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In Vitro Susceptibility of *Coxiella burnetii* to Antibiotics, Including Several Quinolones

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Antibiotic susceptibility testing of the rickettsial Q fever agent *Coxiella burnetii* was performed by using persistently infected L929 fibroblast cells. The efficacies of a variety of antibiotics with different metabolic targets were tested and compared. The most effective antibiotics in bringing about the elimination of the parasite from infected cells included several quinolone compounds and rifampin. Of the quinolone compounds tested, difloxacin (A-56619) was the most effective, followed by ciprofloxacin and oxolinic acid. These three quinolones were apparently rickettsiacidal. After 48 h of exposure to microgram amounts of the compounds (ranging from 2 μg of difloxacin per ml to 5 μg of the other two antibiotics per ml), the number of intracellular parasites markedly declined; after 10 days of treatment, very few intracellular rickettsiae were detected. Rifampin (1 μg/ml) was also very effective in eliminating the parasites. Some of the 13 other antibiotics tested that were somewhat effective included chloramphenicol, doxycycline, and trimethoprim. The persistently infected L929 cells were found to provide a convenient system for the relatively rapid determination of the susceptibility of *C. burnetii* to antibiotics.

Chronic endocarditis and hepatosplenomegaly are occasionally associated with persistent and relapsing infection caused by the obligate intracellular procaryotic rickettsial agent of Q fever, *Coxiella burnetii* (8, 12, 18, 19). Antibiotic treatment of chronic Q fever has not been consistently effective (8, 13, 18); in spite of treatment, the disease can continue or relapse, resulting in life-threatening endocarditis, usually affecting the aortic and mitral valves (13, 17, 18). We have developed an in vitro model of persistent Q fever consisting of cell lines persistently infected with *C. burnetii*. These chronically infected cell lines include L929, J774, and P388D1 (3, 4). The persistently infected L929 cells have been maintained in continuous culture for over 3 years without the addition of normal cells; both the host cells and parasites divide, with *C. burnetii* proliferating within phagolysosomes (2). Recently, we discovered that heavily infected cells are capable of division; those possessing one large parasite-containing vacuole give rise to both infected and uninfected daughter cells (15). This chronically infected model system was employed in this study for examining the efficacies of several antibiotics, including several DNA gyrase inhibitors (16) of the quinolone family. In this report we provide evidence that rifampin and several quinolones are effective in rapidly eliminating the parasite from chronically infected L929 cells. Unlike other host systems (embryonated eggs, guinea pigs) that have been used previously by other investigators (7, 9, 10, 17) to determine the antibiotic susceptibility of *C. burnetii*, the system we describe is more convenient and precise and it allows one to obtain drug efficacy data in a relatively short time.

MATERIALS AND METHODS

Source and propagation of *C. burnetii*. Phase I *C. burnetii* Nine Mile was originally obtained from M. Peacock of the Rocky Mountain Laboratory, Hamilton, Mont. The plaque-purified parasite was obtained in a yolk sac homogenate and was propagated in our laboratory in L929 cells as described previously (4, 15). The infected L929 cells were maintained in suspension culture in 125-ml Erlenmeyer flasks and routinely passaged three times a week. Host cell viability was determined by the dye exclusion technique (11).

Determination of degree of infection. The percentage of cells infected and the degree of infection were determined by direct microscopic examination after the cells were stained by the Gimenez technique (5). Cell smears were prepared for subsequent staining by centrifuging (1,000 rpm; Cytospin 2 centrifuge; Shandon, Cheshire, England) two to three drops of cultured cells onto glass microscope slides. A minimum of 300 cells were examined in each prepared slide to determine the percentage of the population that was infected (1 to 50 rickettsiae per cell) and heavily infected (>50 rickettsiae per cell). Photomicrographs were taken with Ektrachrome film (Eastman Kodak Co., Rochester, N.Y.). Cells were prepared for electron microscopy by standard techniques and procedures described previously (2). Epon was used as the embedding matrix, and thin sections were stained with uranyl acetate and lead citrate. Electron micrographs were made with a transmission electron microscope (EM-109; Zeiss).

