ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2005, p. 2387–2398 Vol. 49, No. 6
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.6.2387–2398.2005

Antimycobacterial Agents Differ with Respect to Their Bacteriostatic versus Bactericidal Activities in Relation to Time of Exposure, Mycobacterial Growth Phase, and Their Use in Combination

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Received 29 June 2004/Returned for modification 4 September 2004/Accepted 3 February 2005

A number of antimycobacterial agents were evaluated with respect to their bacteriostatic activity (growth inhibition) versus the bactericidal activity against a clinical isolate of *Mycobacterium avium* (*Mycobacterium avium* complex [MAC] strain 101) in relation to the time of exposure and the growth phase of the mycobacteria. In terms of growth inhibition the MAC in the active phase of growth was susceptible to clarithromycin, ethambutol, rifampin, amikacin, and the quinolones moxifloxacin, ciprofloxacin, and sparfloxacin. In terms of bactericidal activity in relation to the time of exposure these agents differed substantially with respect to the killing rate. An initial high killing capacity at low concentration was observed for amikacin, which in this respect was superior to the other agents. The bactericidal activity of clarithromycin and ethambutol was only seen at relatively high concentrations and increased with time. Killing by rifampin was concentration dependent as well as time dependent. The bactericidal activity of moxifloxacin was marginally dependent on the concentration or the time of exposure. The activity of clarithromycin in combination with ethambutol was not significantly enhanced compared to single-agent exposure. Only an additive effect was observed. The addition of rifampin or moxifloxacin as a third agent only marginally effected increased killing of MAC. However, by addition of amikacin the activity of the clarithromycin-ethambutol combination was significantly improved. The combination of amikacin and amoxicillin-clavulanic acid exhibited synergistic antimycobacterial activity. Towards MAC at low growth rates, only the quinolones exhibited a bactericidal effect.

Mycobacterial infections (tuberculosis and atypical mycobacterial infections) continue to cause immense morbidity and mortality, particularly in developing countries, and there is clear evidence that the incidence of (multidrug-resistant) mycobacterial infections is increasing (10, 16, 42, 43). The treatment of mycobacterial infections is complicated (12, 39). The intracellular location and the quiescent character of the persistent mycobacteria result in decreased efficacy of antimycobacterial agents (18). Therefore treatment must be continued for months to years. It is generally accepted that long-term exposure of mycobacteria to suboptimal concentrations of antimycobacterial agents facilitates the selection of resistant mutants. Combinations of antimycobacterial agents are used to minimize the emergence of antimicrobial resistance.

Whereas for tuberculosis susceptibility testing is extremely important in guiding treatment (51), for *Mycobacterium avium* complex (MAC) infections a predictable relationship between in vitro susceptibility testing and clinical utility of agents for therapy is marginal (46). The range of susceptibilities of MAC isolates to most antimycobacterial agents, except for macrolides, is very broad (25, 26, 29). There are no controlled clinical trials demonstrating a correlation between in vitro susceptibility tests for MAC and clinical response. A number of studies reviewed by Shafran et al. (46) examining the role of in vitro susceptibilities in predicting the clinical outcome of drug treatment of MAC infection are available but show contradictory results.

Several techniques are applied to determine the antimicrobial susceptibility of mycobacteria in vitro (2, 24, 26, 29). Preferably quantitative assays such as the agar dilution method are used. Heifets suggested giving preference to methods of broth-determined MICs (22). The radiometric BACTEC macrodilution method (radiometric detection of growth) produces a quantitative endpoint in terms of an MIC which can be correlated with the drug concentration attainable in humans (26, 3). For MAC National Committee of Clinical Laboratory Standards (35) are only available for in vitro testing of susceptibility to macrolides because of the correlation between this test and clinical response. Heifets and Iseman recommend susceptibility testing of the patient's isolate, which can serve for better selection of drugs for an individualized treatment regimen (27).

The interpretation of the data of antimicrobial susceptibility of mycobacterial strains in vitro is difficult. Mostly the results of

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the susceptibility tests are red at the end of the incubation period. This in vitro approach does not reflect the in vivo events, as exposure of mycobacteria to maximum achievable concentrations in vivo last for only a short period. In addition, often inhibition of mycobacterial growth is assessed. As stated by the National Committee for Clinical Laboratory Standards (NCCLS), in antimicrobial susceptibility methods a time factor should be included. The rate of killing may have more clinical significance than the degree of killing. However, the antimicrobial killing rate in relation to the time of exposure is also an important characteristic for therapeutic efficacy in the patients with mycobacterial infections. The ideal antimycobacterial agent should exhibit a high killing capacity resulting in a rapid decrease in bacterial load, and hence a reduced risk of development of resistance. Also substantial activity of the agents against mycobacteria at low growth rate is an important determinant. At the site of infection mycobacteria are often localized intracellularly, and their metabolic activity intracellularly is extremely low.

The present study was undertaken to assess the bacteriostatic activity (growth inhibition) versus the bactericidal activity of a number of antimycobacterial agents at various concentrations against a clinical isolate of MAC in relation to the time of exposure. MAC strain 101 serotype 1, one of the three most common serotypes isolated from patients with AIDS (54) was used. In addition, the antimicrobial activity in relation to the bacterial growth rate was investigated. To that aim mycobacteria in the active phase of growth (early logarithmic phase) were exposed to antimycobacterial agents, as well as mycobacteria at the end of the active growth phase (late logarithmic phase) to simulate low metabolic activity of mycobacteria. Agents from various classes of antimicrobials available for the treatment of MAC infections including the macrolide clarithromycin (CLR) as a first-line drug (11, 45) are involved in the study. As macrolide-containing multidrug regimens are often used in the treatment of MAC infection (13, 45, 9, 28, 15, 48, 3, 6), also combinations of CLR with various agents were evaluated. In this regard the potential of fluoroquinolones or aminoglycosides was investigated. In addition, the activity of an aminoglycoside-beta-lactam combination was examined.

MATERIALS AND METHODS

Organism. The *Mycobacterium avium* strain used in this study was MAC strain 101 (serovar 1), originally isolated from the blood of an AIDS patient with disseminated MAC infection, and was kindly supplied by L. S. Young, Kuzell Institute for Arthritis and Infectious Diseases, San Francisco, CA. MAC organisms were cultured on Middlebrook 7H10 agar medium (Difco Laboratories, Detroit, MI) supplemented with oleic acid-albumin-dextrose-catalase enrichment (OADC; Baltimore Biological Laboratories, Baltimore, MD) for 14 days at 37°C. MAC suspensions were prepared in Middlebrook 7H9 broth (Difco) supplemented with OADC and stored at −80°C.

Antimycobacterial agents. Clarithromycin (CLR) was obtained from Abbott (Saint Remy, France); ethambutol (EMB), amikacin (AMK), streptomycin (STR), isoniazid (INH) and pyrazinamide (PZA) were purchased from Sigma Chemical Co. (St. Louis, MO); ciprofloxacin (CIP) and moxifloxacin (MXF) were obtained from Bayer (Leverkusen, Germany); tobramycin (TOB) and gentamicin (GEN) was purchased from Centrafarm (Etten-Leur, The Netherlands); sparfloxacin (SPX) was obtained from Rhone Poulenc Rorer (Vitry sur Seine Cedex, France); rifampin (RIF) was obtained from Hoechst Marion Roussel (Hoevelaken, The Netherlands); and amoxicillin-clavulanic acid (AMC) was obtained from GlaxoSmithKline B.V. (Zeist, The Netherlands). Dilutions of antimicrobial agents were prepared according to the recommendations of the manufacturers.

Susceptibility testing. Drug susceptibility of the MAC strain in terms of MIC was performed using a broth-based method (macrodilution) according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) M24-T2 using 7H9 broth supplemented with OADC, pH 6.8 (35). Antibiotic concentrations ranged from 0.1 to 64 mg/liter (doubling dilutions) for all agents, and 128 and 256 mg/liter for CLR only. The inoculum was prepared from a fresh broth culture and was finally 1.5×10^5 CFU/ml as confirmed by quantitative plate counts. Tubes containing 4 ml were incubated at 37°C and examined at 14 days. The MIC was determined as the lowest concentration that inhibited visible growth.

