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Activities of Roxithromycin against *Mycobacterium avium* Infections in Human Macrophages and C57BL/6 Mice

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The activity of roxithromycin against three clinical isolates of *Mycobacterium avium* was compared with that of clarithromycin both in a model of infection of human monocyte-derived macrophages and in a model of established infection of C57BL/6 mice. In the cell culture model, roxithromycin and clarithromycin were bactericidal for strains MO-1 and N-92159 and bacteriostatic for strain N-93043. For the three strains, the differences between the intracellular activities of roxithromycin and clarithromycin were not significant after 7 days of treatment. Mice were infected with the MO-1 strain. Drugs were given by gavage at a dosage of 200 mg/kg of body weight 6 days per week for 16 weeks starting 5 weeks after infection. At the end of treatment, clarithromycin was more effective than roxithromycin in lungs; roxithromycin was as effective as clarithromycin in spleens. Thus, the activity of roxithromycin was comparable to that of clarithromycin both in vitro and in vivo.

New macrolides are among the most active antibiotics against the *Mycobacterium avium* complex. Clarithromycin, in particular, has been proven to be effective in treating disseminated *M. avium* infection in AIDS patients (6, 10) and is now the reference macrolide for this indication. Among other macrolides, although roxithromycin has been shown to be active in vitro against *M. avium* (1, 2, 11, 14, 15), its activity has been neither compared with that of clarithromycin nor evaluated in vivo. The purpose of the present study was to evaluate the anti-*M. avium* activity of roxithromycin compared with that of clarithromycin both in vitro in a model of infection of human monocyte-derived macrophages and in vivo in a model of established infection in C57BL/6 mice.

MATERIALS AND METHODS

Strains of *M. avium*. Three clinical isolates of *M. avium*, MO-1, N-93043, and N-92159, obtained from the blood of AIDS patients were used for the in vitro studies. Strain MO-1 was used for the in vivo studies; this strain has been used in our previous studies (3, 4, 12, 13). Cell suspensions with predominantly (>95%) transparent colony morphologies cultivated in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) supplemented with ADC enrichment (Difco) were used as described previously (13). The organisms were passaged through C57BL/6 mice to maintain the virulence of the organisms.

Antimicrobial agents. Roxithromycin (Roussel-Uclaf, Romainville, France) and clarithromycin (Abbott France, Rungis, Val de Marne, France) were provided as standard powders by the manufacturers. In the cell model, the lactobionate salt of clarithromycin was used and was dissolved in distilled water; roxithromycin was dissolved in methanol and was subsequently diluted in distilled water. For the in vivo studies, suspensions in carboxymethyl cellulose were used for both antibiotics tested.

In vitro susceptibility. The MICs were determined by the agar macrodilution method by using Mueller-Hinton agar medium supplemented with 10% Middlebrook OADC enrichment (Difco) (17).

Human macrophage model. (i) Human monocyte-derived macrophages. Human monocyte-derived macrophages were obtained from healthy donors as described previously (13). Briefly, heparinized peripheral blood was diluted with RPMI 1640 medium and was centrifuged on Ficoll-Paque to obtain purified mononuclear cells. The cell suspension was distributed into Lab-Tek chambers (Nunc Inc., Naperville, Ill.) to obtain 5×10^5 monocytes per chamber. After sedimentation for 3 h at 37°C in 5% CO_2, the supernatants containing nonadherent cells were removed, and adherent cells corresponding to monocytes were maintained in culture in RPMI 1640 medium supplemented with 10% normal human serum at 37°C in 5% CO_2. On day 6, a homogeneous monolayer of macrophages was obtained.

(ii) Infection of the macrophage monolayers and drug treatment. Macrophages were inoculated on day 6 of culture with a suspension of *M. avium* (3×10^7 CFU per chamber) in RPMI 1640 medium supplemented with 10% human normal serum. After 60 min of incubation, extracellular bacteria were removed by washing with phosphate-buffered saline (PBS). In control chambers, intracellular bacteria were counted as described below. In other chambers, fresh RPMI 1640 medium containing 10% normal human serum with or without antibiotic was added, and macrophages were reincubated at 37°C in 5% CO_2. The concentration of antibiotic used was close to the peak concentration obtained in the serum of humans given the antibiotics orally. This concentration was 10 μg/ml for roxithromycin and 4 μg/ml for clarithromycin.

