

## In Vitro Effectiveness of Azithromycin against Doxycycline-Resistant and -Susceptible Strains of *Rickettsia tsutsugamushi*, Etiologic Agent of Scrub Typhus

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In an effort to find a potential alternative treatment for scrub typhus, we evaluated the effectiveness of the standard drug doxycycline and the new macrolide azithromycin against a doxycycline-susceptible strain (Karp) and a doxycycline-resistant strain (AFSC-4) of *Rickettsia tsutsugamushi*. The antibiotics were tested in an in vitro assay system in which infected mouse fibroblast cells (L929) were incubated for 3 days in various concentrations of the drugs. Rickettsial growth was evaluated by direct visual counts of rickettsiae in Giemsa-stained cells or by flow cytometry. Initial tests were conducted at the concentration of each antibiotic considered to be the upper breakpoint for susceptibility (16 µg/ml for doxycycline and 8 µg/ml for azithromycin). Growth of both Karp and AFSC-4 was strongly inhibited with both antibiotics, as measured by visual counts, although the percentage of cells infected with AFSC-4 in the presence of doxycycline was three times greater than the percentage of cells infected with Karp but was only 60% as great as the percentage of cells infected with Karp in the presence of azithromycin. Flow cytometry confirmed that rickettsial growth occurred in the absence of antibiotics, but it failed to detect it in the presence of high concentrations of either drug. Visual counts of rickettsial growth at lower concentrations of the antibiotics (0.25 to 0.0078 µg/ml) showed that the Karp strain was 16 times more susceptible than the AFSC-4 strain to doxycycline. Azithromycin was much more effective than doxycycline against AFSC-4, inhibiting rickettsial growth at 0.0156 µg/ml to levels below that achieved by 0.25 µg of doxycycline per ml. Azithromycin was also more effective than doxycycline against the Karp strain, causing greater reductions in the number of rickettsiae per cell at lower concentrations. If in vivo testing confirms the in vitro effectiveness of azithromycin, it may prove to be the drug of choice for the treatment of scrub typhus in children and pregnant women, who should not take doxycycline, and in patients with refractory disease from locations where doxycycline-resistant strains of *R. tsutsugamushi* have been found. When tested in an in vitro assay system, azithromycin was more effective than doxycycline against doxycycline-susceptible and -resistant strains of *R. tsutsugamushi*.

Scrub typhus is a disease caused by infection with *Rickettsia tsutsugamushi*. It can vary from asymptomatic (8) to fatal (14) illness, but it usually causes a serious febrile disease outwardly similar to malaria and various arboviral infections (27). The disease is generally associated with disturbed habitats favoring large populations of vector larval trombiculid mites (chiggers). Such habitats include the margins of agricultural lands, the edges of forests, and areas which have been cleared (24). As a result of this distribution, scrub typhus is essentially an occupational disease (2), mainly attacking rural residents engaged in agricultural or gathering activities which bring them into contact with the vectors. Rural women are exposed to the infection when they perform agricultural tasks and may actually experience higher levels of exposure than men (23). Military activity can also result in a significant incidence of scrub typhus (4, 7) because of the inherent disturbance of habitats and the contact of many troops with the vectors. Women in the U.S. military are currently performing many

more duties which were traditionally performed by men (6) and may be expected to have increased contact with scrub typhus.

Unfortunately, current therapy for scrub typhus is suboptimal for children and pregnant women. Chloramphenicol, the first effective treatment for the disease (20, 21), is still commonly used in areas where scrub typhus is endemic. Tetracycline antibiotics, especially doxycycline, are equally effective and are now the treatment of choice (27). Most other antibiotics are considered ineffective (16, 17), although ciprofloxacin has been used experimentally (11). The use of these antibiotics is contraindicated during pregnancy (chloramphenicol, tetracyclines, and ciprofloxacin) and childhood (tetracyclines, ciprofloxacin). What is more, clinical experience supported by in vitro and in vivo (mouse) assays have shown that standard antibiotics are less effective against some strains of scrub typhus in northern Thailand (25). We evaluated the new macrolide antibiotic azithromycin for its activity against *R. tsutsugamushi* because of the low risk that it presents to children and pregnant women and because of the activity of macrolides against other species of *Rickettsia* (16). Here we report that azithromycin is effective in vitro against two strains of *R. tsutsugamushi*.