Time-kill studies. The MAC inocula in the early logarithmic phase of growth or late logarithmic phase of growth were prepared in 7H9 broth supplemented with OADC under shaking conditions at 93 rpm. Early-log-phase MAC inocula at 5×10^5 CFU/ml and late-log-phase MAC inocula of 1.1×10^9 CFU/ml were exposed to the antimycobacterial agent at twofold increasing concentrations (range, 0.05 to 64 mg/liter) for 21 days at 37°C. In 60-ml flasks 20 ml of bacterial suspensions were incubated under shaking conditions at 93 rpm. The initially clear suspensions of early-log-phase MAC inocula allowed establishment of a bacteriostatic as well as a bactericidal effect of the antimicrobial agents. A bacteriostatic effect was expressed as the lowest concentration that inhibited visible growth at day 21. When a bactericidal effect was observed the killing capacity of the agent was expressed as the lowest concentration that resulted in ≥99% killing at day 3, 10, or 21. According to the NCCLS, there are no guidelines available to determine MBC for mycobacteria. Although the interpretive criteria of bactericidal activity of antimycobacterial agents are not uniform, in the majority of studies the MBC designated for mycobacteria is defined as the minimal concentration effectively reducing the bacterial counts by 99%.

For the late-log-phase MAC inocula which are not clear suspensions only a bactericidal effect of the agents could be determined. To determine the bactericidal effect after 1, 2, 3, 10, and 21 days of exposure quantitative cultures were performed as follows. The bacterial suspensions were centrifuged at 2400 × *g* for 10 min, washed in 7H9 broth supplemented with OADC and centrifuged again. Samples were serially diluted and plated onto 7H10 agar supplemented with OADC. After 14 days of incubation at 37°C CFU numbers were counted. In the studies where MAC was exposed to combinations of agents quantitative cultures were performed after 3 and 10 days of incubation.

RESULTS

Susceptibility according to NCCLS. The broth-determined MIC of CLR for the MAC strain 101 at pH 6.8, defined as the lowest concentration that inhibited visible growth, was 4 mg/liter. Interpretation of this MIC according to the interpretive criteria (breakpoints) mentioned in the NCCLS M24-T2 document and based, in part, on a monotherapy trial of disseminated disease in humans is that MAC strain 101 is susceptible to CLR.

The MIC for EMB and RIF was 16 mg/liter and 4 mg/liter, respectively. For the aminoglycosides AMK and STR the MIC was 1 mg/liter and 16 mg/liter, respectively. For the quinolones MXF, CIP, and SPX the MIC was 0.1 mg/liter, 0.25 mg/liter, and 0.25 mg/liter, respectively.

Time-kill studies in relation to the mycobacterial growth phase. In the absence of antimicrobial agent inocula of MAC in the early log phase of growth increased from 5.0×10^5 CFU/ml (day 0) to 6.0×10^7 CFU/ml, 2.0×10^9 CFU/ml, and 1.9×10^9 CFU/ml at day 3, day 10, and day 21, respectively. MAC in the late log phase of growth increased from 1.1×10^9 CFU/ml (day 0) to 2.0×10^9 CFU/ml, 2.2×10^9 CFU/ml, and 2.1×10^9 CFU/ml at day 3, day 10, and day 21, respectively. For five agents representative of the various classes of antimicrobials relevant in the treatment of MAC being CLR, EMB, RIF, AMK, and MXF, the data obtained at all concentrations tested are shown in Fig. 1 to 5. Calculation of the data for these agents and all other agents studied are summarized in Table 1. To clearly gauge the concentration-dependent versus time-

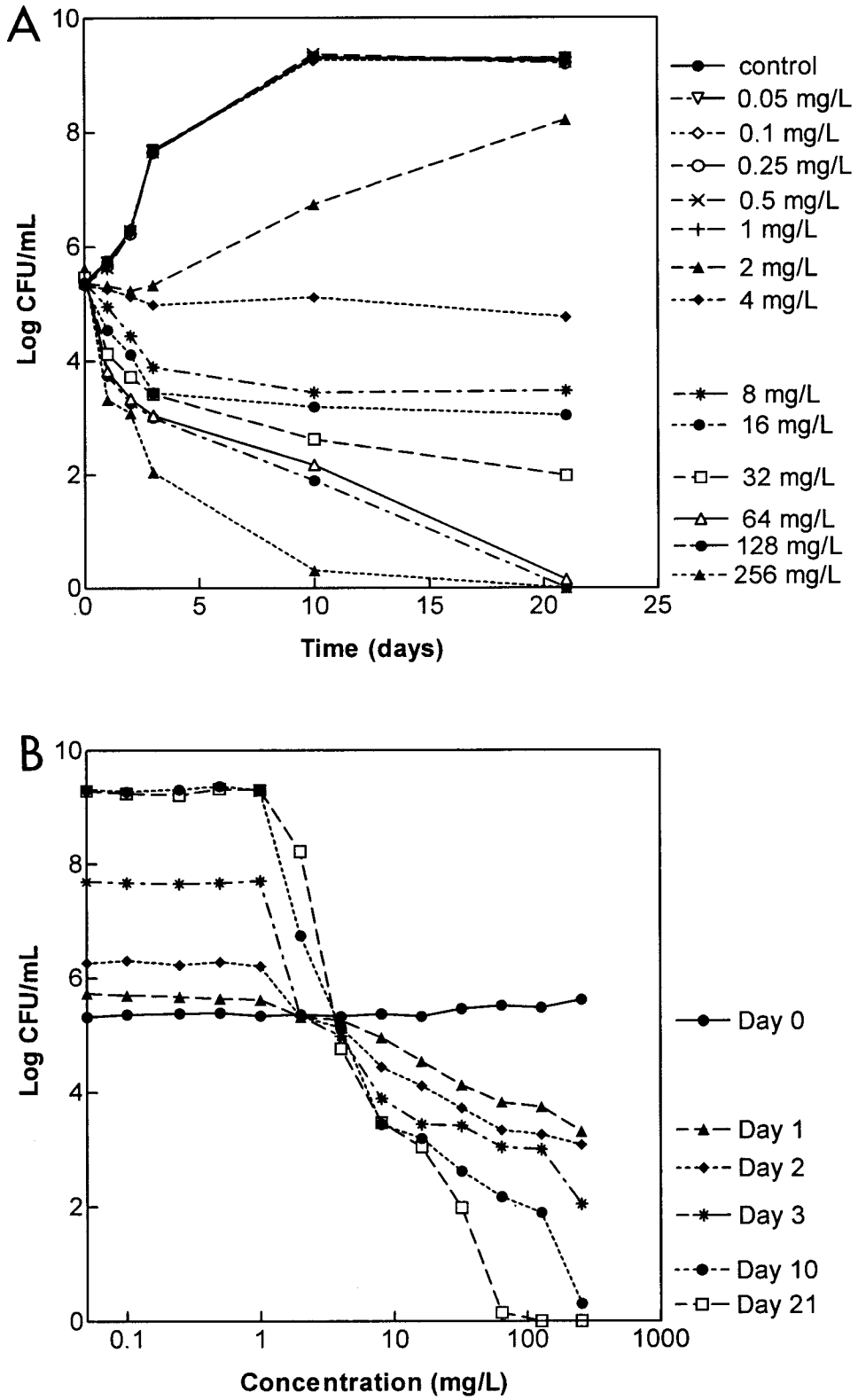

FIG. 1. *Mycobacterium avium* strain 101 in the early logarithmic phase of growth was exposed to clarithromycin at twofold increasing concentrations for 21 days at 37°C. After 1, 2, 3, 10 or 21 days of exposure quantitative cultures were performed.

