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MICs and MBCs of Clarithromycin against *Mycobacterium avium* within Human Macrophages

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The inhibitory and bactericidal activities of clarithromycin were determined quantitatively against the intracellular populations of five *Mycobacterium avium* strains growing in monocyte-derived human macrophages. The MICs were 1.0 μg/ml, and the MBCs ranged from 16.0 to 64.0 μg/ml; these values were similar to the MICs and MBCs found in broth cultures at pH 7.4 and were substantially lower than those found in broth cultures at pHs 6.8 and 5.0. Since the intracellular environment has a neutral or even an acidic pH, relatively low MICs and MBCs found in macrophage cultures can be associated with the fact that the drug concentrations in macrophages are substantially higher than those in the medium in which these cells are cultivated. Pretreatment of the macrophages 2 days prior to infection decreased the MICs twofold in comparison with results of experiments in which the drug was added to already infected macrophages.

The efficacy of clarithromycin in the elimination of *Mycobacterium avium* bacteremia in AIDS patients has been shown in controlled clinical trials (6, 7); this activity corresponds to the activity of this macrolide against *M. avium* in various models, including experiments in culture media (6, 8, 10, 16, 21), in various types of macrophages (17–19, 23), and in mice (8, 13).

Previously (11), we quantitated the bacteriostatic and bactericidal effects of clarithromycin against *M. avium* as MICs and MBCs determined in different types of medium and at various pHs. We reported that agar-determined MICs were two- to eightfold higher than those determined in broth under the same pH conditions and suggested that broth-determined MICs are a better reflection of the concentrations that actually interact with bacteria because of the lower level of absorption and the shorter period of incubation in broth cultures than on agar plates. It was also clear from this and other reports (18, 21) that the inhibitory activity depended on the pH of the medium, being higher at pH 7.4 and lower at pH 5.0. According to our published (11) and current observations, only MICs determined at pH 7.4 in a liquid medium were always 2.0 μg/ml or lower for all tested *M. avium* strains, a concentration below the peak concentration of this drug achievable in blood (1, 8, 12). Therefore, we suggested (11) that the efficacy of clarithromycin in eliminating bacteria from the blood of patients with AIDS is likely associated with its inhibitory activity at pH 7.4.

The activity of clarithromycin against the portion of a patient's bacterial population residing within macrophages may be influenced by two factors that can increase or decrease the effect of the drug. On the one hand, there are indications that clarithromycin can reach concentrations in the cell that are much higher than those in plasma (12). On the other hand, the effect of the drug can be diminished if the interaction takes place in the cytoplasm or within phagosomes at pH 6.8 to 7.0, and the effect of pH may be even more marked if the interaction takes place within phagolysosomes at pH 5.0. The integrated outcome of the possible combined effects of these two factors can be evaluated by testing the effects of various drug concentrations on extra- and intracellular bacterial populations. Previous studies of the activity of clarithromycin against intracellular *M. avium* (17, 18, 23) were done with only one concentration of the drug, imitating the peak concentration achievable in blood, usually 4.0 μg/ml. The lowest concentrations in the extracellular environment necessary for complete inhibition of growth (MIC) or 100- to 1,000-fold killing (MBC) of the bacteria multiplying in human macrophages were not determined in these studies. The MICs for two strains tested in one of the above-cited studies (18) were 4.0 and 8.0 μg/ml when determined on 7H11 agar at pH 6.8, and they were 0.5 and 2.0 μg/ml when determined on Mueller-Hinton agar at pH 7.4. We can assume from our previous in vitro studies that the MICs for these two strains would be within a range of 0.12 to 0.5 μg/ml if tested in pH 7.4 broth, or 1/8 and 1/16 of the concentrations used for macrophages (18).

The aims of the present study were (i) to determine the MIC of clarithromycin against *M. avium* multiplying in monocyte-derived human macrophages, (ii) to determine the MBC and MBC/MIC ratio under the same conditions, and (iii) to compare these data with broth-determined MICs and MBCs.

