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Bactericidal Activities of Various Antimicrobial Agents against Human and Animal Isolates of *Mycobacterium paratuberculosis*

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The MICs and MBCs of various antimicrobial agents for strains of *Mycobacterium paratuberculosis* isolated from animal and human sources were evaluated. The MICs and MBCs of rifabutin, ciprofloxacin, ethambutol, clofazimine, streptomycin, cefazolin, and amikacin were found to be well below therapeutic levels in serum and tissue.

Mycobacterium paratuberculosis is the etiologic agent of paratuberculosis (Johne's disease), a chronic granulomatous ileocolitis of ruminants (3). The disease occurs throughout the world, and epidemiologic surveys of abbattoir dairy cattle have suggested infection rates from 3.6 to 17% in the United States. Despite efforts to treat animals with this disease, chemotherapeutic attempts have failed to clear animals of the infection (3).

In recent years, there has been a surge of interest in the possible public health significance of this organism. Strains of *M. paratuberculosis* genetically identical to each other and to those found in animals have been isolated from the intestinal tissues of human patients with Crohn's disease, a chronic granulomatous ileocolitis of unknown etiology, in Connecticut; Los Angeles, Calif.; Australia; The Netherlands; and France (2). Such findings have suggested some form of etiologic role of *M. paratuberculosis* in at least some cases of Crohn's disease.

Three of the human isolates of *M. paratuberculosis* were previously evaluated for their susceptibilities to 23 antimicrobial agents by a modified broth dilution assay (6). The present study was initiated to reevaluate the activities of antimicrobial agents previously shown to inhibit the growth of *M. paratuberculosis* and determine the bactericidal activities of these agents.

Human isolates of *M. paratuberculosis* examined included strains Linda (ATCC 43015), Dominic (ATCC 43545), and Ben (ATCC 43544) (5); strains Holl-1 (ATCC 49164) and Holl-2 (ATCC 49166) isolated in The Netherlands by J. Haagsma; and strain BB-410 (ATCC 49167) isolated in Los Angeles, Calif., by B. Beaman. Additionally, the neotype strain of *M. paratuberculosis* (ATCC 19698) and an *M. paratuberculosis* isolate from primates (ATCC 43546) (8) were examined. All organisms were identified by standard methods (1) and by genetic analyses (9; R. J. Chiodini, J. Clin. Microbiol., in press).

Organisms were grown in 7H9 broth with Dubo's oleic acid-albumin complex, 0.05% Tween 80, and 2 μg of mycobactin J per ml in 25-cm^2 (30-ml) tissue culture flasks at 37°C without agitation as previously described (4, 6). The antimicrobial agents evaluated are listed in Table 1. Serial twofold dilutions of the antimicrobial agents were freshly prepared in 7H9 broth prior to inoculation.

Samples (6 ml) of broth containing appropriate antimicrobic concentrations were dispensed into culture flasks. Flasks were inoculated with 50 μl of a mid-logarithmic-phase culture containing 2.8×10^6 to 8.0×10^6 CFU/ml as determined spectrophotometrically at 540 nm. Spectrophotometric tubes (10 by 125 mm) were attached to the necks of tissue culture flasks by way of 0.5-in. (1.3-cm) shrinkable tubing (Allied Electric Supply Corp., Hartford, Conn.). Flasks were incubated at 36 to 38°C in a horizontal position without agitation. Assays were performed in duplicate.

Optical density at 540 nm was recorded daily until growth in the control tubes reached a reading of at least 0.04, which represented logarithmic growth. Spectrophotometric monitoring was not performed on all cultures; others were monitored by visual inspection of flasks.

Only broth cultures at and above the MIC were evaluated for bactericidal activity. Broth cultures were mixed, and then 100-μl samples of antimicrobial agent-containing cultures were subjected to three serial 10-fold dilutions and inoculated onto slants of Herrold egg yolk medium with mycobactin J. Cultures were incubated at 36 to 38°C for 12 to 16 weeks or until individual colonies were discernible. At the end of the incubation period, the average numbers of surviving organisms were recorded.

The MIC was the lowest concentration of an antimicrobial agent which prevented growth. The MIC_{50} was the lowest concentration of an antimicrobial agent which prevented growth of at least four of the eight strains (50%). The MBC was the lowest concentration of an antimicrobial agent which caused a loss in viability of 99.9% or more in the original inoculum. The MBC_{50} was the lowest antimicrobial agent concentration which reduced the original inoculum by >99.9% in at least four of eight strains (50%).