TABLE 1. Effect of antimycobacterial agents on *Mycobacterium avium* complex in the early logarithmic phase of growth[a]

Antimycobacterial agent	Lowest concn (mg/liter) resulting in ≥99% killing at day:			Lowest concn (mg/liter) resulting in inhibition of visible growth at day 21
	3	10	21	
Clarithromycin	32	16	16	4
Ethambutol	>64	64	32	16
Rifampin	>64	4	8	4
Amikacin	2	2	2	1
Streptomycin	16	16	16	16
Tobramycin	16	>64	>64	>64
Gentamicin	16	>64	>64	>64
Moxifloxacin	8	1	0.5	0.1
Ciprofloxacin	64	32	2	0.25
Sparfloxacin	64	2	2	0.25
Isoniazid	>64	64	64	64
Pyrazinamide	>64	>64	>64	>64
Amoxicillin-clavulanic acid	>64	64	>64	>64

[a] *Mycobacterium avium* strain 101 at 5×10^5 CFU/ml was exposed to the antimycobacterial agent at twofold increasing concentrations (range, 0.05 to 64 mg/liter) for 21 days at 37°C. After 3, 10, or 21 days of exposure, quantitative cultures were performed.

dependent effects, the data obtained for MAC in the early log phase of growth have been presented as viable count versus time at all concentrations tested (panels A in all figures) and as viable count versus concentration at all days assessed (panels B in all figures).

Exposure of early-log-phase MAC to CLR resulted in a concentration-dependent as well as time-dependent effect (Fig. 1A and B, Table 1). The lowest concentration of CLR effecting complete inhibition of growth was 4 mg/liter. At concentrations of 8 mg/liter or higher MAC organisms were killed, and ≥99% reduction of the inoculum was obtained after exposure to 16 mg/liter CLR for 10 days. A maximum bactericidal effect was obtained at the relatively high concentration of 256 mg/liter. Substantial killing by CLR was not observed when MAC was in the late log phase of growth. At the relatively high concentration of 64 mg/liter about 87% of the MAC population was killed after 10 days of incubation, and bacterial numbers did not further decrease. At the concentrations tested ≥99% killing of the inoculum was never achieved (data not shown).

EMB also showed killing of MAC in the early log phase of growth which effect was primarily time-dependent (Fig. 2A and B, Table 1). Inhibition of visible growth was obtained at 16 mg/liter. At higher concentrations a relatively slow decrease in bacterial numbers was observed, and ≥99% killing of the inoculum was obtained after exposure to 32 mg/liter EMB for 21 days. The bactericidal killing capacity of EMB was decreased when the MAC was in the late log phase of growth. At a concentration of 64 mg/liter EMB 90% killing of the inoculum was obtained after exposure of 10 days (data not shown).

RIF exhibited bactericidal activity on early-log-phase MAC which effect was concentration-dependent as well as time-dependent (Fig. 3A and B, Table 1). A concentration of 4 mg/liter resulted in inhibition of visible growth. Initially bacterial killing capacity of RIF was low, ≥99% killing was not achieved after 3 days of exposure but killing capacity increased with time. After 10 days of exposure ≥99% killing was obtained at 4 mg/liter. Killing beyond 10 days of exposure was not observed. Occurrence of resistant mycobacteria was excluded. A bactericidal effect of RIF towards late-log-phase MAC was not seen. RIF at 64 mg/liter effected 91% killing of the inoculum within 10 days (data not shown).

AMK appeared highly and rapidly bactericidal against MAC in the early log phase of growth (Fig. 4A and B, Table 1). Inhibition of visible growth was obtained at 1 mg/liter. A concentration of only 2 mg/liter AMK effected ≥99% killing of the inoculum within 3 days. The bactericidal effect was concentration-dependent as well as time-dependent. However the high bacterial killing capacity was not observed when MAC was in the late log phase of growth. A concentration of 16 mg/liter or 64 mg/liter AMK resulted in 96% and 98% killing, respectively, after 10 days of incubation (data not shown).

Exposure to MXF at a concentration of only 0.1 mg/liter resulted in inhibition of visible growth of early-log-phase MAC (Fig. 5A and B, Table 1). A concentration of only 1 mg/liter MXF was needed to achieve ≥ 99% killing of the inoculum within 10 days. The bactericidal effect was marginally concentration-dependent within the therapeutic range, or time-dependent. MXF also showed substantial killing capacity for MAC in the late log phase of growth, ≥99% of the inoculum was killed at 32 mg/liter within 10 days and at 16 mg/liter within 21 days (data not shown).

With respect to the aminoglycosides compared to AMK STR was far less active whereas TOB and GEN were not active at all at the concentrations tested (Table 1). Regarding the quinolones compared to CIP and SPX, MXF exhibited higher killing capacity towards MAC in the early log phase of growth (Table 1) or the late log phase of growth (data not shown).

Among the agents effective in the therapy of tuberculosis and known to be ineffective against MAC isolates, only INH inhibited visible growth at high concentrations which are clinically irrelevant, whereas PZA was not active at all at the concentrations tested.

Time-kill studies of combinations of antimycobacterial agents. It was investigated whether the combination of EMB to CLR resulted in increased bactericidal activity against early-log-phase MAC, and whether the addition of RIF, AMK, or MXF to the CLR-EMB combination further reduced mycobacterial numbers (Table 2). All agents were used at the concentration that inhibited visible growth (Table 1). For CLR, MIC as well as sub-MIC concentrations were used.

The addition of EMB to CLR did not substantially enhance the bactericidal activity. Exposure to CLR alone at 4 mg/liter or EMB alone at 16 mg/liter resulted in 86% killing and 83% killing, respectively of the MAC population within 3 days, whereas the combination of both agents killed 86% of MAC within 3 days. After 10 days of exposure killing percentages were 63%, 51%, and 81% for CLR, EMB and CLR-EMB, respectively, and were not significantly different. In the presence of CLR at 2 mg/liter MAC numbers 10-fold increased from 4.9×10^5 CFU/ml to 5.1×10^6 CFU/ml within 10 days. With addition of EMB at 16 mg/liter, 77% killing of MAC was achieved, whereas exposure to EMB alone resulted in 51% killing. Addition of RIF or MXF to both CLR-EMB combi-