Antibiotic preparation and use. Ciprofloxacin (Bayer, Leverkusen, Federal Republic of Germany) and difloxacin (A-56619; Abbott Laboratories, North Chicago, Ill.) were obtained in powder form; oxolinic acid was synthesized by one of us (L.A.M.). Tetracycline (HCl), doxycycline (HCl), rifampin, penicillin (Na), polymyxin B, trimethoprim, sulfamethoxazole, chloramphenicol, streptomycin (SO_4), gentamicin (SO_4), nalidixic acid (Na), novobiocin (Na), and erythromycin were obtained from Sigma Chemical Co., St. Louis, Mo. Stock solutions of each antibiotic were freshly prepared before they were added to cell cultures. Stock concentrations varied from 3.5 to 10.0 mg/ml. Solvents used in the

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TABLE 1. Antibiotic efficacies in the elimination of *C. burnetii* from persistently infected L929 cells

Antibiotic	% of cells infected in experimental/control cultures at[a]:				MIC (μg/ml)	
	0 h	24 h	48 h	10 days	50%	90%
Novobiocin	92 (60)/93 (62)			93 (64)/95 (67)		
Nalidixic acid	90 (61)/89 (59)			92 (69)/89 (60)		
Difloxacin	91 (68)/91 (66)	58 (39)/93 (74)	33 (15)/93 (72)	2 (0)/93 (66)	1.2	1.8
Ciprofloxacin	92 (65)/90 (64)	48 (28)/90 (58)	26 (12)/92 (60)	2 (0)/93 (60)	1.6	3.6
Oxolinic acid	90 (67)/92 (62)	81 (52)/91 (70)	42 (32)/92 (75)	7 (0)/93 (60)	3.2	4.5
Rifampin	82 (58)/83 (60)	40 (18)/85 (52)	34 (16)/83 (47)	4 (1)/79 (55)		
Tetracycline	83 (37)/85 (42)			90 (39)/90 (54)		
Doxycycline	89 (46)/92 (55)			75 (28)/90 (51)		
Chloramphenicol	91 (70)/89 (62)			53 (12)/90 (66)		
Erythromycin	92 (61)/93 (62)			89 (56)/94 (66)		
Gentamicin	90 (60)/93 (61)			92 (60)/95 (65)		
Streptomycin	89 (72)/82 (56)			76 (18)/88 (60)		
Sulfamethoxazole	91 (51)/88 (52)			91 (44)/89 (44)		
Trimethoprim	91 (47)/86 (51)			52 (22)/90 (51)		
Penicillin G	89 (63)/89 (71)			86 (41)/90 (54)		
Polymyxin B	82 (53)/86 (56)			93 (72)/92 (65)		

[a] Values are mean results compiled from duplicate experiments, each of which was sampled independently in triplicate. Nonparenthetic values indicate the total percentage of cells infected (1 or more rickettsiae); values in parentheses are the percentage of heavily infected cells (more than 50 rickettsiae). Results for experimental cultures (antibiotic treated) and control (nontreated) cultures are given. All values (except for those for rifampin [1 μg/ml]) were from experiments in which cultures were treated with 10 μg of antibiotic per ml.

stock antibiotic preparations included equal volumes of 95% ethanol and dimethyl sulfoxide (rifampin and chloramphenicol), 47% ethanol (pH 8.0; ciprofloxacin, difloxacin, novobiocin, and oxolinic acid), 57% ethanol (erythromycin), 95% ethanol-propylene glycol (5:2; nalidixic acid), and 9.5% ethanol (tetracycline, doxycycline, penicillin, polymyxin B, sulfamethoxazole, trimethoprim, and streptomycin). At the concentrations used, the solvents were not toxic to normal and infected L929 cells. With the exceptions of rifampin and chloramphenicol, all antibiotic stock solutions were sterilized via filtration (pore size, 2 μm). The ethanol-dimethyl sulfoxide solvent used for preparing the rifampin and chloramphenicol stock solutions was also an excellent autosterilizing agent. After sterilization antibiotic stock solutions were stored at 4°C in approximately 4-ml fractions. An appropriate volume (10 to 25 μl) of stock antibiotic was added aseptically to 10-ml cell cultures and at subsequent cell passages to maintain the same concentration of antibiotic during the test period. An equal volume of solvent with no antibiotic was added to control infected cultures.

Calculation of MIC. MICs for 50% (MIC_{50}) and 90% (MIC_{90}) of strains tested were determined by the graphical analytical methods described by Goldstein (6) and are expressed in micrograms per milliliter. The MICs were determined after 10 days of antibiotic treatment of the persistently infected L929 cells.