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## MATERIALS AND METHODS

The effectiveness of antibiotics against *R. tsutsugamushi* was measured by infecting mouse fibroblast cells (L929) with the rickettsiae, incubating the cells for 3 days in the desired concentration of antibiotic, and then harvesting the cells for evaluation of rickettsial growth. Experiments were performed in two stages: assays at screening dilutions intended to expose the pathogens to the highest reasonable physiological antibiotic concentration (8 µg/ml for azithromycin, 16 µg/ml for doxycycline) and assays of azithromycin and doxycycline at much lower concentrations (0.25, 0.125, 0.0612, 0.0313, 0.0156, and 0.0078 µg/ml) intended to determine the MICs.

**Sources of antibiotics.** (i) **Doxycycline.** A quality control reference standard of doxycycline hyclate (lot 9E150-58QCS-10; Pfizer Central Research, New York, N.Y.) with a purity of 86.6% was prepared in aliquots of 0.5 ml at an active ingredient concentration of 1,000 µg/ml in sterile distilled water. These aliquots were stored frozen at -70°C until use.

(ii) **Azithromycin.** A quality control standard of azithromycin dihydrate (lot OM341-64OCS-10; Pfizer) with a purity of 94.1% was prepared as described for doxycycline, except that the powder was dissolved in a minimal quantity of absolute ethanol prior to dilution in distilled water.

**Sources of *R. tsutsugamushi* isolates.** Two strains of *R. tsutsugamushi* were used. The first was the Karp strain, which has arbitrarily been considered the most typical strain and has therefore received the most study. Our isolate had been plaque purified three times in mouse fibroblast cells (L929) from the 52nd yolk sac passage (13), passed 11 times in yolk sacs, and used as the 9th subsequent L929 passage (designated Karp L9 A15/16 23 Nov 93 Semi-Purified). The other strain, AFSC-4, was isolated by the Royal Thai Army in 1990 from a human patient in Kanchanaburi, a province in western Thailand. This strain was chosen because of what appeared to be a sharply reduced susceptibility to doxycycline in previous *in vitro* trials and because of its genetic distinctness from the Karp strain (5). The isolate used in the present study had been passed twice in CD-1 mice (Charles River Laboratories, Wilmington, Mass.) and nine times in tissue culture and was used as the 10th L929 passage (designated AFSC-4 L10 A5/6 22 Nov 93 Semi-Purified).

The inocula for the present trials were processed to produce rickettsial particles free of their cellular host so that infection of cells would be as uniform as possible. The procedure for purification began by harvesting cells from a monolayer of irradiated L929 cells in which the rickettsiae had been cultured for 5 (AFSC-4) or 6 (Karp) days by using medium 199 (Quality Biologicals, Inc., Gaithersburg, Md.) with 1% L-glutamine, 2.5% fetal bovine serum (The Salzman Corp., Davenport, Iowa), and 5% tryptose phosphate broth (Quality Biologicals, Inc.) in an atmosphere of 5% carbon dioxide at 35°C. The cells were dislodged from the flasks in K36 buffer (19.5 mM KH<sub>2</sub>PO<sub>4</sub>, 33.5 mM K<sub>2</sub>HPO<sub>4</sub>, 100 mM KCl, 15.0 mM NaCl) by using 5-mm-diameter glass beads and were pooled with the culture medium. Following centrifugation at 10,500 × g for 30 min at 4°C, the supernatant was discarded and the pellet was resuspended in sucrose-Renografin-magnesium buffer (26) (SRM; 4.8 mM L-glutamic acid, 3.6 mM KH<sub>2</sub>PO<sub>4</sub>, 7.1 mM K<sub>2</sub>HPO<sub>4</sub>, 218 mM sucrose, 4.9 mM MgCl<sub>2</sub>, 1.3% [vol/vol] Renografin [diatrizoate meglumine and diatrizoate sodium injection, MD-76; Mallinckrodt, St. Louis, Mo.] [pH 7.2]) and frozen at -70°C. The suspension was thawed, disrupted with a motorized Teflon-glass homogenizer, and then spun at low speed (500 × g, 10 min, 4°C), and the supernatant was passed through a glass fiber filter (AP-25; Millipore Corp., Bedford, Mass.). The filtrate was centrifuged at high speed, the pellet was then suspended by homogenization, and the concentrated suspension was diluted with SRM, divided into aliquots, and frozen at -70°C.