MATERIALS AND METHODS

Antimicrobial agent. Clarithromycin was obtained from Abbott Laboratories (Abbott Park, Ill.). To make a stock solution, we dissolved 50 mg of the drug in 5 ml of methanol and then diluted the solution to a volume of 25 ml with phosphate buffer (pH 6.5). This stock solution, 2 mg/ml, was diluted further with phosphate buffer to make appropriate working solutions, which were kept refrigerated for not more than 2 weeks.

Test strains. Five test strains identified by the Gen-Probe (San Diego, Calif.) technique as *M. avium* were used as target strains for these studies; they had been used in the previous study to determine the in vitro activity of clarithromycin (11). These strains, isolated from blood samples obtained from patients with AIDS, were preserved in frozen

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7H9 broth culture aliquots at −70°C and represented smooth transparent colonies. For each experiment, a subculture in 7H9 broth was made from a frozen aliquot. After 2 to 5 days of incubation at 37°C on a constantly rotating roller drum, the bacterial suspension was forced through a 27-gauge needle and then centrifuged at 250 × *g* for 5 min to remove large clumps of bacteria. A smear examination of the supernatant showed that the bacterial suspension consisted mostly of single cells, and only a few small clumps could be found.

Macrophage cultures. Peripheral blood from healthy purified protein derivative-negative donors was collected in a 60-ml syringe that had been pretreated with approximately 0.06 ml of a preservative-free solution containing 10,000 IU of heparin per ml (GIBCO Laboratories, Grand Island, N.Y.). This pretreatment resulted in 10 IU/ml of blood. We used the techniques of Crowle et al. (2, 4), with slight modifications, for the separation and cultivation of the monocytes into a macrophage monolayer. The 60-ml blood sample was placed in a tube containing a Ficoll-Hypaque gradient (Sigma Diagnostics, St. Louis, Mo.) and centrifuged at 800 × *g* for 15 min at room temperature, in accordance with the manufacturer's instructions. The mononuclear cell band was collected, transferred to a 50-ml Falcon tube, and diluted with RPMI 1640 (GIBCO), containing 10 IU of heparin per ml, to a total volume of 40.0 ml. The suspension was centrifuged at 250 × *g* for 10 min at room temperature. The cells were washed once in 10 ml of RPMI 1640 containing heparin, and the pellet was resuspended in RPMI 1640 and adjusted to 10^7 cells per ml. This suspension was placed in 35-mm plastic petri plates (Becton Dickinson Labware, Lincoln Park, N.J.) in two spots, 1 drop (approximately 0.05 ml) for each, resulting in monolayers containing about 5×10^5 cells. The plates were incubated at 37°C for 1 h to allow the cells to adhere and were then washed twice with RPMI 1640. Then, 1.5 ml of RPMI 1640 containing 3% human autologous nonheated fresh serum was added to each plate for a 7-day period of incubation at 37°C in the presence of 7% CO_2. The pH of the medium was 7.2 to 7.3.

***M. avium* infection in macrophages.** The above-described bacterial suspension was centrifuged at 3,500 × *g* for 30 min in a refrigerated centrifuge, and the pellet was resuspended in 2.0 ml of RPMI 1640. To estimate the numbers of acid-fast bacilli per milliliter of this suspension, we placed 0.01-ml samples of the suspension on Reich counting slides (Bellco Biotechnology, Vineland, N.J.) with a known number of fields (under ×1,000 magnification) per circle. The slides were fixed and stained by the Kinyoun method (22). The numbers of acid-fast bacilli per milliliter estimated from the counts on these slides proved to be accurate, as shown previously (15, 20). On the basis of these counts, the bacterial suspension in RPMI 1640 was adjusted to contain 10^6 acid-fast bacilli per ml.