RESULTS

A number of selected antibiotics were tested for their capacity to control L929 fibroblast cells persistently infected with *C. burnetii*. The cells were continuously infected for a minimum of 720 days. Efficacies were determined by direct microscopic examination of Gimenez-stained cells and subsequently by calculating the percent reduction (or nonreduction) of infection. For each experimental and control infected flask, a total of 300 cells were examined each time that the flasks were sampled. In all experiments performed, the viabilities of treated and control infected L929 cell populations were greater than 95%. The maximum antibiotic concentration tested was 10 μg/ml; concentrations above 10 μg/ml were considered nonphysiological and, therefore, nonrelevant.

The efficacies of all the antibiotics tested are summarized in Table 1. The most effective antibiotics for eliminating the parasite from the chronically infected cells were three quinolone compounds (difloxacin, ciprofloxacin, oxolinic acid) and rifampin. The drop in the number of infected L929 cells during 10 days of continuous treatment with difloxacin (2 μg/ml), ciprofloxacin (5 μg/ml), and oxolinic acid (5 μg/ml) are depicted in Fig. 1 to 3, respectively. By day 10 of treatment with the three quinolones, almost no parasites associated with the L929 cells could be detected. The electron micrographs (Fig. 4) of infected L929 cells treated with difloxacin are representative of the effects of the quinolones after 10 days of exposure to the antibiotics. A

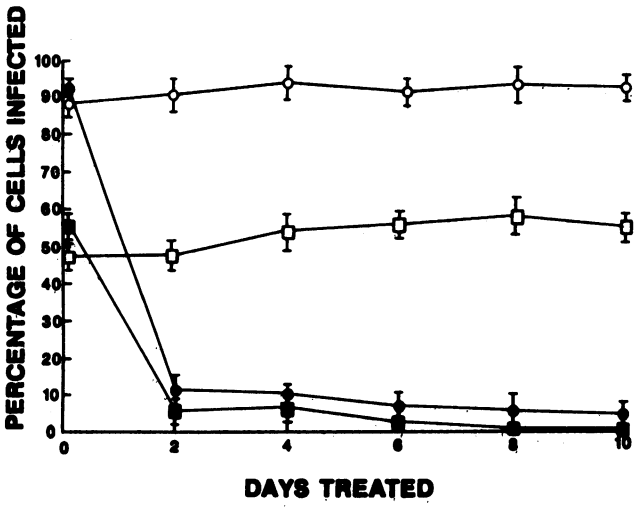

FIG. 1. Effect of difloxacin on L929 cells persistently infected with *C. burnetii* for 948 days at time zero. Values for untreated (○, □) and antibiotic-treated (●, ■) cells containing >1 (○, ●) and >50 (□, ■) parasites are shown.

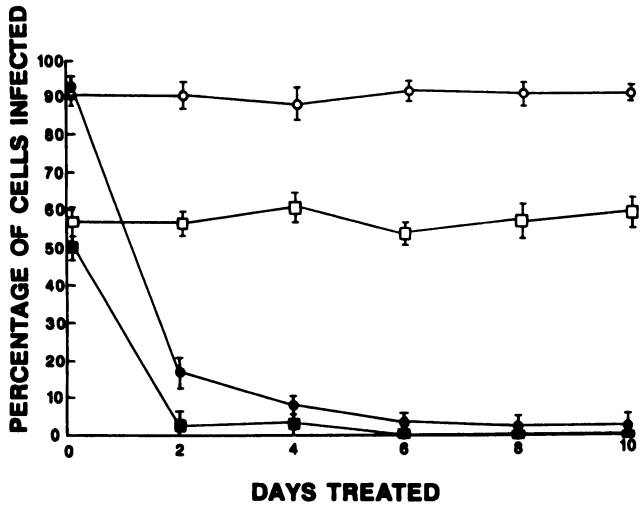

FIG. 2. Effect of ciprofloxacin on L929 cells persistently infected with *C. burnetii* for 952 days at time zero. Values for untreated (○, □) and antibiotic-treated (●, ■) cells containing >1 (○, ●) and >50 (□, ■) rickettsiae are shown.