**Preparation and infection of cells.** L929 cells were grown in monolayers in RPMI 1640 supplemented with volume dilutions of 5% fetal bovine serum, 5% tryptose phosphate broth, and 1% L-glutamine (200 mM), and the cells were harvested by exposing them for 5 to 10 min to a solution of 0.1% trypsin (Gibco Laboratories, Grand Island, N.Y.) and 0.1% EDTA in Hank's balanced salt solution (Quality Biologicals, Inc.). The suspended cells were washed in tissue culture medium and were then irradiated with a total of 3,000 rads by using a gamma radiation source (<sup>137</sup>Cs). The cells were replated in tissue culture flasks for 3 days. The cells were then trypsinized again, and cells shown to be viable by trypan blue staining (Gibco Laboratories) were counted in a hemocytometer to determine the number of cells in the suspension.

For infection, the number of viable cells was adjusted to 6.67 × 10<sup>6</sup> cells per ml, and 0.6 ml of the suspension was added to a 2-ml plastic vial. To this vial was added 0.3 ml of a rickettsial suspension, which was diluted to achieve 20 to 30% infected cells after 6 h (data not shown). The tightly capped vials were rotated 360° at 10 rpm at 34°C for 60 min to initiate the infection process, and the infected cell suspension was diluted to a concentration of 10<sup>6</sup> cells per ml. A culture flask (25 cm<sup>2</sup>) containing 2.5 ml of the appropriate antibiotic dissolved in tissue culture medium received 2.5 ml of the diluted, infected cell suspension. The cells were incubated at 37°C (35°C for the first screening assay) in an atmosphere of 5% carbon dioxide.

**Preparation of cells following incubation.** Cells were harvested after 3 days of incubation by aspirating the culture medium and replacing it with 2 ml of the trypsin-EDTA solution described above. The cells were incubated with the trypsin solution for exactly 5 min, and the flasks were then firmly tapped in order to loosen the cells. This process must be done without too much delay, because

overexposure to trypsin was particularly detrimental to subsequent evaluation of rickettsial growth. The suspended cells were pipetted into a tube with 0.5 ml of fetal bovine serum (in order to neutralize the trypsin), and the mixture was then diluted with 2 ml of phosphate-buffered saline (PBS; pH 7.4; Sigma Chemical Co., St. Louis, Mo.) containing 0.1% sodium azide (PBSA), which had been used to rinse the flask. This suspension was centrifuged at low speed (500 × g, 5 min, 4°C), the supernatant was poured off, and the pellet was resuspended in 3 ml of PBSA. After another low-speed centrifugation and discarding the supernatant, the pellet was resuspended in 0.75 ml of PBSA.

A slide of the infected cells was prepared from this suspension by centrifugal application of cells (Cytospin 2; Shandon Lipshaw, Pittsburgh, Pa.) for 5 min at 500 rpm from 100 µl of the suspension on one spot of the slide and 50 µl of the suspension on another spot of the slide. The two spots were necessary because of the difficulty in predicting the density of the cells. After air drying, the slide was fixed for at least 5 min in methanol and was then stained with Giemsa stain (EM Science, Gibbstown, N.J.).

The remainder of the suspension was prepared for flow cytometry as described previously (9). The detection antibody was fluorescein isothiocyanate (FITC)-labelled rabbit anti-Karp immunoglobulin G for both Karp and AFSC-4 strain-infected cultures. FITC-labelled normal rabbit immunoglobulin G was used as a control antibody. Cell suspensions were analyzed by flow cytometry with a FAC-Scan flow cytometer (Becton Dickinson, Mansfield, Mass.) equipped with a 15-mW argon laser. Data for 5,000 cells were collected, and analysis was performed by using LYSIS II software (Becton Dickinson).