On day 4 after inoculation, the numbers of CFU in the supernatants were counted. In half of the chambers, the intracellular numbers of CFU were counted; in the other half, culturing was prolonged to day 7. In these latter chambers, supernatant was replaced on day 4 by the same fresh medium containing the antibiotic at the same concentration.

(iii) Quantitation of CFU. Extracellular bacteria were removed by washing with PBS. Intracellular bacteria were recovered by hypotonic lysis of the macrophages with sterile distilled water. Serial dilutions of the bacterial suspensions were plated onto 7H11 agar medium. The plates were incubated at 37°C, and the colonies were counted after 14 days of culturing.

(iv) Expression of CFU count results. At 60 min after inoculation, only intracellular CFU counts, corresponding to the intracellular inocula, were expressed. In this model, the multiplication of bacteria is mainly intracellular (5). The bacterial load of macrophages that detached from the chambers remained in the supernatant (supernatant bacteria) and was counted with the extracellular bacteria. Thus, the total multiplication of bacteria on day 4 was expressed by adding the intracellular and supernatant CFU counts on day 4. Since supernatant bacteria were removed from the chambers on day 4 when the medium was replaced, the total multiplication of bacteria on day 7 was expressed by adding the intracellular and supernatant CFU counts on day 7 to the supernatant CFU counts on day 4. Crowle et al. (5) showed that adding the supernatant counts to the intracellular counts was more relevant than counting only intracellular bacteria. Supernatant bacteria include true extracellular bacteria (released by lysed macrophages) and intracellular bacteria inside detached (but not lysed) macrophages. Because the supernatant is removed on day 4 in order to replace the culture medium, the number of supernatant bacteria obtained at day 7 is artificially reduced, overestimating the activity of the drug. It is impossible to know precisely what would be the supernatant counts obtained on day 7 had the supernatant not been removed on day 4. The addition of the supernatant counts

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obtained on day 4 to the total counts obtained on day 7 is an approximation that cannot overestimate but that could underestimate the activity of the drug. Because there is no ideal solution, we chose the conservative solution to the dilemma, as we did in our previous work (4, 13).

For each strain and each antibiotic, the results of CFU counts were expressed as the means of three experiments, each of which was performed in duplicate. A drug was considered bactericidal intracellularly if it reduced the CFU counts by at least 1 log unit compared with the CFU counts in the control at the time of drug addition (60 min postinoculation) (16).

C57BL/6 mice model. (i) Established infection of mice. Healthy 6-week-old female C57BL/6 mice from Iffa Credo, L'Arbresle, Rhône, France, were challenged intravenously with 1.5×10^7 CFU of *M. avium* MO-1. To better mimic the infection in humans, antimicrobial agents were tested against an established infection (3). Therefore, treatments were started only 5 weeks after inoculation and were given over a prolonged period of 16 weeks.

(ii) Administration of drugs. Mice were randomly assigned to an untreated control group or roxithromycin- or clarithromycin-treated groups. Drugs were given by gavage at a dosage of 200 mg/kg of body weight six days per week for 16 weeks. Control mice received saline.

(iii) Quantitation of mycobacteria in spleens and lungs. Treated and control mice were sacrificed at weeks 2, 6, 8, 12, and 16 of treatment. Control mice were also sacrificed on the first day of treatment. At least four animals were sacrificed at each time point. Spleens and lungs were aseptically removed, weighed, and ground in a tissue homogenizer. Serial dilutions were plated onto 7H11 agar, and the colonies were counted after 14 days of incubation at 37°C. The number of CFU per gram of tissue was calculated.

(iv) Resistance frequency. At each time point, homogenates of spleens were also plated onto 10% OADC-enriched Mueller-Hinton agar containing one of the drugs tested at a concentration of four times the MIC, i.e., 16 μg of roxithromycin per ml and 8 μg of clarithromycin per ml. Colonies were counted after 14 days at 37°C.

(v) Pharmakokinetics. The levels of antibiotics in the sera, spleens, and lungs of C57BL/6 mice were determined by microbiological assay, with *Sarcina lutea* ATCC 9341 used as the test strain. The sensitivity of the method allowed us to assay concentrations as low as 0.05 μg/ml for roxithromycin and 0.5 μg/ml for clarithromycin.

Statistical analysis. At each time point in both the cell and the animal models, results of CFU counts were compared by a one-way analysis of variance. If the F ratio was significant, pairwise comparisons between treatments were made by the Fisher protected least significant difference test. For each group of mice, the average rate of decrease in the number of CFU per week was also determined by measuring the slope of the line fitted to the data by using a linear regression model (9).