comparison of the MIC_{50}s and MIC_{90}s of those compounds (Table 1) indicates that difloxacin is the most effective of the quinolones in reducing the level of infection (MIC_{50}, 1.2). Other quinolone compounds that were examined included nalidixic acid and the related coumarin derivative novobiocin. At 10 μg/ml, neither antibiotic caused a perceptible decrease in the number of parasites during 10 days of continuous exposure to the antibiotics (Table 1). At a concentration of 1 μg/ml, rifampin reduced the number of infected cells to almost 0 (Fig. 5); of all the antibiotics tested, it was the most effective in controlling the parasite (MIC_{50}, 0.5 μg/ml; Table 1). Chloramphenicol, doxycycline (Fig. 6), and trimethoprim also reduced the level of infection but not to the extent that either the quinolones or rifampin did (Table 1). At the maximum concentrations employed (10 μg/ml), the antibiotics tetracycline, gentamicin, streptomycin, erythromycin, sulfamethoxazole, penicillin G, and polymyxin B produced no significant decrease in the percentage of infected host cells (Table 1).

DISCUSSION

The most effective antibiotics for eliminating *C. burnetii* from persistently infected host cells were the inhibitors of nucleic acid synthesis: rifampin and the quinolones difloxacin, oxolinic acid, and ciprofloxacin. With the exception of the quinolone compounds that were previously unavailable, the pattern of antibiotic susceptibilities of *C. burnetii* determined with infected L929 cells is similar to the results obtained by others with embryonated eggs (7, 9, 10, 17); the effective range of antibiotic concentrations was comparable. These other investigators have found that *C. burnetii* is relatively resistant to erythromycin (9) and streptomycin (7). They also reported that chloramphenicol is ineffective (9), whereas we found that it did have some effect on the agent (Table 1). Spicer et al. (17) have reported that the most effective antibiotics for protecting chicken embryos from four isolates of the Q fever agent, including the Nine Mile strain, are rifampin, trimethoprim, doxycycline, and oxytetracycline; clindamycin, erythromycin, viomycin, cycloserine, and cephalothin were found to be ineffective. A fifth *C. burnetii* isolate (from Cyprus) was somewhat resistant to the tetracyclines (17). It was concluded (17) that the effective antibiotics are rickettsiastatic because subculturing of the treated parasites resulted in their reappearance. While that may be the case, it must be kept in mind that *C. burnetii* can be found outside cells (Fig. 4) and that exposure to antibiotics might have no effect on such presumably nonmetabolizing extracellular parasites; subsequently, they may remain fully infectious.

Because of the rapid disappearance of the parasite from the cells soon after exposure to the antibiotics (Fig. 1 to 3 and 5), it appears that rifampin and the effective quinolone compounds ciprofloxacin, difloxacin, and oxolinic acid are directly or indirectly rickettsiacidal. As early as 24 h after initial exposure to difloxacin, the percentage of infected cells decreased from 91 to 58%, and after 48 h of treatment it decreased to 33% (Table 1). Because the generation times of the infected populations were approximately 36 h (data not shown), the drop in rickettsial numbers was not just the result of the fact that inhibited rickettsiae were simply diluted out by the dividing host cells. The host cells apparently play an active role in eliminating the parasites after antibiotic treatment and parasite growth inhibition. The growth-inhibited rickettsiae might subsequently become highly susceptible to the toxic metabolites generated by the host cell and its lysosomal enzymes. It seems reasonable to conclude that the parasite must remain metabolically active to defend itself from the lysosomal contents of the host cell. The defensive properties of the parasite that allow it to survive within the hostile confines of the phagolysosome are not fully identified; however, it has been demonstrated (1) that *C. burnetii* possesses superoxide dismutase and catalase, which are probably not synthesized in antibiotic-treated and inhibited parasites, making them susceptible to host-produced hydrogen peroxide and superoxide anion.

That difloxacin, ciprofloxacin, and oxolinic acid are effective against *C. burnetii* is consistent with the report that these substituted quinolones are most active at pH 4.5 to 6.5 (16) and that the pH of the *C. burnetii*-containing phagolysosome is approximately 5.2 (2). Another member of the family