**Evaluation of rickettsial growth.** Slides containing infected cells were examined for the presence of darkly staining, intracellular rickettsial particles. The intensity of infection was estimated by counting the number of particles in 50 infected cells or as many particles that could be observed in a total of 300 cells. Up to 50 particles were counted per cell, with a greater number of rickettsiae scored as "greater than 50." An analysis of variance (ONEWAY procedure; SPSS/PC+ Statistics, Chicago, Ill.) was performed on the numbers of rickettsiae per cell, counting those with greater than 50 particles as having 51. Differences between means were interpreted from the 95% confidence intervals calculated in the analysis.

The extent of infection was estimated by counting the number of infected cells after examination of at least 300 cells. Differences in the percentage of infected cells and in the percentage of infected cells with greater than 50 rickettsiae were compared between treatment groups by calculating the 95% confidence intervals on the basis of the binomial distribution (22).

An index combining both the intensity and the extent of cellular infection was calculated from the data, producing an estimate of the number of rickettsiae per cell if the infection were completely uniform. The calculated number of rickettsiae per cell was determined by the formula CRPC = [(a/100) × b] + [(c/100) × 51], where CRPC is the calculated number of rickettsiae per cell, *a* is the percentage of cells infected with <51 rickettsiae, *b* is the mean number of rickettsiae per cell for those infected cells with <51 rickettsiae, and *c* is the percentage of cells with >50 rickettsiae. The 95% confidence interval for the calculated number of rickettsiae per cell was estimated by performing the calculations with the lower and upper 95% confidence limits from the three measurements of rickettsial infection (parameters *a*, *b*, and *c*).

Flow cytometry produced a graph of fluorescence versus the number of cells for uninfected and infected cells. When the peaks of the two groups of cells did not coincide, it was considered that significant rickettsial growth had occurred.

## RESULTS

At the high screening concentrations of the antibiotics, doxycycline and azithromycin significantly inhibited both strains of *R. tsutsugamushi* (Table 1). The extent of infection with the Karp strain, expressed as a percentage of infected cells, was reduced about equally by both antibiotics (8 to 12 times reduction), whereas the extent of infection with the AFSC-4 strain was reduced more by azithromycin (16-fold reduction) than by doxycycline (4-fold reduction). The intensity of infection, measured by counting the number of rickettsiae per infected cell, was also markedly reduced by both antibiotics. A mean of 4 to 9 rickettsiae were counted in infected cells incubated with antibiotics, whereas a mean of 40 rickettsiae were counted in infected cells incubated without antibiotics. A majority of cells incubated without antibiotics were maximally infected (i.e., they contained >50 rickettsiae per cell), whereas no cells were maximally infected in the presence of antibiotics.

Assays performed at lower concentrations of the antibiotics showed that Karp and AFSC-4 were different in their responses to doxycycline (Table 2). The percentage of cells infected after 3 days of incubation in the presence of the lowest

TABLE 1. Extent and intensity of infection of L929 mouse fibroblast cells with *R. tsutsugamushi* following 3 days of incubation with high concentrations of doxycycline (16 µg/ml) or azithromycin (8 µg/ml)

Strain and antibiotic	% Infected cells (95% confidence interval)	Mean no. of rickettsiae/infected cell <sup>a</sup>	% Cells with >50 rickettsiae
<b>Karp</b>			
None	84 (81.5–85.8) <sup>b</sup>	39.5 (35.0–43.7)	60 (57.2–62.8)
Doxycycline	7 (5.8–8.8)	4.5 (3.3–5.9)	0
Azithromycin	10 (8.0–11.4)	8.9 (5.1–12.8)	0
<b>AFSC-4</b>			
None	95 (93.3–97.1)	40.4 (35.7–45.2)	68 (64.1–72.4)
Doxycycline	22 (19.3–23.8)	5.1 (4.1–6.0)	0
Azithromycin	6 (4.9–7.7)	4.0 (1.9–5.8)	0

<sup>a</sup> Counting those with >50 rickettsiae as 51. Analysis of variance:  $F = 104.8$ , degrees of freedom = 5/228,  $P < 0.0001$ .

concentration of doxycycline (0.0078 µg/ml) was reduced 78% compared with the no-antibiotic control for Karp but only 13% for AFSC-4. With the exception of doxycycline at a concentration of 0.0625 µg/ml, increases in the concentration of doxycycline did not significantly decrease the percentage of cells infected with Karp. In contrast, the percentage of cells infected with AFSC-4 was successively decreased by increases in the concentration of antibiotic, but a level of inhibition equivalent to that of Karp was not achieved until a concentration of 0.25 µg/ml was used. By using the percentage of cells infected as a measure of rickettsial growth, these results suggest that the MIC of doxycycline for Karp was below 0.0078 µg/ml and that the MIC for AFSC-4 was 0.25 µg/ml, a difference of at least 32-fold.