After 7 days of incubation, the monocytes were considered to have matured into a macrophage monolayer (2, 4, 5). The medium was removed from the plates and replaced with the bacterial suspension at 1.5 ml per plate. After incubation for 1 hour, the plates were washed three times to remove the extracellular bacteria. The infected macrophages were incubated in RPMI 1640 supplemented with 1% human autologous serum, and various concentrations of clarithromycin were added to some cultures, while others remained drug-free controls. It was previously shown (5) that in the described system, mycobacteria do not multiply in the extracellular medium. We confirmed this fact in the preliminary experiments in this study; the bacterial counts used in the experiments described below represented only intracellular bacteria. At days 0, 4, and 7, the medium from alternate plates was discarded, and the monolayers were lysed by exposure for 10 min to a 0.25% solution of sodium dodecyl sulfate at 1.0 ml per plate. After the suspension was transferred to a tube, the plate was rinsed with 1.0 ml of 7H9 broth containing 20% bovine albumin, and the rinse was then added to the same tube. Tenfold serial dilutions were made to inoculate 7H11 agar plates for subsequent colony counts. The results were expressed as the number of CFU per monolayer.

TABLE 1. Multiplication of five *M. avium* strains in monocyte-derived macrophages

Strain	No. of expt	No. of CFU/ml ± SD at day: 0	4	7
3010	3	$(1.4 \pm 1.9) \times 10^5$	$(6.5 \pm 1.3) \times 10^6$	$(1.2 \pm 1.0) \times 10^7$
453	5	$(2.2 \pm 1.5) \times 10^5$	$(3.0 \pm 0.7) \times 10^6$	$(2.7 \pm 0.9) \times 10^7$
3354	3	$(6.0 \pm 3.0) \times 10^4$	$(9.0 \pm 2.0) \times 10^5$	$(8.0 \pm 1.7) \times 10^6$
1435	2	$(2.5 \pm 0.7) \times 10^4$	$(5.9 \pm 2.8) \times 10^5$	$(3.5 \pm 0.7) \times 10^6$
3011	2	$(4.0 \pm 0.1) \times 10^4$	$(1.3 \pm 1.0) \times 10^6$	$(8.0 \pm 3.0) \times 10^6$

MIC determination. The MIC was defined as the lowest concentration that inhibited more than 99% of the bacterial population over 7 days of cultivation. Practically, it was the lowest concentration in the presence of which no increase in the viable counts occurred, but some decrease in the number of CFU per milliliter may have taken place, usually not more than 10%. The following concentrations of clarithromycin were used to determine the MIC: 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/ml. For determination of the MIC, the drug was added to the monolayers 1 h after infection, at the time of the removal of the extracellular, nonphagocytosed bacteria. The MIC was determined in two or three experiments with each of five *M. avium* strains.

In each experiment, the mycobacterial viable counts per monolayer were determined by colony counting on 7H11 agar plates inoculated with the serial dilutions of the macrophage lysate. Each measurement of the numbers of viable bacteria represented the average of counts obtained in triplicate. In addition to these basic experiments, we also tested the effect of clarithromycin as a prophylactic agent by adding the drug to the macrophages 2 days before infection. These experiments were done with three strains.

MBC determination. The MBC was defined as the lowest concentration that decreased the mycobacterial viable counts in macrophages more than 100-fold over 7 days of

TABLE 2. MICs and MBCs of clarithromycin (μg/ml)[a]

Strain	MIC in: 7H12 broth at pH: 5.0	6.8	7.4	Macrophages: When drug was added after infection	When drug was added 2 days before infection	MBC in macrophages
3010	8.0	2.0	0.5	1.0, 1.0, 1.0		32.0, 32.0
453	8.0	2.0	0.5	0.5, 1.0, 1.0	0.5	16.0, 16.0
3354	32.0	8.0	1.0	1.0, 1.0, 1.0		32.0, 32.0
3011	16.0	1.0	0.25	1.0, 1.0	0.5, 0.5	64.0
1435	32.0	8.0	0.5	1.0, 1.0	1.0, 0.5	32.0

[a] Each value represents one experiment done in triplicate. Multiple values represent multiple experiments.