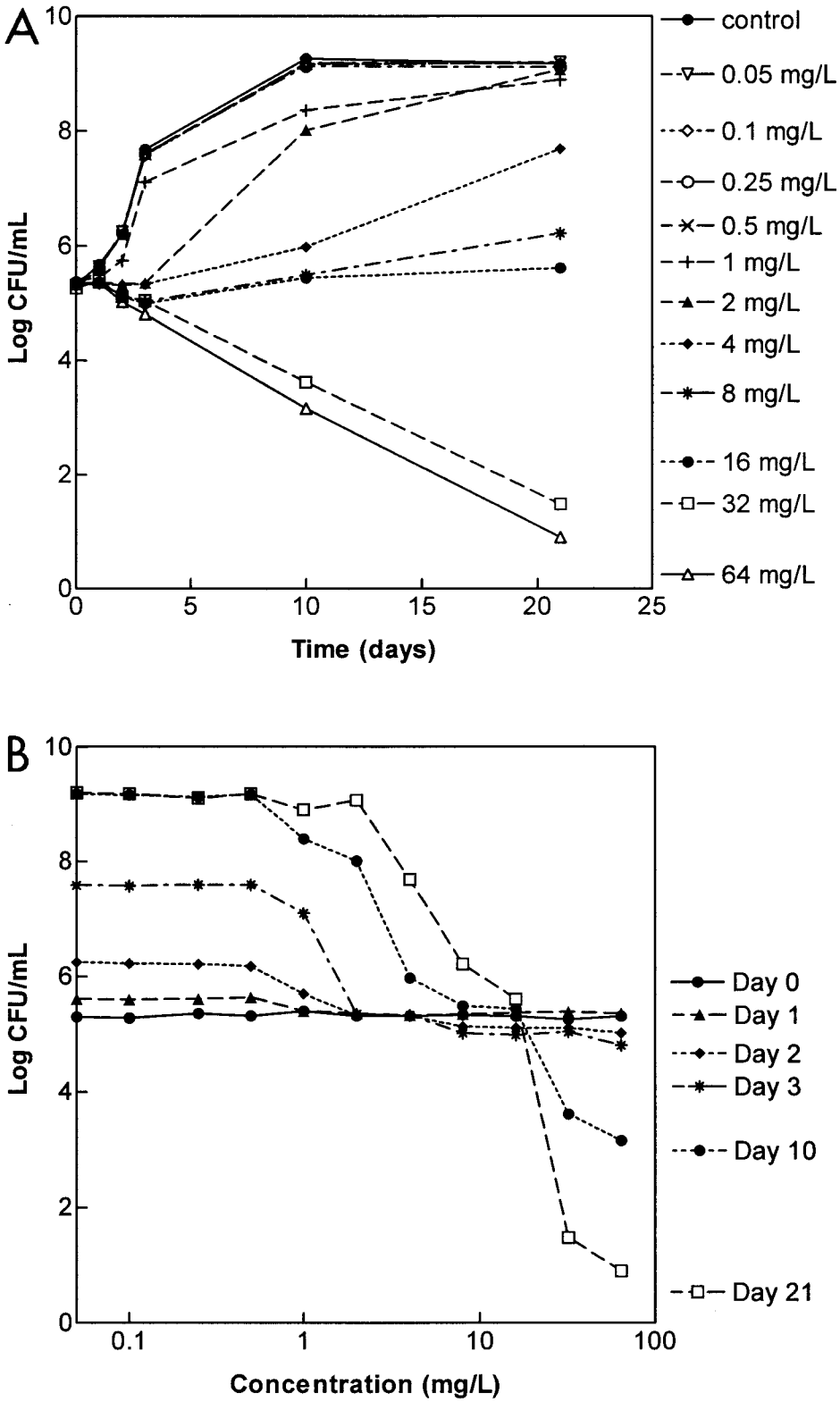

FIG. 2. *Mycobacterium avium* strain 101 in the early logarithmic phase of growth was exposed to ethambutol at twofold increasing concentrations for 21 days at 37°C. After 1, 2, 3, 10 or 21 days of exposure quantitative cultures were performed.

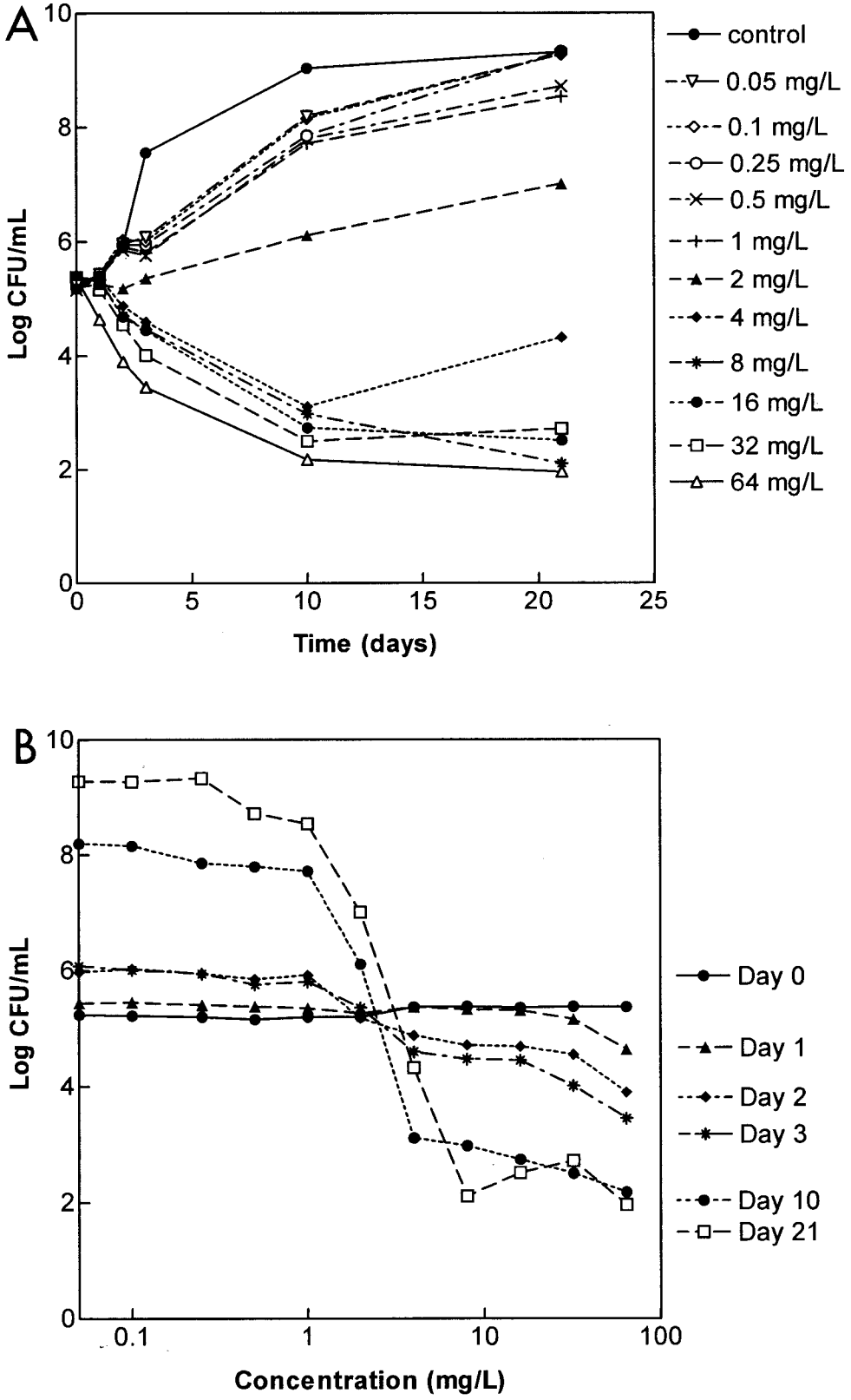

FIG. 3. *Mycobacterium avium* strain 101 in the early logarithmic phase of growth was exposed to rifampin at twofold increasing concentrations for 21 days at 37°C. After 1, 2, 3, 10 or 21 days of exposure quantitative cultures were performed.