The intensity of infection in doxycycline-treated cells was also different for Karp and AFSC-4 (Table 2). The number of the Karp strain of rickettsiae per cell was maximally reduced by doxycycline at a concentration of 0.0625 µg/ml and, by this measure, achieved a level of inhibition similar to that observed at 16 µg/ml (Table 1). The mean number of AFSC-4 rickettsiae per cell was reduced in the presence of doxycycline, but even at 0.25 µg/ml the number was not as low as that in the presence of 16 µg/ml. Presumably, the MIC measured by determining the mean number of rickettsiae per cell would be 0.0625 µg/ml for Karp and at least 0.5 µg/ml for AFSC-4, implying a difference of at least eight-fold. Similarly, the percentage of infected cells with at least 50 rickettsial particles was reduced to zero at 0.0625 µg/ml for the Karp strain, whereas 12% of the infected cells were maximally infected at 0.25 µg/ml for the AFSC-4 strain.

As a combination of intensity and extent of infection, the index of the calculated number of rickettsiae per cell also reflected the difference in susceptibility between Karp and AFSC-4 (Fig. 1). Karp was nearly completely inhibited by 0.0625 µg of doxycycline per ml, but AFSC-4 was able to continue at least some growth in the presence of doxycycline at concentrations up to 0.25 µg/ml. Sixteen- to 32-fold greater concentrations of doxycycline were required to inhibit AFSC-4 to the growth levels of Karp occurring at 0.0078 µg/ml.

At a screening concentration of 8 µg/ml, azithromycin was also effective against both strains (Table 1), although inhibition was marginally greater for AFSC-4 than for Karp. At lower concentrations (Table 2), Karp was significantly inhibited, as measured by both the extent and the intensity of infection, at the lowest concentration (0.0078 µg/ml). Only the extent of infection (percentage of infected cells) decreased with dosage,

going down to a low of 3.7% at 0.25 µg/ml. Maximally infected cells were detected at two of the higher concentrations, but at 0.0625 µg/ml there was only one such cell and at 0.125 µg/ml there were only two such cells. AFSC-4 was significantly inhibited at the second concentration (0.0156 µg/ml), implying a twofold difference in susceptibility compared with that of Karp. At higher concentrations, however, AFSC-4 was more inhibited than Karp. Only 2% of the cells were infected with AFSC-4 in the presence of azithromycin at 0.125 and 0.25 µg/ml, and no cells were maximally infected. The graph of the calculated number of rickettsiae per cell (Fig. 1) also shows that Karp was inhibited at all azithromycin concentrations and that AFSC-4 was inhibited at azithromycin concentrations of 0.0156 µg/ml and higher.

Results of flow cytometry corroborated the direct rickettsial cell counts for screening dilutions of antibiotics for both strains (data not shown). However, this technique did not distinguish

TABLE 2. Measurements of *R. tsutsugamushi* growth in L929 cells incubated for 3 days in dilution series of either doxycycline hydrochloride or azithromycin chloride