RESULTS

In vitro susceptibility. The MICs for strains MO-1, N-92159, and N-93043 were, respectively, 4, 2, and 16 μg/ml for roxithromycin and 2, 0.5, and 2 μg/ml for clarithromycin.

Human macrophage model. The comparative activities of roxithromycin and clarithromycin evaluated by CFU counts are given in Fig. 1. For strains MO-1 and N-92159, the CFU counts decreased significantly between 60 min and day 7 with roxithromycin ($P < 0.0001$ for strain MO-1 and $P < 0.01$ for strain N-92159) and clarithromycin ($P < 0.0001$ for strain MO-1 and $P < 0.003$ for strain N-92159). For strain N-93043, the CFU counts decreased significantly between 60 min and day 7 with clarithromycin ($P < 0.02$) but not with roxithromycin. Roxithromycin and clarithromycin were bacteriostatic only for strain N-93043 (less than 1 log unit of intracellular killing).

On days 4 and 7, compared with the untreated controls, roxithromycin ($P < 0.003$) and clarithromycin ($P < 0.002$) significantly slowed the intracellular replication of the three strains. For strain MO-1, clarithromycin was more effective than roxithromycin ($P < 0.03$) on day 4, but the difference was not significant on day 7 ($P > 0.05$). For strains N-93043 and N-92159, the difference between the intracellular activities of roxithromycin and clarithromycin was not significant ($P > 0.05$) on days 4 and 7.

C57BL/6 mouse model. (i) Weights of spleens. Splenomegaly was observed in untreated mice. The average spleen weight was 66.4 mg on the day of inoculation and 348 mg 5 weeks after the inoculation. After 16 weeks of treatment, the average spleen weight was 486 mg in untreated controls, 98 mg in clarithromycin-treated mice, and 114 mg in roxithromycin-treated mice.

