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Activity of Clarithromycin Compared with Those of Other Drugs against *Mycobacterium paratuberculosis* and Further Enhancement of Its Extracellular and Intracellular Activities by Ethambutol

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Radiometric MICs of clarithromycin, a new macrolide drug, were determined against five mycobactin-dependent strains of *Mycobacterium paratuberculosis* (including two Crohn's disease clinical isolates) and compared with those of other drugs which included rifampin, ethambutol, amikacin, ofloxacin, ciprofloxacin, and sparfloxacin. Among the drugs screened, clarithromycin was the drug for which MICs were lowest against the five strains tested. As MICs were significantly below the reported C_{max} levels (about 4 μg/ml), the intracellular activity of clarithromycin against the type strain of *M. paratuberculosis* maintained in cultured macrophages was screened. Clarithromycin was able to kill the initial inoculum by more than 1 log within 7 days, and this activity was further potentiated by ethambutol. Extracellular drug combination screened by using sublethal concentrations of the drugs showed that ethambutol was able to enhance clarithromycin activity in three out of four *M. paratuberculosis* strains instead of only one out of four strains (or none in the case of ofloxacin) when associated with other drugs. These results suggest that clarithromycin may be fruitful to treat human disease in which *M. paratuberculosis* may be etiologically involved.

Chemotherapeutic efforts to treat *Mycobacterium paratuberculosis*, the etiologic agent of paratuberculosis (Johne's disease) in ruminants, have failed to clear animals of the infection (2). Recently, mycobacteria isolated from human cases of chronic granulomatous ileocolitis of unknown etiology (Crohn's disease) have been found to be genetically identical to *M. paratuberculosis* (9). DNA-DNA hybridization studies have established that *M. paratuberculosis* (including the Crohn's disease isolates), and *M. avium* belong to a single genomic group (20, 25).

Similarities between the pathogenesis of *M. avium* complex (MAC) infections in AIDS, Johne's disease of ruminants, and Crohn's disease in humans, all of which are contracted by the fecal-oral route and involve the gastrointestinal tract, have been underlined (3, 10, 21). These observations, as well as recent suggestions that *M. paratuberculosis* may be etiologically involved in human disease (1), prompted us to examine comparative in vitro activities of antituberculous drugs against *M. paratuberculosis*.

Among the macrolides, the newer erythromycin derivative clarithromycin, which possesses a methyl group at C-6 (6), was shown to be highly active against multiple-drug-resistant MAC organisms (4, 11, 15, 17, 24). Its further screening against 18 mycobacterial species showed that against 13 out of 18 species, MICs of the drug were below reported C_{max} levels at pH 6.8. Following these observations, we decided to compare the radiometric MICs of clarithromycin against *M. paratuberculosis* with those of rifampin, ethambutol, amikacin, ofloxacin, ciprofloxacin, and sparfloxacin.

As mycobacteria are intracellular pathogens (5, 12, 13), we also decided to compare the intracellular activity of clarithromycin by using cultured murine macrophages. In accordance with our previous macrophage studies (17–19), the infected macrophages were fed C_{max} levels of the drugs studied. Potentiation of the intracellular drug activity by ethambutol, which decreases the MAC cell wall barrier by disrupting the wall outer layer (16) by inhibiting both the biosynthesis of arabinogalactan (23) and the transfer of mycolic acids in the mycobacterial cell envelope (22), was also investigated. The drug combinations were also tested at sublethal concentrations by using Bactec radiometric methodology to correlate the drug enhancement results obtained using extracellular and intracellular systems used.

The mycobactin-dependent *M. paratuberculosis* strains used in this investigation (see Table 1) were grown in complete 7H9 broth (supplemented with Middlebrook ADC enrichment; Difco Laboratories, Detroit, Mich.) containing 0.05% (vol/vol) Tween 80 to avoid clumping and 2 μg of mycobactin-J (Rhône-Mérieux, France) per ml at 37°C. Bacteria were harvested at their mid-logarithmic phase at an optical density of 0.15 (measured at 650 nm with a Coleman Junior II spectrophotometer) which corresponded to about 10^8 CFU/ml. All the *M. paratuberculosis* strains and Crohn's disease clinical isolates identified as *M. paratuberculosis* were kindly provided by M. F. Thorel, Laboratoire Central de Recherches Vétérinaires, Maisons-Alfort, France.

Radiometric determination of MICs by using the Bactec 460-TB apparatus (Becton Dickinson, Towson, Md.) was performed as reported earlier at pH 6.8 ± 0.2 (15, 17, 18), except that the commercially available 12B vials were supplemented with 2 μg of mycobactin-J per ml because of the mycobactin dependence of these strains (Fig. 1). Parallel experiments in the case of the type strain of *M. paratuberculosis* (ATCC 19698) showed that the strain grew extremely slowly in the 7H12 broth without mycobactin-J, with MICs interpretable only after 21 days. Although MICs of the drugs did not change in mycobactin-supplemented medium, the data were interpretable at least 1 week earlier.