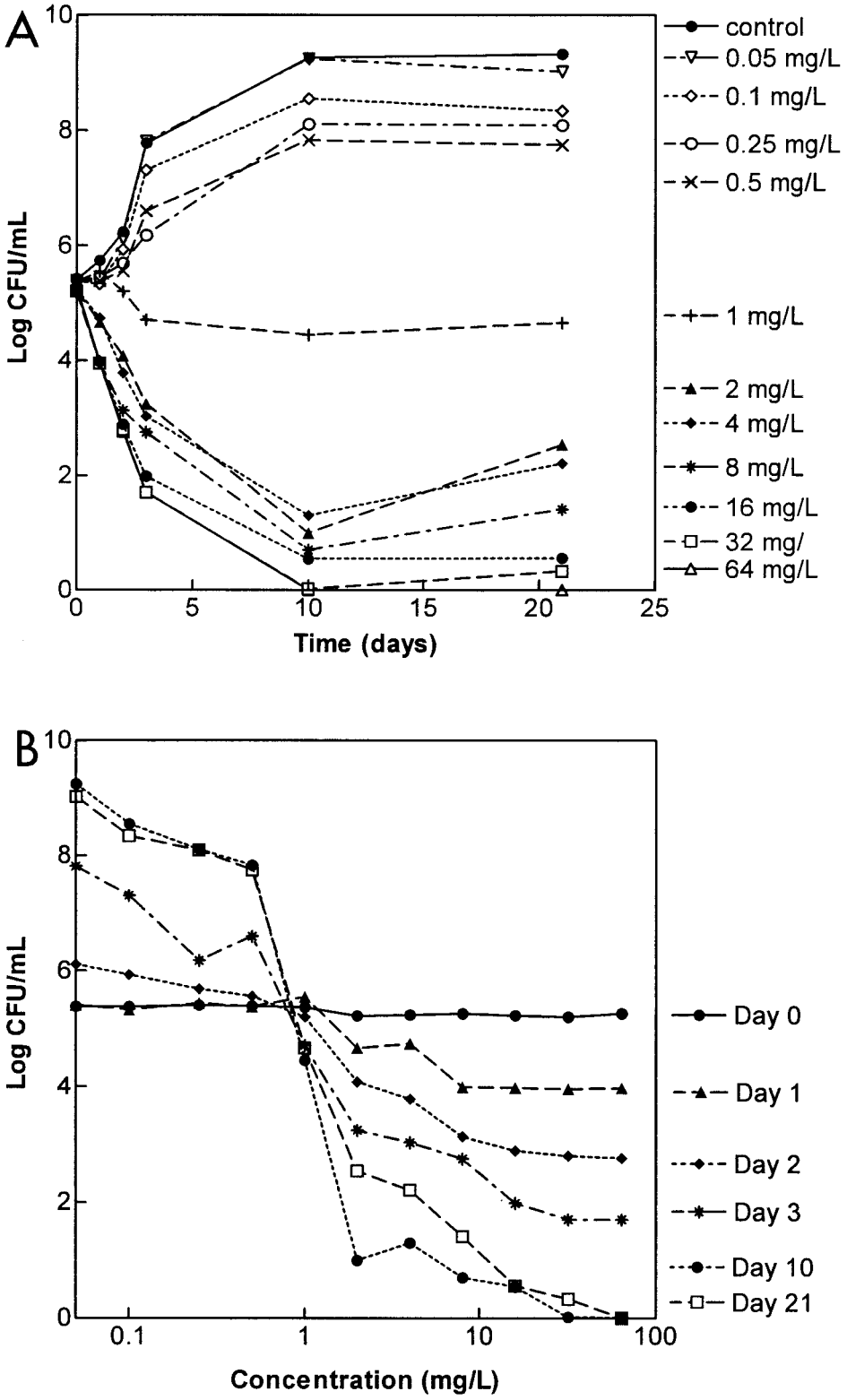

FIG. 4. *Mycobacterium avium* strain 101 in the early logarithmic phase of growth was exposed to amikacin at twofold increasing concentrations for 21 days at 37°C. After 1, 2, 3, 10 or 21 days of exposure quantitative cultures were performed.

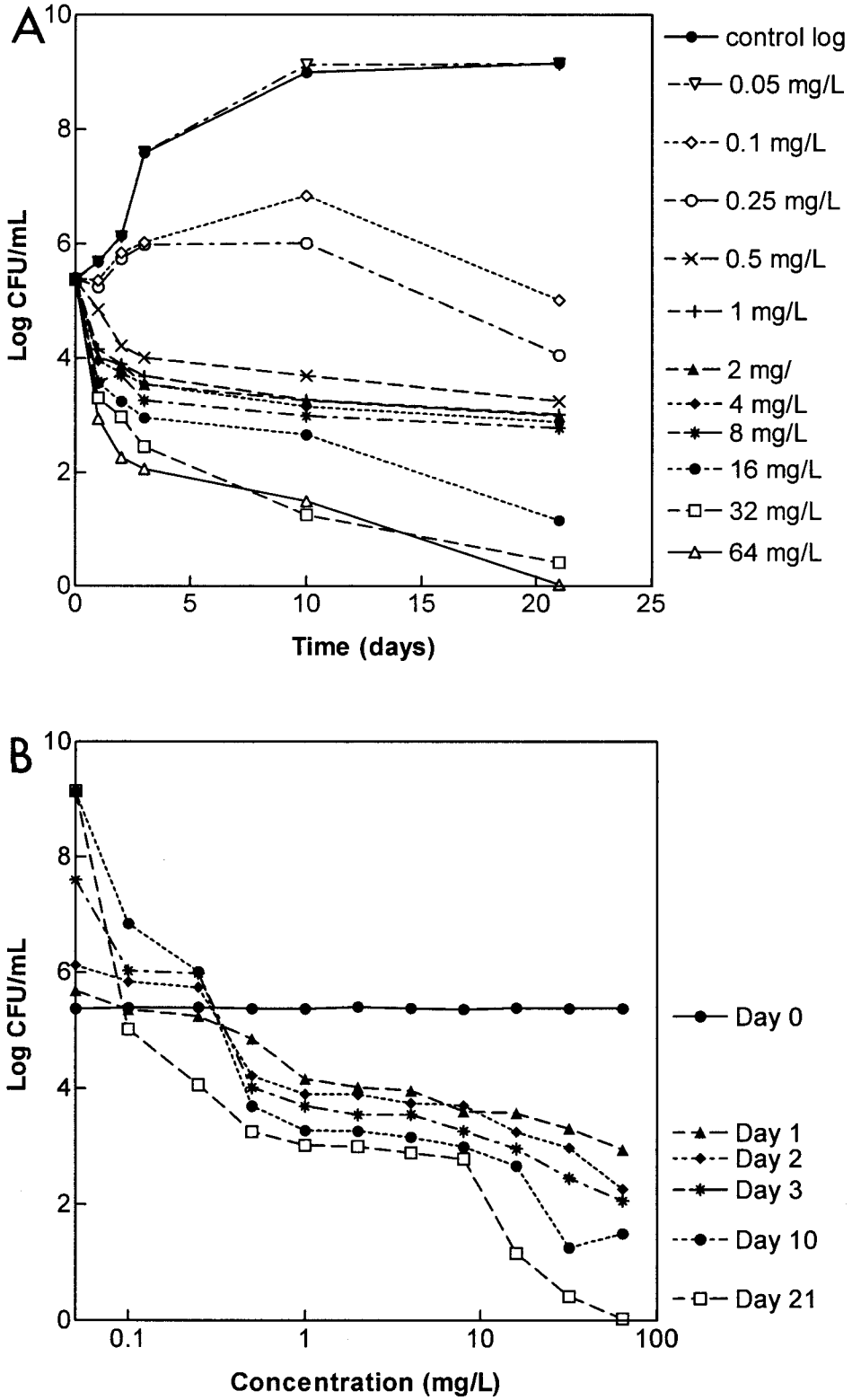

FIG. 5. *Mycobacterium avium* strain 101 in the early logarithmic phase of growth was exposed to moxifloxacin at twofold increasing concentrations for 21 days at 37°C. After 1, 2, 3, 10 or 21 days of exposure quantitative cultures were performed.

TABLE 2. Effect of combinations of antimycobacterial agents on *Mycobacterium avium* complex in the early logarithmic phase of growth[a]

Concn (mg/liter)					Log CFU/ml at day:		
Clarithromycin	Ethambutol	Rifampin	Amikacin	Moxifloxacin	0	3	10
0	0	0	0	0	5.7	7.6	9.1
2						5.2	6.7
2	16					4.5	5.0
2		4				4.4	3.0
2			1			4.3	3.9
2				0.1		5.1	6.6
2	16	4				3.9	2.5
2	16		1			3.6	4.9
2	16			0.1		4.4	5.0
4						4.8	5.3
4	16					4.8	5.0
4		4				4.5	3.0
4			1			4.5	4.3
4				0.1		4.7	4.8
4	16	4				4.5	2.7
4	16		1			4.4	4.3
4	16			0.1		4.6	5.1
	16					4.9	5.4
		4				4.5	3.1
			1			4.7	4.4
				0.1		6.1	6.9

[a] *Mycobacterium avium* strain 101 at 5×10^5 CFU/ml was exposed to the antimycobacterial agent for 10 days at 37°C. After 3 or 10 days of exposure, quantitative cultures were performed.

nations resulted in slightly increased killing of MAC. However compared to either exposure alone a decrease in mycobacterial numbers of 1 log was never obtained. The most effective combination was AMK added to CLR 2 mg/liter and EMB 16 mg/liter, resulting in a tenfold enhanced bactericidal activity within 3 days of exposure compared to AMK or CLR-EMB ($P < 0.01$).