Drug, strain, and drug concn (µg/ml)	% Cells infected <sup>a</sup>	No. of rickettsiae/cell <sup>b</sup>	% Cells maximally infected <sup>c</sup>
<b>Doxycycline</b>			
<b>Karp</b>			
0	83.7 (79.5–87.9) <sup>d</sup>	39.4 (35.0–43.7)	60.0 (46.4–73.6)
0.0078	18.7 (14.3–23.1)	20.1 (15.4–24.8)	16.0 (5.8–26.2)
0.0156	13.0 (9.2–16.8)	23.1 (16.9–29.2)	20.5 (7.8–33.2)
0.0313	15.7 (11.6–19.8)	18.7 (14.2–23.3)	12.8 (3.2–22.4)
0.0625	5.0 (2.5–7.5)	11.7 (5.5–18.0)	0
0.125	14.7 (10.7–18.7)	8.2 (6.5–9.9)	0
0.25	13.3 (9.5–17.2)	6.8 (4.8–8.8)	0
<b>AFSC-4</b>			
0	95.2 (91.5–99.0)	40.4 (35.7–45.2)	68.0 (55.1–80.9)
0.0078	83.0 (78.7–87.2)	39.8 (34.9–44.8)	68.0 (55.1–80.9)
0.0156	72.2 (65.2–79.1)	22.0 (17.2–26.9)	20.0 (8.9–31.1)
0.0313	72.2 (67.4–77.0)	20.6 (16.2–25.0)	16.0 (5.8–26.2)
0.0625	52.1 (46.5–57.7)	21.2 (17.5–24.9)	12.0 (3.0–21.0)
0.125	43.0 (37.4–48.6)	13.1 (9.5–16.7)	6.0 (0–12.6)
0.25	13.7 (9.8–17.6)	17.7 (12.3–23.0)	12.0 (2.1–21.9)
<b>Azithromycin</b>			
<b>Karp</b>			
0	83.7 (79.5–87.9)	39.4 (35.0–43.7)	60.0 (46.4–73.6)
0.0078	14.3 (10.4–18.3)	10.8 (7.8–13.9)	0
0.0156	9.3 (6.0–12.6)	9.5 (6.6–12.3)	0
0.0313	8.7 (5.5–11.9)	10.8 (7.3–14.4)	0
0.0625	8.7 (5.5–11.9)	10.3 (5.7–15.0)	3.8 (0–11.1)
0.125	9.0 (5.8–12.2)	13.0 (7.5–18.5)	7.4 (0–17.3)
0.25	3.7 (1.5–5.8)	8.3 (3.8–12.8)	0
<b>AFSC-4</b>			
0	95.2 (91.5–99.0)	40.4 (35.7–45.2)	68.0 (55.1–80.9)
0.0078	89.0 (85.5–92.5)	35.6 (30.8–40.4)	50.0 (36.1–63.9)
0.0156	9.7 (6.3–13.0)	5.9 (4.7–7.1)	0
0.0313	6.0 (3.3–8.7)	9.6 (5.5–13.7)	0
0.0625	5.7 (3.1–8.3)	4.2 (1.5–6.9)	0
0.125	2.0 (0.4–3.6)	9.2 (1.3–17.1)	0
0.25	2.0 (0.4–3.6)	15.7 (0–34.6)	0

<sup>a</sup> On the basis of counts of approximately 300 cells.

<sup>b</sup> Mean for all infected cells; cells with >50 rickettsiae were counted as having 51 cells. Analysis of variance for all data:  $F = 28.2$ , degrees of freedom = 27/985,  $P < 0.0001$ .

<sup>c</sup> Percentage of infected cells.

<sup>d</sup> Ninety-five percent confidence intervals are given in parentheses.