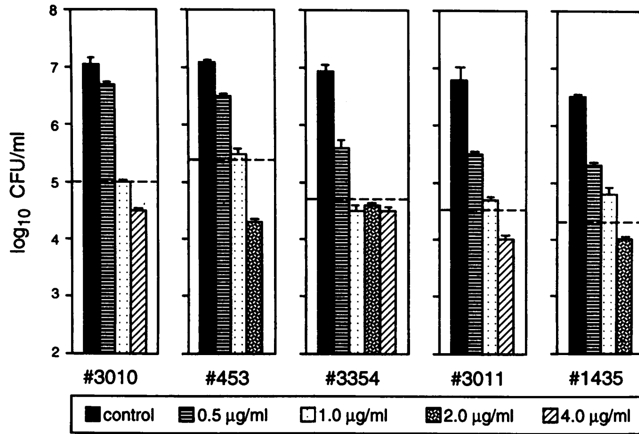

FIG. 1. CFU per milliliter in experiments with five *M. avium* strains at day 0 (dotted lines) and after 7 days of incubation in drug-free controls and in the presence of various concentrations of clarithromycin: 0.5, 1.0, 2.0, and 4.0 µg/ml. Each measurement represents an average from two or three experiments.

incubation. The MBC was determined simultaneously with the MIC in eight experiments (see Table 2). The following drug concentrations were used: 16.0, 32.0, and 64.0 µg/ml. Each measurement of the viable counts was taken in triplicate. The variations between the monolayers were minimal, with a standard error not exceeding 5% of the mean.

RESULTS

Multiplication of *M. avium* within human macrophages. In a total of 15 experiments with five strains, the initial numbers of intracellular viable bacteria ranged from 3×10^4 to 6×10^5 per monolayer, and a two-log increase took place within the 7-day period of incubation (Table 1).

MIC of clarithromycin. All five strains selected for this study were isolated from patients who had AIDS and who had never been treated with clarithromycin or any other macrolide. The broth-determined MICs for these strains ranged from 0.25 to 1.0 µg/ml at pH 7.4, 1.0 to 8.0 µg/ml at pH 6.8, and 8.0 to 32.0 µg/ml at pH 5.0 (Table 2).

The changes in the viable counts of *M. avium* in macrophages exposed to different concentrations of clarithromycin during the 7-day period of incubation are shown in Fig. 1. On the basis of these data, the MICs of clarithromycin, determined in experiments repeated two or three times with each of five strains, were 1.0 µg/ml in 12 of 13 experiments and 0.5 µg/ml in the other experiment (Table 2). In five additional experiments, the drug was added to the macrophages two days before infection, and the MICs were determined during the 7-day period after the infection. In four of these experiments, the MIC was 0.5 µg/ml, and in the other, it was 1.0 µg/ml (Table 2).

MBC of clarithromycin. The lowest concentrations that decreased the intracellular bacterial population by 100-fold ranged from 16.0 to 64.0 µg/ml (Table 2), values comparable to the MBCs that we determined by using 7H12 broth at pH 7.4 (11). The changes in the numbers of viable intracellular bacteria in drug-free macrophage cultures and in the presence of the MIC and MBC are shown for three of the tested strains in Fig. 2.

DISCUSSION

The MICs and MBCs of clarithromycin for five *M. avium* strains growing in monocyte-derived human macrophages were within the same ranges as the MICs and MBCs determined in 7H12 broth at pH 7.4 but were substantially lower than those determined in broth at pHs 6.8 and 5.0. The environment of the bacteria in macrophages depends on their specific location: the pH is 6.8 to 7.0 in the cytoplasm or within phagosomes and acidic (5.0) within phagolysosomes. Despite the recent suggestion (3) that the environment in which mycobacteria, particularly *M. tuberculosis*, reside in macrophages is neutral rather than acidic, there are no clear data on whether lysosome-phagosome fusion takes place in macrophages infected not with *M. tuberculosis* but