The addition of the beta-lactam AMC to AMK significantly enhanced the antimicrobial activity as shown in Table 3. AMC alone at 4 mg/liter or 16 mg/liter only inhibited early-log-phase MAC growth. In the presence of AMK at 0.5 mg/liter alone MAC numbers increased from 5×10^5/ml to 2.5×10^7/ml within 10 days. Exposure of MAC to a combination of AMK 0.5 mg/liter and AMC 4 mg/liter or 16 mg/liter resulted in 98% killing within 10 days ($P < 0.01$). After exposure to AMK 1 mg/liter alone MAC numbers were reduced about 9-fold within 10 days, whereas addition of AMC at 4 mg/liter or 16 mg/liter to AMK 1 mg/liter resulted in enhanced killing, being 99% and 99.9%, respectively after 10 days of exposure ($P < 0.01$).

TABLE 3. Effect of combination of antimycobacterial agents on *Mycobacterium avium* complex in the early logarithmic phase of growth[a]

Concn (mg/liter)		Log CFU/mL at day:		
Amikacin	Amoxicillin-clavulanic acid	0	3	10
0	0	5.7	7.8	9.3
0.5	4		3.9	3.9
0.5	16		4.0	3.9
0.5			6.2	7.4
	4		5.7	6.0
	16		5.6	5.5
1.0			4.8	4.8
1.0	4		3.9	3.5
1.0	16		2.9	2.7

[a] See Table 2, footnote *a*.

DISCUSSION

The antimicrobial treatment of MAC infections, particularly in patients with AIDS, is difficult. This is in part due to the severely depressed state of host defense mechanisms in these patients, resulting in reduced macrophage antimicrobial capacity (4). In addition, the variability of MAC isolates in susceptibility to most antimycobacterial agents, except for macrolides, is high (29, 25, 26).

A number of agents have been suggested for treatment of MAC infections (3). Mostly macrolide-containing multidrug treatment regimens are applied (3, 6, 9, 13, 15, 28, 45, 48). In clinical studies the macrolide CLR showed high efficacy against disseminated MAC infections in patients with AIDS or non-AIDS patients with localized pulmonary disease (2, 4, 9, 11, 25). The macrolides (particularly CLR) are the only antimicrobial agents for which a correlation between in vitro susceptibility tests (broth dilution) for MAC and clinical response has been demonstrated in controlled clinical trials (25, 26). Empirical regimens in the treatment of MAC infections are recommended (3, 30).

As clinical studies show a high risk for relapse and emergence of resistant isolates during monotherapy with macrolides, combinations of agents are used primarily in order to reduce the incidence of macrolide resistance. However, uncertainty persists regarding the optimal drugs that should accompany a macrolide (6). EMB is recommended as a second drug (31). It has been shown in vitro (34) as well as in vivo in a mouse model of disseminated MAC infection (8) that the combination of CLR and EMB is effective at reducing the incidence of CLR resistant mutants. However, the addition of EMB appeared to have no significant effect on the reduction of bacterial numbers (19). Also, a clinical study of Dube et al. shows that the addition of EMB to a CLR regimen results in a reduction in the development of resistance but does not enhance clearance rates of MAC from the blood (15).

Rifamycins, including RIF, have the best potential as a third agent, although their role is not fully clarified. In addition, RIF, by inducing the hepatic cytochrome P450 pathways, results in substantial reductions in the bioavailability of CLR (37, 52). For severe cases of MAC disease, other antimycobacterial drugs such as quinolones and aminoglycosides are suggested. The potential of fluoroquinolones or aminoglycosides is of considerable interest because of the proven clinical activity of these agents against other mycobacterial species, including *Mycobacterium tuberculosis* (1, 48, 14).

Quinolones including CIP, SPX, and MXF, have been used in relatively effective MAC multidrug treatment regimens (7, 13, 45, 1). MXF particularly had significant anti-MAC activity

in beige mice (7). Still, the antimicrobial activity of each of the quinolones is not yet fully understood. The aminoglycosides AMK and STR are highly active and reduce the incidence of resistance in macrolide-containing regimens in animal models of MAC (33). Aminoglycosides have been used in clinical studies in successful treatment regimens, whereas other investigators failed to show a beneficial effect of aminoglycosides (36). However, Fattorini et al. demonstrated in in vitro assays that AMK significantly improved the activity of the CLR-EMB combination (17). The potential risks and benefits of treatment with aminoglycosides should be weighed carefully. These agents should be considered especially as part of short-term induction therapy in view of toxicity observed with long-term administration.

Although the macrolides are recognized to be the most active of the drugs available for MAC treatment, important questions remain in defining optimal treatment. The relative contribution in terms of antimicrobial activity of each of the components in the multidrug regimen is not clear. Some patients receiving multidrug macrolide-containing regimens who initially respond to therapy develop relapse of infection, which is often the result of insufficient antimicrobial activity (assuming adherence to the drug regimen and no further decline of the host's cellular immune response).

The MAC strain 101 used in the present study, originally isolated from the blood of an AIDS patient with disseminated MAC infection, appeared CLR susceptible following the breakpoints for determining susceptibility and resistance according to the NCCLS guidelines (35, 53). The data show that five relevant antimycobacterial agents, CLR, EMB, RIF, AMK, and MXF, all inhibited mycobacterial growth at clinically relevant concentrations but differed with respect to their bactericidal activity during 21 days of exposure of the early-log-phase MAC. At the end of the 21-day incubation period, compared to the concentrations resulting in growth inhibition, the concentrations needed to achieve a bactericidal effect ($\geq$99% killing) were always twofold or fourfold higher. CLR, RIF, and AMK particularly showed concentration-dependent killing.

However, with respect to the bacterial killing rate, the agents differed markedly. For AMK the high bactericidal activity ($\geq$99% killing) observed after 21 days was already obtained after 3 days of exposure. In contrast, for MXF the concentration needed to achieve bactericidal activity within 3 days was 16-fold higher compared to the concentration needed at 21 days of exposure. At the end of the incubation period of 21 days MXF was superior over AMK, whereas after 3 days of incubation AMK appeared superior over MXF. Actually, the high killing rate of AMK is unique, and in this respect AMK is superior to MXF as well as CLR, EMB, and RIF. RIF showed concentration-dependent as well as time-dependent bactericidal activity. Initially bacterial killing by RIF was low but increased with time. The killing rate of RIF was higher compared to that of CLR that showed extremely low killing rate. Whereas after 21 days of exposure RIF and CLR were similar with respect to their bacteriostatic activity, both agents differ regarding the rate of bactericidal activity. After 3 days of exposure the bactericidal activity of RIF was less compared to that of CLR, whereas after 21 days RIF appeared superior over CLR. The low killing rate as seen for CLR was also observed for EMB.

It can be concluded that the antimicrobial agents differ with respect to time-dependent bacterial killing capacity. After long-term exposure of 21 days the comparative bactericidal capacity is highest for MXF, followed by AMK, RIF, CLR, and EMB, respectively. After short-term exposure of 3 days the comparative bactericidal capacity is highest for AMK, followed by MXF, CLR, and RIF or EMB. The rate of bacterial killing may have more clinical significance than the degree of killing, as in vivo after administration of the agents exposure to clinically achievable concentrations lasts for only a limited period of time. In addition, a rapid decrease in mycobacterial load is needed for therapeutic efficacy and also may result in a reduced risk of development of drug resistance. In this respect AMK seems to be superior, as it was the only agent that was rapidly bactericidal at relatively low concentrations which are far below the achievable plasma concentrations. These are important parameters which may compensate in part for the low intracellular penetrating capability of this agent.

RIF and MXF are bactericidal at concentrations below the concentrations achievable in plasma and tissues and inside infected cells. CLR inhibited bacterial growth at plasma concentrations attainable in vivo. Bacterial killing was only achieved at relatively high concentrations, and the killing rate of CLR is extremely low. As CLR concentrates intracellularly in macrophages and achieves excellent tissue penetration, it is bactericidal in mice and humans. Compared to CLR, higher concentrations of EMB were needed to obtain a bacteriostatic or bactericidal effect, which are far above the concentrations achievable in vivo. The relatively high concentration of EMB needed to obtain growth inhibition is within the range of MICs of EMB for most MAC isolates (20 to 32 mg/liter). EMB is considered bacteriostatic, and its principal role has been as a companion drug to prevent macrolide resistance.