The combined drug action against *M. paratuberculosis* was studied radiometrically as reported previously, using the *X/Y* quotient methodology (16–18). For these studies, all the drugs were used at sublethal concentrations as indicated in the figure legends. The reason for this choice was that at this

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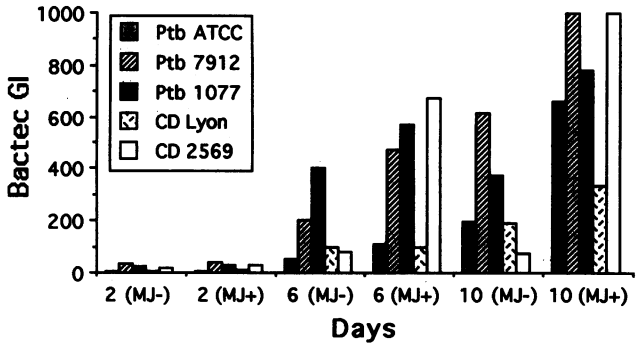

FIG. 1. Effect of mycobactin-J on growth of mycobactin-dependent mycobacteria. The growth is expressed in terms of the Bactec growth index (GI) measured by using vials containing 7H12B broth with a pH of 6.8. The cultures were performed without or with 2 μg of mycobactin-J per ml. The vials were inoculated with 0.1 ml of a culture pregrown to a Bactec GI of about 500, and growth was monitored for 10 consecutive days. Symbols: Ptb, *M. paratuberculosis*; CD, mycobacteria isolated from Crohn's disease patients and identified as *M. paratuberculosis*; (MJ-), growth in vials without addition of mycobactin-J; (MJ+), growth with 2 μg of mycobactin-J per ml.

concentration, the drugs used alone were unable to significantly reduce the initial inoculum added in the Bactec vials. In such a case, any significant drug enhancement observed could eventually suggest a reproducible effect in infected host cells, where the drugs are available in much higher concentrations.

Monolayers containing about 10^6 mouse bone marrow-derived macrophages per well from *Bcg*^{s} 6- to 13-week-old female C57BL/6 mice were cultured and infected with mycobacteria as described recently (18). After 4 h of phagocytosis, all the extracellular bacteria were washed away with Hanks balanced salt solution and the macrophages were refed fresh growth medium (supplemented with 2 μg/ml of mycobactin-J) containing the desired antibiotics. The bacteria were enumerated at various time points by lysing the macrophages with 0.25% (wt/vol) sodium dodecyl sulfate (SDS), doing immediate serial dilutions, and plating the lysates on 7H11 agar medium containing 2 μg of mycobactin-J per ml. Addition of 0.25% SDS to parallel bacterial cultures which were immediately serially diluted for viability assessment in parallel control experiments showed that it did not lower the bacterial viable counts. Results were expressed as mean viable counts ± standard error per well.

In accordance with our experimental model for determining the intracellular action of drugs (17–19), drugs were used at their reported C_{max} in humans, i.e., 4 μg/ml for clarithromycin (7) and 6 μg/ml for ethambutol (8). Sparfloxacin (Rhône-D.P.C. Europe, Antony, France), clarithromycin (Abbott Laboratories, North Chicago, Ill.), amikacin (Bristol, Paris, France), ethambutol (Lederle, Oullins, France), ofloxacin (Laboratoire Diamant, Puteaux, France), and ciprofloxacin (Bayer Pharma, Puteaux, France), were kindly provided by their manufacturers, whereas rifampin was purchased from Sigma Chemical Co., St. Louis, Mo.

The results obtained are summarized in Tables 1 and 2 and Fig. 1 and 2. When clarithromycin activity was screened against five strains of mycobactin-dependent *M. paratuberculosis* and compared with activities of six other drugs used in parallel (i.e., rifampin, ethambutol, amikacin, ofloxacin, ciprofloxacin, and sparfloxacin), clarithromycin MICs were lowest against all the strains, with MICs of 0.25 μg/ml for four strains and 0.5 μg/ml for one strain. Origin of strains (type strain, livestock, or Crohn's disease isolates) did not change the overall drug susceptibility profile. The mycobactin dependence of all the *M. paratuberculosis* strains used in this study was verified by using solid growth media (results not shown) or radiometrically as illustrated in Fig. 1.

When the enhancement of clarithromycin activity by ethambutol was measured by the *X/Y* quotient calculations, clarithromycin activity was enhanced in three out of four strains of *M. paratuberculosis* (Table 2). This drug enhancement effect was confirmed in the case of the ATCC 19698 strain by plating the cultures from Bactec vials for viable count determinations (Fig. 2A). All the drugs in radiometric enhancement experiments were used at sub-MIC levels, i.e.,

TABLE 2. Comparative enhancement of various drugs used at sub-MIC levels against *M. paratuberculosis* strains by ethambutol (1 μg/ml) according to the radiometric *X/Y* quotient criteria

Drug (μg/ml)	Enhancement of activity[a] for:			
	ATCC 19698	7912	1077	CD-2569
Rifampin (1.0)	−	−	+	−
Amikacin (0.25)	−	−	+	−
Ofloxacin (1.0)	−	−	−	−
Ciprofloxacin (1.0)	−	−	+	−
Sparfloxacin (0.25)	−	−	+	−
Clarithromycin (0.1)	+	−	+	+

[a] Enhancement of drug activity was calculated by *X/Y* quotients radiometrically as described in the text. *X* is the Bactec growth index obtained with the combination of drugs, and *Y* is the minimal growth index value for any of the drugs used alone. An *X/Y* quotient of <0.5 in case of a two-drug combination indicates enhanced drug action.