A representative graph of optical density monitoring of *M. paratuberculosis* cultures exposed to an antimicrobial agent in broth is shown in Fig. 1. The incubation period required to make a determination averaged 8 to 10 days. In the example provided in Fig. 1, complete inhibition of growth occurred at rifabutin concentrations from 1 to 0.125 μg/ml, and no inhibition was present at 0.03 μg/ml. At 0.06 μg of rifabutin per ml, growth occurred after about 9 days but was less than that of the control, suggesting over 99% growth inhibition by the agent.

In all cases, duplicate test results were identical. All antimicrobial agents evaluated inhibited the growth of *M. paratuberculosis* at well below therapeutic concentrations, except for cefoxitin, which was dropped from further eval-

TABLE 1. MICs and MBCs for isolates of *M. paratuberculosis*

Test agent	MIC (μg/ml)		MBC (μg/ml)	
	Range	50%	Range	50%
Rifabutin	0.03–0.25	0.06	0.12–1.0	1.0
Ciprofloxacin	0.12–0.25	0.12	0.25–1.0	0.5
Ethambutol	5.0–10.0	5.0	5.0–>10.0	10.0
Clofazimine	0.03–0.12	0.06	0.12–1.0	2.5
Cefoxitin	20.0–>40.0	ND[a]	ND	ND
Streptomycin	0.25–1.0	0.5	0.25–1.0	0.5
Cefazolin	1.2–>10.0	2.5	10.0–>10.0	>10.0
Amikacin	1.2–2.5	1.2	1.2–5.0	2.5

[a] ND, Not determined.

uation after three of five strains failed to be inhibited at the highest concentration evaluated. Generally, the growth of strains Linda and ATCC 43546 was inhibited by the lowest antimicrobial concentration, and strain BB-410 required the highest concentration for growth inhibition.

All antimicrobial agents also had bactericidal activity at well below therapeutic levels in serum and tissue (Table 1). In general, most antimicrobial agents were bactericidal at 2 to 4 times the MIC, except for cefoxitin, for which the MBC for five strains was not achieved at the concentrations tested, and rifabutin, for which the MBC_{50} was 16 times higher than the MIC_{50}.

With the increased occurrence in immunocompromised patients of mycobacterial infections (11) which require treatment with bactericidal rather than inhibitory or bacteriostatic concentrations, the determination of bactericidal activities of antimicrobial concentrations has greater significance. In other mycobacterial diseases in which bacterial proliferation is also uninhibited, such as paratuberculosis and lepromatous leprosy, the use of bactericidal antimicrobial concentrations is also more likely to be therapeutically effective. Although standard and accepted methods exist for the determination of bactericidal activities of antimicrobial agents for a variety of microorganisms (10, 12), none exists for such determinations for mycobacteria. Methods similar to those accepted for other microorganisms were used in the present study with modifications required to support the growth of *M. paratuberculosis*.

By the methods described herein, MICs of antimicrobial agents were obtained in approximately 8 to 10 days by the broth assay, and bactericidal data were obtained by reinoculation of antimicrobial agent-containing broths onto fresh solid media. The ability to determine growth or inhibition of *M. paratuberculosis* in 8 to 10 days is comparable to detection rates reported with the use of the BACTEC system (7) but without the need for expensive laboratory equipment. Determinations of bactericidal activity, however, required 12 to 16 weeks of growth on solid media. The methods described here provide a reliable procedure for the determination of bactericidal activities of antimicrobial agents against *M. paratuberculosis* and other slow-growing mycobacteria.

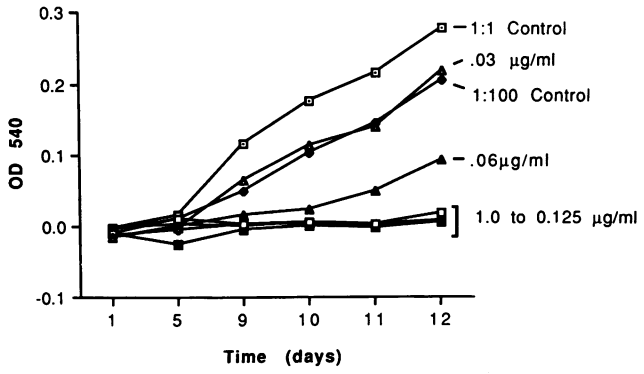

FIG. 1. Changes in optical density (OD) at 540 nm of *M. paratuberculosis* Ben exposed to various concentrations of rifabutin in broth. Complete inhibition of growth was observed with rifabutin concentrations from 1.0 to 0.125 μg/ml, partial inhibition was observed at 0.06 μg/ml, and <99% inhibition was observed at 0.03 μg/ml.

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