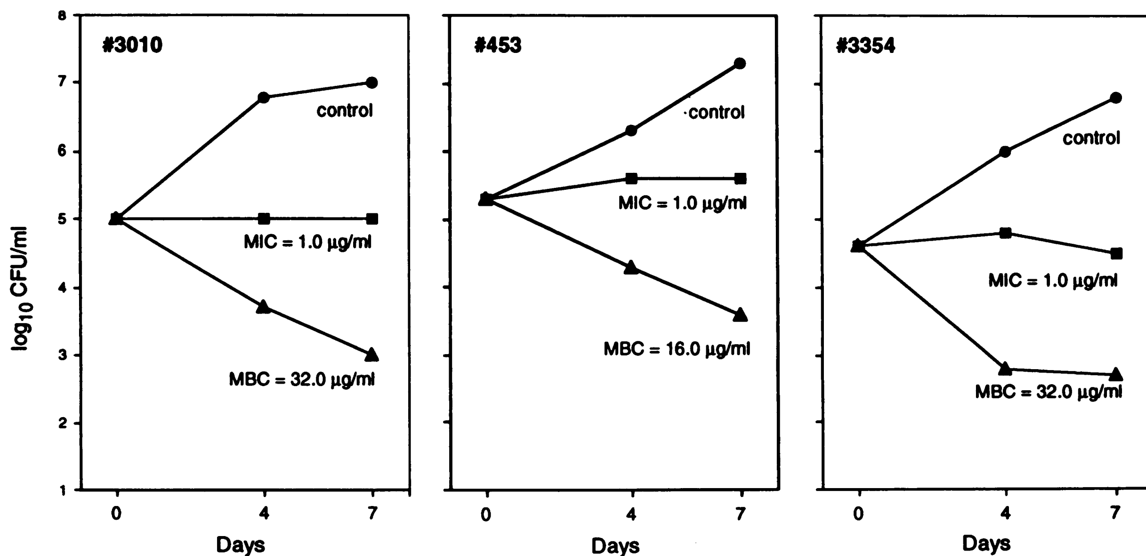

FIG. 2. Numbers of viable bacteria found during 7 days of cultivation in macrophages in drug-free cultures (control) and in the presence of clarithromycin concentrations equivalent to the MIC and the MBC.

with *M. avium*. If such fusion does take place, then at least part of the intracellular bacterial population resides at pH 5.0, the most favorable environment for *M. avium* multiplication (9, 14). Nevertheless, it is obvious that the bacteria do not grow in macrophages at pH 7.4 but rather grow at pH 6.8 to 7.0 within phagosomes or at pH 5.0 within phagolysosomes. The fact that the MICs and MBCs against the intracellular mycobacteria were much lower than those found in broth cultures at pHs 6.8 and 5.0 is an indirect indication of an accumulation of clarithromycin in macrophages at concentrations exceeding those in the extracellular medium. This observation corresponds to the finding that clarithromycin accumulates at concentrations 16.4-fold higher than those in the extracellular fluid (12).

It is not known in what compartments of the intracellular environment the interaction between the bacterial population and clarithromycin takes place, but it is reasonable to assume that the MIC of clarithromycin, the lowest drug concentration in the extracellular medium that produces inhibition of growth (>99%) in macrophages, is probably the result of the balanced effect of two opposing factors: the intracellular accumulation of the drug and the diminution of the effect of the drug by the acidic environment of the phagolysosomes (pH 5.0), in which a substantial portion of the bacterial population may reside. The actual intracellular interactions may be even more complicated, since some portions of the bacterial population may reside in different compartments of the macrophages. Regardless of the actual events in the macrophages, it is clear that the intracellular multiplication of the five tested *M. avium* strains was completely inhibited in the presence of 1.0 μg of clarithromycin per ml in the extracellular fluid, a concentration substantially lower than the peak concentration attainable in blood, usually 4.0 μg/ml (1, 8, 12). Therefore, it is fair to assume that the effect of clarithromycin in patients with disseminated *M. avium* infections is associated with the portion of the bacterial population found not only in blood but also in macrophages.

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