoints. Concentrations of rifampin, ciprofloxacin, and co-trimoxazole ranged from 4 to 0.125 μg/ml, concentrations of doxycycline and chloramphenicol ranged from 16 to 0.5 μg/ml, and concentrations of erythromycin ranged from 8 to 0.25 μg/ml.

Antibiotic assays. We used 96-well microtiter plates (Cell-Cult; Sterilin, Ltd., Feltham, England) as described previously (5). Briefly, when 50% of the DH82 cells in the flask were observed to be infected, approximately 10^4 cells were introduced into each well and incubated overnight in a CO_2 incubator at 37°C to obtain a confluent layer. The medium was then removed and replaced by 100 μl of the various concentrations of antibiotics or antibiotic-free medium, which was used as a positive control. One well per antibiotic concentration per test was used. Percentages of infected cells were then evaluated 24, 48, and 72 h after treatment for each antibiotic at each concentration. The cells were sampled by gently scraping the bottom of the well with a 100-μl pipette, and the level of infection was assessed by Diff-Quik (Dade, Düdingen, Germany) staining of the infected cells after cytospin centrifugation of 100 μl of the harvested cells onto a slide. Cells were considered infected when one or more elementary bodies or morulae were seen. The same samples were also reacted sequentially with human anti-*E. chaffeensis* positive serum diluted 1:200 in phosphate-buffered saline (a gift from J. S. Dumler, Department of Pathology, University of Texas Medical Branch, Galveston, Tex.) and a 1:200 dilution of fluorescein-labelled goat anti-human immunoglobulin G (Biomerieux, Marcy l'Etoile, France). Three days after antibiotics were added, the medium was removed, and the infected cells were washed three times and refed with fresh, antibiotic-free medium. The percentage of infected cells after the removal of antibiotics was evaluated on day 2 post-antibiotic removal (PAR), day 4 PAR, and day 6 PAR to assess the regrowth of ehrlichiae. The extracellular MIC (ECMIC) was defined as the lowest extracellular concentration of antibiotic capable of preventing the growth of ehrlichiae as reflected by the proportion of infected cells at 72 h posttreatment. The extracellular MBC (ECMBC) was defined as the lowest concentration capable of reducing the number of ehrlichiae present in the cells by day 3 after treatment. The bactericidal effect of an antibiotic was confirmed by the lack of regrowth of ehrlichiae by day 6 PAR. The postantibiotic effect was assessed by determining whether the number of infected cells continued to decrease after the antibiotic was removed. When the observed differences in the number of infected cells were small, the level of significance was assessed by Fisher's exact test.

RESULTS

Under these conditions, *E. chaffeensis* was found to be highly susceptible to doxycycline and rifampin (Fig. 1). In doxycycline-treated cells, the proportion of infected cells decreased from 62 to 20% during the first 24 h of treatment. The ECMIC and the ECMBC were less than 0.5 μg/ml. Between days 3 and 5 (day 2 PAR), a slow decrease was observed. No regrowth was detected, regardless of the concentration used. The differences observed in the percentage of infected cells on day 3 when 8 μg/ml and when the other concentrations were used were not significant. Rifampin was also very effective against *E. chaffeensis*, with an ECMBC of 0.125 μg/ml. After 3 days of treatment, rifampin at 0.125 μg/ml was found to be significantly less effective against ehrlichiae than were higher concentrations. However, the percentage of infected cells continued to decrease after removal of the antibiotic, and no regrowth was noted. When rifampin- and doxycycline-treated cells were observed by light microscopy on day 2, morulae appeared heterogeneous by reduced Diff-Quick staining, and only a few intact organisms were seen. However, chloramphenicol, erythromycin, penicillin, and co-trimoxazole were ineffective against *E. chaffeensis*, as the infection increased in the same manner as in the untreated controls, regardless of the concentration used. In chloramphenicol-treated cells, the morulae appeared bigger and more numerous than in the untreated control. When ciprofloxacin was tested at 4 μg/ml, the growth of ehrlichiae was significantly inhibited ($P = 0.0807 \times 10^{-4}$), as reflected by the lack of increase in the number of infected cells. After 3 days of incubation in ciprofloxacin at a concentration of 2 μg/ml, the number of infected cells was significantly lower ($P = 0.03 \times 10^{-3}$) than in the untreated control, giving an ECMIC of 2 μg/ml. However, after the removal of the antibiotic, for concentrations of less than 4 μg/ml, the number of infected cells increased to the same levels as in the untreated control. In the cells treated with 4 μg of ciprofloxacin per ml, the percentage of infected cells remained at the same level until day 6 PAR. In the gentamicin-treated cells, the infection increased during the first 24 h of treatment, and then the number of infected cells decreased slowly, even after the removal of the antibiotic, until less than 10% of the cells were infected. In each of these samples, very few cells were observed in mitosis, confirming the very low turnover rate of this cell line. However, for the cells treated with penicillin, a few intact cells were observed on day 4 PAR, which indicated that not all of the cells were killed by the bacteria. When a blind evaluation of uninfected cells stained by Diff-Quik was performed, the false-positive rate of cells interpreted as infected was 3.6%. This may be explained by the fact that the DH82 cells compose a highly pleomorphic neoplastic cell line containing a high number of cellular structures which may be confused with small morulae. When the number of infected cells was assessed by immunofluorescence assay, none of the antibiotics tested were very active on day 3 after antibiotic treatment (data not shown).