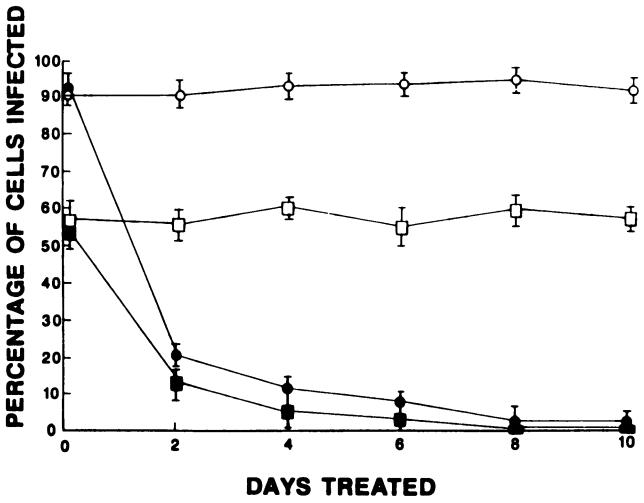

FIG. 3. Effect of oxolinic acid on L929 cells persistently infected with *C. burnetii* for 952 days at time zero. Values for untreated (○, □) and antibiotic-treated (●, ■) cells containing >1 (○, ●) and >50 (□, ■) rickettsiae are shown.

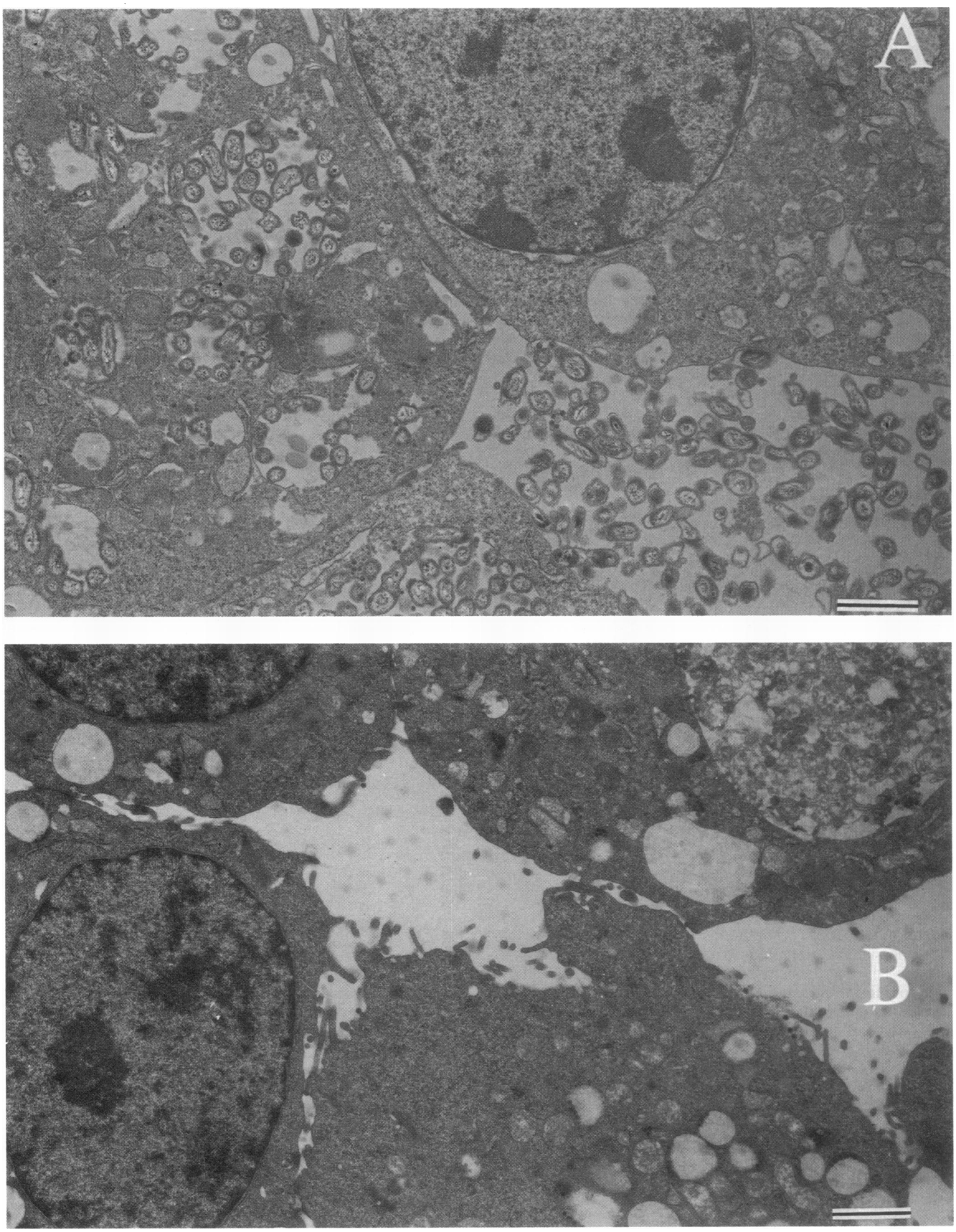

FIG. 4. Electron micrographs illustrating the elimination of *C. burnetii* (B) from L929 cells persistently infected with *C. burnetii* (A) after treatment with difloxacin (5 μg/ml) for 10 days. The cells were infected for 952 days at the onset of treatment. Bar, 1.0 μm.