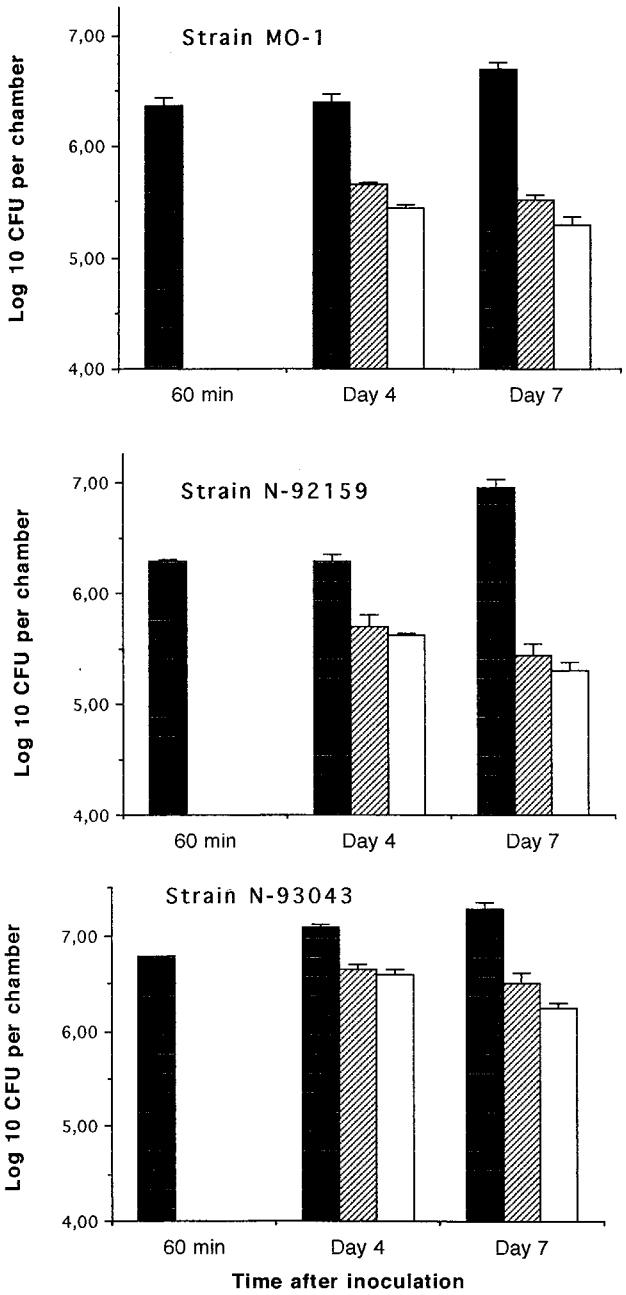

FIG. 1. Comparison of intracellular growth of three strains of *M. avium* in Lab-Tek chambers without antibiotics (control) and in the presence of roxithromycin (10 μg/ml) or clarithromycin (4 μg/ml). Results on day 4 represent intracellular plus supernatant CFU counts on day 4. Results on day 7 represent intracellular plus supernatant CFU counts on day 7 plus supernatant CFU counts on day 4, since bacteria were removed on day 4 when the medium was replaced. Results are the means ± standard errors of the means of three experiments (each performed in duplicate). ■, control; ▨, roxithromycin; □, clarithromycin.

(ii) Comparative activities of roxithromycin and clarithromycin. CFU counts in the lungs and spleens are shown in Fig. 2. Compared with the untreated controls, roxithromycin and clarithromycin significantly decreased the level of infection in the spleens and lungs at each time point ($P < 0.001$). Clarithromycin was more effective than roxithromycin on weeks 6,

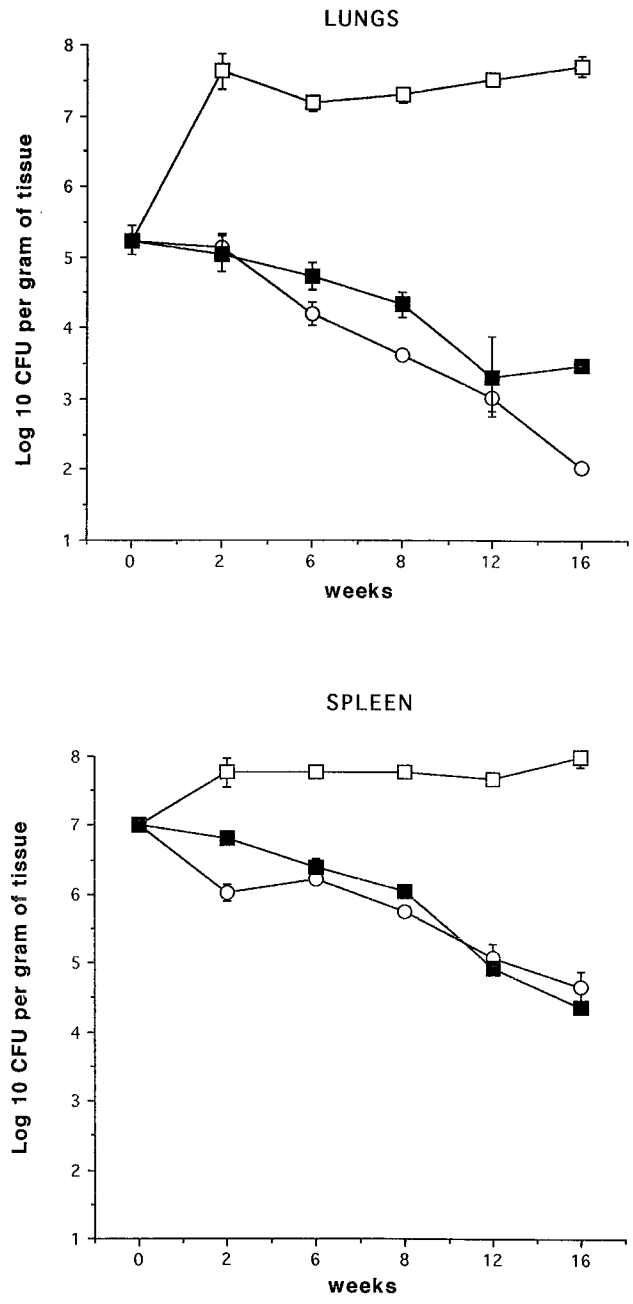

FIG. 2. Time course of CFU of *M. avium* in the lungs and spleens of C57BL/6 mice treated with roxithromycin or clarithromycin. Drugs were given by gavage at a dosage of 200 mg/kg six times weekly for 16 weeks. Control mice received saline. Treatments were started 5 weeks after intravenous challenge with the MO-1 strain of *M. avium*. Results are presented as means ± standard errors of the means. □, control; ○, clarithromycin; ■, roxithromycin.