DISCUSSION

The problem with testing ehrlichiae is that, like chlamydiae, they are suspected of having a life cycle with an intracellular infectious stage (20, 24). In the model described for *Ehrlichia* species, the ECMIC was defined as the lowest extracellular concentration of antimicrobial agent that inhibited growth as reflected by the proportion of infected cells. The ECMBC was determined both by the ability of the antibiotic to diminish the number of infected cells and by the lack of regrowth after removal of the antibiotic (5, 23). In this model, cell growth is restricted either by the addition of cycloheximide or through the use of a slowly growing cell line such as DH82 to avoid underevaluation of infected cells because of dilution. Moreover, we think that immunofluorescence staining should be avoided, because it does not indicate whether the identified bacteria are dead or alive. For this reason, evaluation of infected cells by the Diff-Quik method is probably more reliable, giving a better reflection than immunofluorescence assay of the bactericidal activities of antibiotics.

The activity of doxycycline was not surprising, because of its reported success in the treatment of human ehrlichiosis.

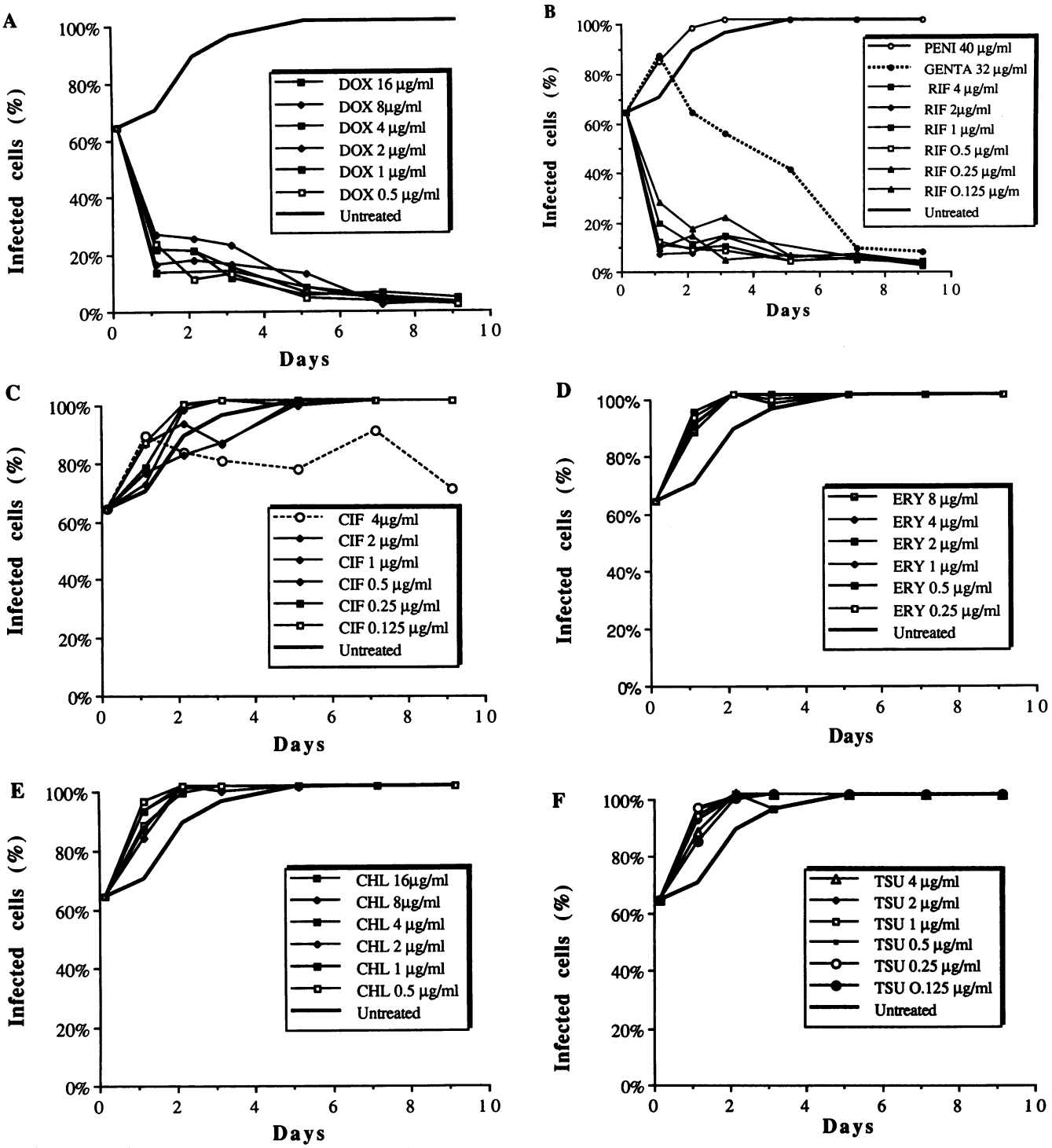

FIG. 1. Effects of antibiotics on percents of cells infected with *E. chaffeensis*. The antibiotics used were doxycycline (DOX) (A); rifampin (RIF), penicillin (PENI), and gentamicin (GENTA) (B); ciprofloxacin (CIF) (C); erythromycin (ERY) (D); chloramphenicol (CHL) (E); and co-trimoxazole (trimethoprim-sulfamethoxazole [TSU]) (F).

In fact, doxycycline has been shown to be effective against all of the rickettsiae, including the ehrlichiae, with an ECMIC of as low as 0.125 µg/ml (5, 6, 23). Doxycycline is also bactericidal at this concentration. This bactericidal effect is confirmed by the lack of regrowth after removal of the antibiotic, and some authors assume that ehrlichial killing is due to the ability of doxycycline to restore the phagosome-lysosome fusion that is actively blocked by the ehrlichiae (7, 30). Rifampin was found to be effective against most rickettsiae, including *E. sennetsu* and *E. risticii* (5, 23).

At concentrations of greater than 1 μg/ml, rifampin is bactericidal rather than bacteriostatic for ehrlichiae, as reflected by the lack of regrowth after removal of the antibiotic. *E. chaffeensis* is not susceptible, as defined by the NCCLS, to ciprofloxacin. However, at a concentration of ≥2 μg/ml, ciprofloxacin is bacteriostatic against *E. chaffeensis*. The reason for this continued bacteriostatic effect after removal of the antibiotic is unclear. Some discrepancies have been observed in the activities of quinolones against ehrlichiae. Although ciprofloxacin has been shown to