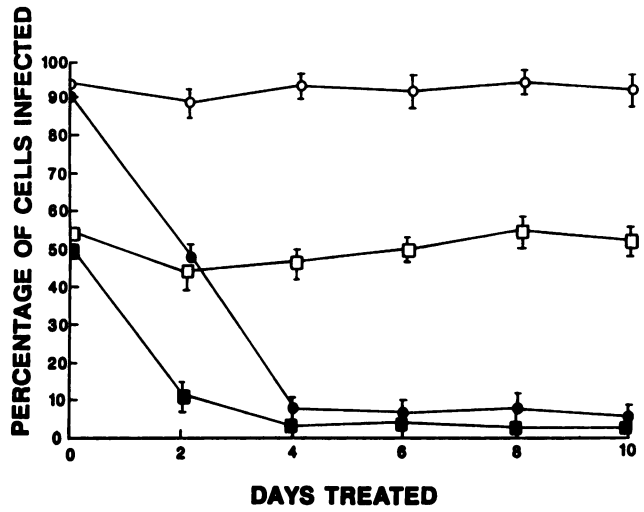

FIG. 5. Effect of rifampin on L929 cells persistently infected with *C. burnetii* for 720 days at time zero. Values for untreated (○, □) and antibiotic-treated (●, ■) cells containing >1 (○, ●) and >50 (□, ■) parasites are shown.

Rickettsiaceae, *Rickettsia conorii*, has recently been shown to be susceptible to ciprofloxacin (14); it proliferates and resides in the cytoplasm, not in vacuoles, as does *C. burnetii*.

Other antibiotics that were somewhat effective in controlling *C. burnetii* included trimethoprim, doxycycline, and chloramphenicol. To a limited degree, they too caused the disappearance of the agent from the host cell. The target of these antibiotics is the dihydrofolate reductase pathway (trimethoprim) or the protein synthesis apparatus.

Results of this investigation have shown that L929 cells persistently infected with *C. burnetii* provide a convenient system for in vitro determination of antibiotic efficacy. The time required for determining susceptibility is considerably less (as early as 24 to 48 h after the tests are initiated) than it is with other in vitro systems (7, 9, 10, 17) (embryonated eggs and animals), which take more than a week. Antibiotic concentrations were more precisely controlled in our system, whereas with embryonated eggs and experimental animals they could only be estimated.

Results of this study suggest that several members of the quinolone family may be useful in treating acute and chronic Q fever.

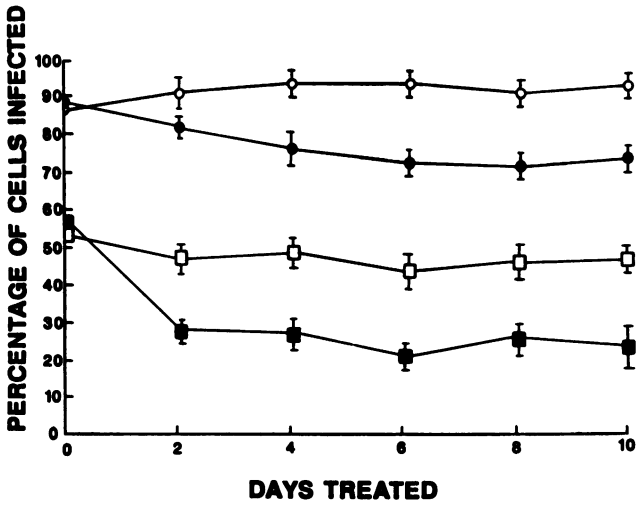

FIG. 6. Effect of doxycycline on L929 cells persistently infected with *C. burnetii* for 923 days at time zero. Values for untreated (○, □) and antibiotic-treated (●, ■) cells containing >1 (○, ●) and >50 (□, ■) rickettsiae are shown.

ACKNOWLEDGMENT

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