TABLE 1. Comparative Bactec MICs of clarithromycin and other drugs against bacteria classified as *M. paratuberculosis*

Organism	MIC (μg/ml)[a]						
	Rifampin	Ethambutol	Amikacin	Ofloxacin	Ciprofloxacin	Sparfloxacin	Clarithromycin
M. paratuberculosis							
ATCC 19698	10.0	2.5	2.5	5.0	>5.0	1.5	0.25
7912	5.0	2.5	2.5	>5.0	5.0	0.5	0.25
1077	10.0	2.5	1.0	>5.0	5.0	1.5	0.25
Crohn's disease isolates							
CD-Lyon	10.0	2.5	1.0	>5.0	5.0	1.0	0.25
CD-2569	5.0	2.5	2.5	>5.0	5.0	1.0	0.5

[a] The MICs were determined in 7H12B medium (pH 6.8 ± 0.2) supplemented with 2 μg of mycobactin-J per ml. The drug-containing Bactec vials were inoculated with 0.1 ml of the bacterial preculture grown to a Bactec growth index of about 500, and the results were interpreted in comparison to a 1:100 diluted parallel control.

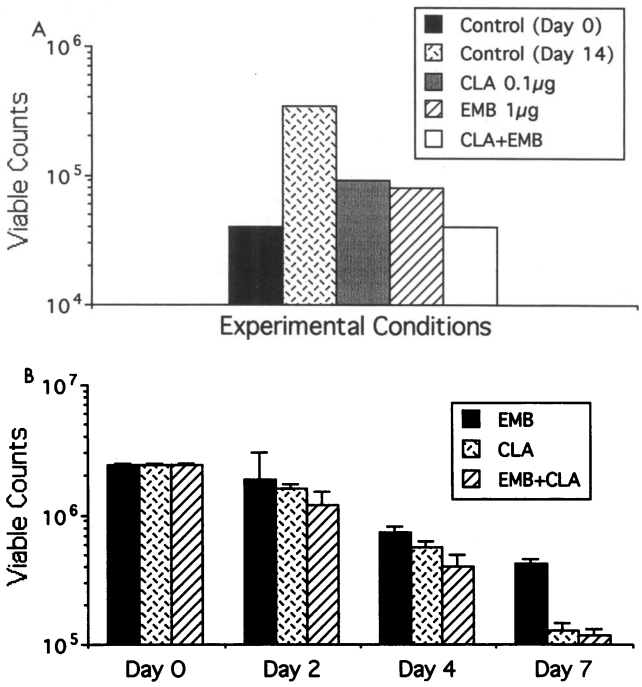

FIG. 2. Extracellular (A) and intracellular (B) action of clarithromycin against *M. paratuberculosis*. (A) Viable count data showing the enhancement of sub-MIC levels of clarithromycin (CLA, 0.1 μg/ml) by sub-MIC levels of ethambutol (EMB, 1 μg/ml) against the ATCC 19698 strain of *M. paratuberculosis*. The experiment was performed as reported for *X/Y* quotient calculations in Table 2, and the Bactec vials were plated onto 7H11 agar for viable count determinations. (B) Intracellular action of clarithromycin against *M. paratuberculosis* ATCC 19698 phagocytized by murine bone marrow-derived macrophages and potentiation of its activity by ethambutol. All the drugs were used at their reported C_{max} levels for humans, i.e., 4 μg/ml for clarithromycin and 6 μg/ml for ethambutol. Please refer to text for further information.

1 μg/ml for rifampin, ofloxacin, and ciprofloxacin; 0.25 μg/ml for amikacin and sparfloxacin; and 0.1 μg/ml for clarithromycin. These results are in agreement with previously published data with MAC organisms (14, 16–18).

When tested against *M. paratuberculosis* ATCC 19698 phagocytized by murine macrophages, clarithromycin used at its C_{max} level of 4 μg/ml possessed significant bactericidal activity, as it was able to kill the initial inoculum by more than 1 log within 7 days, and this activity was further potentiated by ethambutol used at a C_{max} level of 6 μg/ml (Fig. 2B).

In summary, the results obtained showed that clarithromycin possessed promising in vitro MICs against both animal and human isolates of *M. paratuberculosis*, with significant bactericidal activity against intracellular bacteria. In agreement with our previous observations for *M. avium* (17), the present study showed that ethambutol was further able to enhance both the extracellular and intracellular activities of clarithromycin against *M. paratuberculosis*. Clarithromycin, therefore, may prove to be as effective in treating *M. paratuberculosis* infections as it was recently shown to be in treating *M. avium*-infected AIDS patients (4).

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