be very effective against *E. sennetsu* (5), *E. canis* (6) and *E. chaffeensis* were found to be less susceptible to this compound at ECMICs of ≥2 μg/ml. At these concentrations, ciprofloxacin was only bacteriostatic, as nalidixic acid was against *E. risticii* (23). Although *E. chaffeensis* was found to be resistant, as defined by the NCCLS, to gentamicin (ECMIC, ≥32 μg/ml), note that at the concentration used in this study, gentamicin is slowly bactericidal even after it has been removed. This result might be explained by the fact that aminoglycosides penetrate very slowly but may concentrate at high levels within the cell (4). However, this in vitro activity of gentamicin should prompt one to limit the use of gentamicin-penicillin in the cell culture medium when one is attempting to isolate the organism.

The first patient with human ehrlichiosis reported in the United States (18) was initially treated with chloramphenicol. This was switched to tetracycline when the clinical illness and the hematological laboratory data did not improve. The patient subsequently recovered. Almost all reported patients were treated with tetracycline or its analogs. Among five symptomatic patients with serologic evidence of human ehrlichiosis in Texas (28), four became afebrile within 24 h after doxycycline or tetracycline therapy was begun, and for the fifth patient, fever resolved slowly over 10 days. In a study of human ehrlichiosis, seven of eight patients were treated with tetracycline or its analogs (14). Defervescence (temperature of <37.2°C) occurred after an average of 3 days, regardless of the delay in initiation of the treatment. The untreated patient had a fever for 19 days. All patients, including the untreated one, eventually recovered without complications. The efficacy of chloramphenicol against these organisms is still disputed (1, 22). Although some patients recovered with chloramphenicol therapy (2, 3, 11, 13), other patients were switched to doxycycline therapy (18) or died (12). Fishbein et al. (14) and Pearce et al. (21) reported spontaneous recoveries of some patients with human ehrlichiosis. The fact that chloramphenicol is ineffective in vitro and in vivo against *E. sennetsu* (5, 17), *E. canis* (6, 26), and *E. chaffeensis* should make one question the future use of this antibiotic for human ehrlichiosis. Human ehrlichiosis appears to respond in vitro and in vivo to tetracycline therapy, which should be the treatment of choice. A possible alternative drug, on the basis of our in vitro results, is rifampin. For some patients, especially children and pregnant women, this antimicrobial agent might be a useful alternative. However, clinical trials are needed to confirm these results.

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