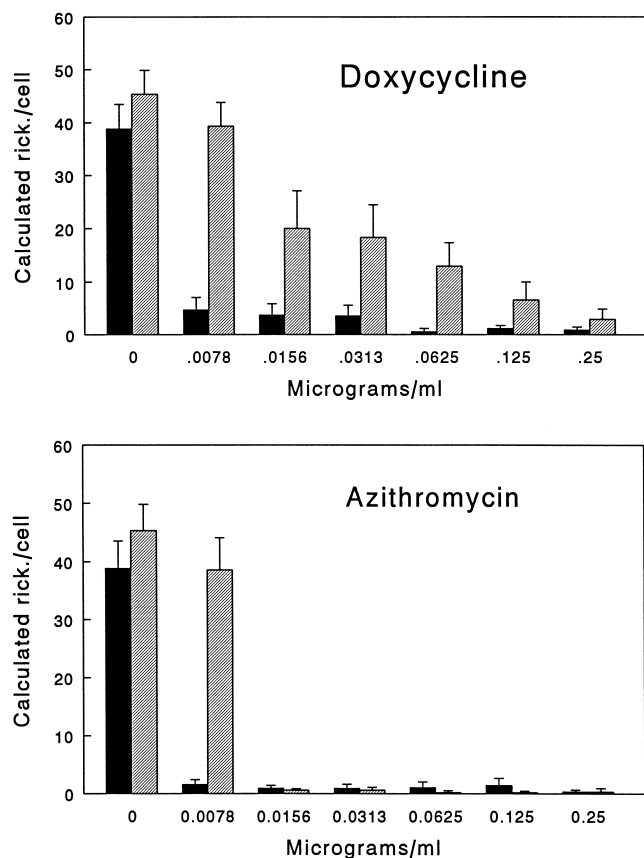


FIG. 1. Calculated number of *R. tsutsugamushi* Karp (■) and AFSC-4 (▨) organisms per cell following incubation for 3 days in various concentrations of doxycycline or azithromycin. Error bars represent the upper 95% confidence limits.

effectively between cells with low rickettsial numbers and uninfected cells at lower concentrations of antibiotics.

## DISCUSSION

This is the first study to demonstrate that azithromycin suppresses the growth of *R. tsutsugamushi* in a cell culture system and that growth is suppressed at least as effectively by azithromycin as by doxycycline. For the doxycycline-susceptible Karp strain, a substantial reduction in the extent of infection (measured by the percentage of cells infected after 3 days of incubation) was achieved with either doxycycline or azithromycin at 0.0078 µg/ml. The intensity of infection (measured by the number of rickettsiae per infected cell) was suppressed by an eight times lower concentration of azithromycin (0.0078 µg/ml) than of doxycycline (0.0625 µg/ml). These values are somewhat lower than a tetracycline MIC of 0.15 to 0.31 µg/ml for the Karp strain reviewed by Raoult and Drancourt (17) and an MIC of 0.1 µg/ml for the Karp strain reported by Barker (3).

Against the recent Thai isolate, strain AFSC-4, azithromycin was clearly more effective than doxycycline. The extent of infection was substantially reduced by 16 times less azithromycin (0.0156 µg/ml) than doxycycline (0.25 µg/ml). The intensity of infection remained high (12% of infected cells with more than 50 rickettsiae per cell) even at 0.25 µg of doxycycline per ml, whereas the intensity of infection was very low (no infected cells with greater than 50 rickettsiae per cell) at 0.0156 µg of azithromycin per ml.

Our concentrations were well below the low breakpoint (12) of 4 µg of doxycycline per ml and the observed levels in serum of 1 µg of azithromycin per ml following oral therapy with azithromycin (19). Given that the concentrations that we tested were low, it might be expected that both azithromycin and doxycycline would be effective against a clinical infection with the Karp strain and that at least azithromycin would be effective against AFSC-4.

Flow cytometry results supported those of cell counts for screening the activities of both drugs against the Karp and AFSC-4 strains. These were the first tests of the technique for a strain other than Karp. In the future, it will be simpler to rapidly screen antibiotics at higher concentrations by flow cytometry without using supporting cell counts. However, cell counts will probably continue to be necessary to determine the MICs of those antibiotics which suppress rickettsial growth because our flow cytometry technique was not sufficiently sensitive to distinguish quantitatively between low levels of infection. Another limitation of the technique is that it uses antibodies against a particular strain of *R. tsutsugamushi* and is likely to be less sensitive for the detection of antigenically disparate strains.

Azithromycin may prove to have clinical usefulness in the treatment of scrub typhus. Azithromycin's longer half-life of 68 h compared with a half-life of 18 to 22 h for doxycycline (1) may permit shorter, more convenient dosing schedules than those now available. For strains which are less susceptible to doxycycline in vitro and for those which respond less well clinically, azithromycin may provide a more effective alternative. Azithromycin causes no known harm to the developing fetus (Class B) (1) and, as a macrolide, is among those antibiotics considered safer than other antibiotics for use in young children and pregnant women (15). In contrast, doxycycline binds to developing skeletal tissue, stunting long bones (18), causes discoloration and enamel hypoplasia in developing teeth at 16 weeks postconception, and sometimes can cause liver, pancreas, or renal damage (10). Therefore, azithromycin may be a safer drug for treating pregnant women and young children. Future work will concentrate on assessing the effectiveness of azithromycin against scrub typhus in an animal model and in clinical trials.

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