8, and 16 in the lungs and weeks 2 and 8 in the spleens. There was no significant difference in activity between roxithromycin and clarithromycin on weeks 2 and 12 in the lungs and weeks 6, 12, and 16 in the spleens.

When considering the average rate of change in CFU counts per week, in the lungs the rate of decrease in the CFU counts in clarithromycin-treated mice (−0.19 CFU per week) was higher than that in roxithromycin-treated mice (−0.12 CFU per week; $P < 0.001$). In the spleens, the rate of decrease in the CFU counts in roxithromycin-treated mice (−0.15 CFU per week) was higher than that in clarithromycin-treated mice (−0.12 CFU per week; $P < 0.001$).

TABLE 1. Concentrations of roxithromycin and clarithromycin in the sera, lungs, and spleens of C57BL/6 mice

Compound	Sample	Dose[a]	Concn at the following sampling times[b]:	
			1 h	24 h
Roxithromycin	Serum	sd	11.03 ± 1.19	<0.05
		md	11.84 ± 2.81	<0.05
	Lung	sd	89.68 ± 14.99	<0.2
		md	93.74 ± 16.18	<0.2
	Spleen	sd	102.8 ± 24.06	0.49 ± 0.06
		md	122.73 ± 38.8	<0.4
Clarithromycin	Serum	sd	4.46 ± 0.60	0.87 ± 0.09
		md	3.66 ± 0.59	0.5 ± 0.04
	Lung	sd	28.63 ± 1.03	6.93 ± 1.32
		md	30.32 ± 2.58	5.05 ± 2.75
	Spleen	sd	200.9 ± 41.66	18.23 ± 8.08
		md	193 ± 20.38	13.07 ± 11.49

[a] sd, single oral dose of 200 mg/kg; md, multiple oral doses of 200 mg/kg/day for 7 days.
[b] Concentrations in sera are in micrograms per milliliter; concentrations in lungs and spleens are in micrograms per gram. Values are the means ± standard errors of the means for five mice.

(ii) Resistance frequency. No *M. avium* colonies were selected on plates containing four times the MICs of the drugs during the 16 weeks of treatment in either untreated or treated mice.

(iii) Pharmacokinetics. The concentrations of roxithromycin and clarithromycin in the sera, spleens, and lungs after administration of a single oral dose of 200 mg/kg and after 7 days of continuous therapy are given in Table 1. The levels of roxithromycin and clarithromycin were close to those found in humans given standard oral doses.

DISCUSSION

The purpose of the present study was to compare the activities of two macrolides, roxithromycin and clarithromycin, against *M. avium* infection in human monocyte-derived macrophages and C57BL/6 mice. The MICs of roxithromycin were higher than those of clarithromycin for the three *M. avium* isolates tested. However, the activity of roxithromycin against *M. avium* was comparable to that of clarithromycin both in vitro against infections in human monocyte-derived macrophages and in vivo against established infections in C57BL/6 mice. The difference in efficacy was statistically significant in favor of clarithromycin at some time points, but further evaluation would be necessary to determine if this difference is clinically relevant in the treatment of disseminated *M. avium* infection in humans. In a pilot study evaluating the efficacy of roxithromycin in the primary prevention of pneumocystosis and cerebral toxoplasmosis in 52 human immunodeficiency virus-infected patients, none of the patients in the roxithromycin group developed mycobacterial infections (7). Although the number of patients tested was limited, these results are encouraging for the continuation of clinical evaluation of the

prophylactic or therapeutic potential of roxithromycin against *M. avium* infections.

The bacterial loads in the spleens and lungs of roxithromycin- or clarithromycin-treated mice continued to decrease over the 16 weeks of treatment without the selection of resistant mutants. Ji et al. (8) estimated the frequency of clarithromycin-resistant mutants to be between 10^{-8} and 10^{-9} in untreated beige mice. The lack of selection of resistant mutants in our model was likely due to the fact that the bacterial load remained less than 10^8 CFU per gram of tissue.

Finally, in our animal model, neither roxithromycin nor clarithromycin was able to completely eliminate the organisms from the lungs and spleens, despite continuous treatment for 16 weeks. These results emphasize that there is a clear need for an additional bactericidal agent against *M. avium*. However, for the near future, roxithromycin deserves further evaluation to determine whether it could be an alternative regimen to clarithromycin in treating *M. avium* infection in humans.

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