Compared to the other aminoglycosides AMK appeared superior over STR, TOB, and GEN, which were active at only relatively high concentrations. This finding is in agreement with the general observation that AMK is the most active aminoglycoside against the nontuberculous mycobacteria (20). Regarding the quinolones, MXF, particularly during the first 3 days of exposure, appeared superior over SPX and CIP. After 21 days of exposure, SPX and CIP showed similar bactericidal activity, but in terms of killing rate SPX is superior over CIP. The other agents investigated, INH, PZA, and AMC, were far less active. PZA was not bactericidal at all at the concentrations tested, whereas INH and AMC were bactericidal only at high concentrations that are not clinically relevant. It is generally known that these agents are not useful for treatment of MAC infections.

Based on the results of bacteriostatic capacity in terms of growth inhibition, CLR, RIF, AMK, and MXF all show substantial activity. According to the thresholds for interpretation of growth inhibition (23), MAC strain 101 is considered "susceptible" to these agents. However, the present study demonstrates that the assay of time-dependent bactericidal capacity is more discriminative. The data show that these agents differ substantially with respect to their bactericidal capacity, particularly the killing rate, demonstrating a superior and rapid killing activity of AMK and high killing activity of MXF.

In the treatment of MAC, combinations of antimycobacterial agents are necessary to reduce the incidence of drug resistance. It is not well understood whether their use also results in improved efficacy in terms of enhanced eradication of mycobacteria. In the present study it was investigated whether addition of a number of antimycobacterial agents to CLR resulted in an increased level of bactericidal activity after 3 or 10 days of exposure in vitro against early-log-phase MAC. Significant enhancement of activity of CLR by combination with EMB could not be demonstrated. Only an additive effect has been shown. The addition of RIF or MXF as a third agent to the CLR-EMB combination did not significantly promote enhanced bacterial killing. Although the three-drug combinations resulted in higher bactericidal effect than any of the exposures used alone, these combinations did never reach a 1 log further decrease in mycobacterial numbers. Only AMK had a substantial effect by improving the activity of the combination CLR-EMB tenfold within only 3 days of exposure. This is in agreement with the findings of Fattorini et al., demonstrating that the addition of AK made the combination of CLR-EMB synergistic against a number of MAC strains in vitro (17).

Other studies investigating the in vitro activity of CLR in combination with various antimycobacterial agents against MAC show discrepancy in results. Besides claims by some authors about a synergistic interaction between CLR in combination with EMB (20), RIF (49, 47), EMB and RIF (41), AMK (38), or various quinolones (38), only additive effects have been observed (20, 32). In addition, antagonism has been reported between CLR in combination with AMK (17) or quinolones (49). As the studies on the in vitro activities of CLR in two-drug combinations or three-drug combinations against MAC are controversial, these studies do not clearly provide insight into the rational design of combinations of agents with potent therapeutic activity.

Beta-lactam activity against mycobacteria has been described. However, the doses required to obtain efficacy in infection are not feasible. Synergistic activity of aminoglycoside–beta-lactam combinations towards bacterial strains is well known but was not investigated for mycobacteria. In the present study the addition of the beta-lactamase-stable AMC to AMK resulted in a higher level of MAC killing. In view of the low intracellular penetrating capability of aminoglycosides and beta-lactams, the clinical relevance of this finding with respect to intracellular MAC is questionable. However, as a result of their high antimicrobial activity, an aminoglycoside alone or in combination with a beta-lactam may be efficacious in eliminating MAC organisms which are growing extracellularly in the cavitating lesions in advanced stages of MAC infection.

There are many factors influencing the activity of antibiotics in vivo. Besides the antibiotic susceptibility of the infectious agent, the concentration profile of the antibiotic in serum, other body fluids, and tissues, and at the site of infection in relation to the dosing regimen is of importance. With respect to mycobacterial infections, antimicrobial activity against mycobacteria at the low growth rate is also an important determinant for therapeutic efficacy in view of the low metabolic activity of mycobacteria residing in tissues in the dormant state. The data from the present study show that, towards MAC that are not actively growing, only the quinolones exhibited bactericidal activity, although the absolute killing of these was substantially less compared to that of actively growing MAC. Even AMK, which was highly bactericidal against actively growing MAC, appeared not to be effective against MAC at the low growth rate. Whether this superior activity of quinolones for dormant *Mycobacterium avium* implies that quinolones should be used in the first line regimen for *Mycobacterium avium* disease will have to be clinically evaluated.

REFERENCES

1. **Alangaden, G. J., and S. A. Lerner.** 1997. The clinical use of fluoroquinolones for the treatment of mycobacterial diseases. Clin. Infect. Dis. **25:**1213–1221.
2. **American Thoracic Society.** 1997. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. Am. J. Respir. Crit. Care Med. **156:**S1–S25.
3. **Amsden, G. W., C. A. Peloquin, and S. E. Berning.** 1997. The role of advanced generation macrolides in the prophylaxis and treatment of *Mycobacterium avium* complex (MAC) infections. Drugs **54:**69–80.
4. **Benson, C. A.** 1996. Treatment of disseminated *Mycobacterium avium* complex disease: a clinician's perspective. Res. Microbiol. **147:**16–24.
5. **Benson, C. A.** 1998. Disseminated *Mycobacterium avium* complex infection: implications of recent clinical trials on prophylaxis and treatment. AIDS Clin. Rev. **1998:**271–287.
6. **Benson, C. A., P. L. Williams, J. S. Currier, F. Holland, L. F. Mahon, R. R. MacGregor, C. B. Inderlied, C. Flexner, J. Neidig, R. Chaisson, G. F. Notario, R. Hafner, and the AIDS Clinical Trial Group 223 Protocol Team.** 2003. A prospective randomized trial examinig the efficacy and safety of clarithromycin in combination with ethambutol, rifabutin, or both for the treatment of disseminated *Mycobacterium avium* complex disease in persons with acquired immunodeficiency syndrome. Clin. Infect. Dis. **37:**1234–1243.
7. **Bermudez, L. E., C. B. Inderlied, P. Kolonoski, M. Petrofsky, P. Aralar, M. Wu, and L. S. Young.** 2001. Activity of moxifloxacin by itself and in combination with ethambutol, rifabutin, and azithromycin in vitro and in vivo against *Mycobacterium avium*. Antimicrob. Agents Chemother. **45:**217–222.
8. **Bermudez, L. E., K. A. Nash, M. Petrofsky, L. S. Young, and C. B. Inderlied.** 1996. Effect of ethambutol on emergence of clarithromycin-resistant *Mycobacterium avium* complex in the beige mouse model. J. Infect. Dis. **174:**1218–1222.
9. **Chaisson, R. E., C. A. Benson, M. P. Dubé, L. B. Heifets, J. A. Korvick, S. Elkin, T. Smith, J. C. Craft, and F. R. Sattler.** 1994. Clarithromycin therapy for *Mycobacterium avium* complex bacteremia in patients with AIDS. Ann. Intern. Med. **121:**905–911.
10. **Chan, I. S. F., J. D. Neaton, L. D. Saravolatz, L. R. Crane, and J. Osterberger.** 1995. Frequencies of opportunistic diseases prior to death among HIV-infected persons. AIDS **9:**1145–1151.
11. **Dautzenberg, B.** 1994. Clinical trials of disseminated *Mycobacterium avium* therapy: lessons to take home. Res. Microbiol. **145:**197–206.
12. **Davies, P. D.** 2003. Recent developments in the treatment of tuberculosis. Expert Opin. Investig. Drugs **12:**1297–1312.
13. **De Lalla, F., R. Maserati, P. Scarpellini, P. Marone, R. Nicolin, F. Caccamo, and R. Rigoli.** 1992. Clarithromycin-ciprofloxacin-amikacin for therapy of *Mycobacterium avium–Mycobacterium intracellulare* bacteremia in patients with AIDS. Antimicrob. Agents Chemother. **36:**1567–1569.
14. **Donald, P. R., F. A. Sirgel, A. Venter, E. Smit, D. P. Parkin, B.W. van de Wal, and D. A. Mitchison.** 2001. The early bactericidal activity of amikacin in pulmonary tuberculosis. Int. J. Tuberc. Lung Dis. **5:**533–538.
15. **Dube, M. P., F. R. Sattler, F. J. Torriani, D. See, D. V. Havlir, C. A. Kemper, M. G. Dezfuli, S. A. Bozzette, A. E. Bartok, J. M. Leedom, J. G. Tilles, and J. A. McCutchan.** 1997. A randomized evaluation of ethambutol for prevention of relapse and drug resistance during treatment of *Mycobacterium avium* complex bacteremia with clarithromycin-based combination therapy. California Collaborative Treatment Group. J. Infect. Dis. **176:**1225–1232.
16. **Espinal, M. A.** 2003. The global situation of MDR-TB. Tuberculosis **83:**44–51.
17. **Fattorini, L., B. Li, C. Piersimoni, E. Tortoli, Y. Xiao, C. Santoro, M. L. Ricci, and G. Orefici.** 1995. In vitro and ex vivo activities of antimicrobial agents used in combination with clarithromycin, with or without amikacin, against *Mycobacterium avium*. Antimicrob. Agents Chemother. **39:**680–685.
18. **Fréhel, C., C. Offredo, and C. de Chastellier.** 1997. The phagosomal environment protects virulent *Mycobacterium avium* from killing and destruction by clarithromycin. Infect. Immun. **65:**2792–2802.
19. **Furney, S. K., P. S. Skinner, J. Farrer, and I. M. Orme.** 1995. Activities of rifabutin, clarithromycin, and ethambutol against two virulent strains of *Mycobacterium avium* in a mouse model. Antimicrob. Agents Chemother. **39:**786–789.
20. **Gevaudan, M. J., C. Bollet, M. N. Mallet, and P. de Micco.** 1993. In-vitro evaluation of clarithromycin, temafloxacin, and ethambutol in combination

against *Mycobacterium avium* complex. J. Antimicrob. Chemother. **31:**725–730.
21. **Grosset, J., and B. Ji.** 1997. Prevention of the selection of clarithromycin-resistant *Mycobacterium avium-intracellulare* complex. Drugs **54**(Suppl. 2)**:** 23–27.
22. **Heifets, L. B.** 1991. Dilemmas and realities in drug susceptibility testing of *M. avium–M. intracellulare* and other slowly growing nontuberculous mycobacteria, p. 123–146. *In* L. B. Heifets (ed.), Drug susceptibility in the chemotherapy of mycobacterial infections. CRC Press, Inc., Boca Raton, Fla.
23. **Heifets, L. B.** 1994. Quantitative cultures and drug susceptibility testing of *Mycobacterium avium* clinical isolates before and during the antimicrobial therapy. Res. Microbiol. **145:**188–196.
24. **Heifets, L. B.** 1996. Drug susceptibility testing. Clin. Mycobacteriol. **16:**641–656.
25. **Heifets, L.** 1996. Clarithromycin against *Mycobacterium avium* complex infection. Tuberc. Lung Dis. **77:**19–26.
26. **Heifets, L.** 1996. Susceptibility testing of *Mycobacterium avium* complex isolates. Antimicrob. Agents Chemother. **40:**1759–1767.
27. **Heifets, L. B., and M. D. Iseman.** 1991. Individualized therapy versus standard regimens in the treatment of *Mycobacterium avium* infections. Am. Rev. Respir. Dis. **144:**1–2.
28. **Heifets, L., N. Mor, and J. Vanderkolk.** 1993. *Mycobacterium avium* strains resistant to clarithromycin and azithromycin. Antimicrob. Agents Chemother. **37:**2364–2370.
29. **Inderlied, C. B., C. A. Kemper, and L. E. M. Bermudez.** 1993. The *Mycobacterium avium* complex. Clin. Microbiol. Rev. **6:**266–310.
30. **Iseman, M. D.** 2002. Medical management of pulmonary disease caused by *Mycobacterium avium* complex. Clin. Chest Med. **23:**633–641.
31. **Kaplau, J. E., H. Masure, and K. K. Holmes.** 2002. Guidelines for preventing opportunistic infections among HIV-infected persons-2002: recommendations of the US Public Health Service and the Infectious Diseases Society of America. Morb. Mortal. Wkly Rep. **51:**1–52.
32. **Kent, R. J., M. Bakhtiar, and D. C. Shanson.** 1992. The in-vitro bactericidal activities of combinations of antimicrobial agents against clinical isolates of *Mycobacterium avium-intracellulare*. J. Antimicrob. Chemother. **30:**643–650.
33. **Lounis, N., B. Ji, C. Truffot-Pernot, and J. Grosset.** 1995. Selection of clarithromycin-resistant *Mycobacterium avium* complex during combined therapy using the beige mouse model. Antimicrob. Agents Chemother. **39:** 608–612.
34. **Nash, K. A.** 2001. Effect of drug concentration on emergence of macrolide resistance in *Mycobacterium avium*. Antimicrob. Agents Chemother. **45:**1607–1614.
35. **National Committee for Clinical Laboratory Standards.** 2000. Susceptibility testing of mycobacteria, Nocardia, and other aerobic actinomycetes; tentative standard M24–T2, 2nd ed. National Committee for Clinical Laboratory Standards, Wayne, PA.
36. **Parenti, D. M., P. L. Williams, R. Hafner, M. R. Jacobs, P. Hojczyk, T. M. Hooton, T. W. Barber, G. Simpson, C. van der Horst, J. Currier, W. G. Powderly, M. Limjoco, and J. J. Ellner.** 1998. A phase II/III trial of antimicrobial therapy with or without amikacin in the treatment of disseminated *Mycobacterium avium* infection in HIV-infected individuals. AIDS Clinical Trials Group Protocol 135 Study Team. AIDS **12:**2439–2446.
37. **Peloquin, C. A., and S. E. Berning.** 1996. Evaluation of the drug interaction between clarithromycin and rifampin. J. Infect. Dis. Pharmacother. **2:**19–35.
38. **Piersimoni, C., E. Tortoli, M. T. Mascellino, C. Passerini Tosi, G. Sbaraglia, F. Mandler, F. Bistoni, S. Bornigia, G. De Sio, A. Goglio, E. Iona, M. B. Pasticci, and M. T. Simonetti.** 1995. Activity of seven antimicrobial agents, alone and in combination, against AIDS-associated isolates of *Mycobacterium avium* complex. J. Antimicrob. Chemother. **36:**497–502.
39. **Quy, H. T., N. T. Lan, M. W. Borgdorff, J. Grosset, P. D. Link, L. B. Tung, D. van Soolingen, M. Raviglione, N. V. Co, and J. Broekmans.** 2003. Drug resistance among failure and relapse cases of tuberculosis: is the standard re-treatment regimen adequate. Int. J. Tuberc. Lung Dis. **7:**607–608.
40. **Rastogi, N., K. S. Goh, A. Bryskier, and A. Devallois.** 1996. Spectrum of activity of levofloxacin against nontuberculous mycobacteria and its activity against the *Mycobacterium avium* complex in combination with ethambutol, rifampin, roxithromycin, amikacin and clofazimine. Antimicrob. Agents Chemother. **40:**2483–2487.
41. **Rastogi, N., and V. Labrousse.** 1991. Extracellular and intracellular activities of clarithromycin used alone and in association with ethambutol and rifampin against *Mycobacterium avium* complex. Antimicrob. Agents Chemother. **35:**462–470.
42. **Raviglione, M. C.** 2003. The TB epidemic from 1992 to 2002. Tuberculosis **83:**4–14.
43. **Samuel, R., R. L. Bettiker, and B. Suh.** 2002. AIDS related opportunistic infections, going but not gone. Arch. Pharm. Res. **25:**215–228.
44. **Schiavano, G. F., A. G. Celeste, L. Salvaggio, M. Sisti, and G. Brandi.** 2001. Efficacy of macrolides used in combination with ethambutol, with or without other drugs, against *Mycobacterium avium* within human macrophages. Int. J. Antimicrob. Agents **18:**525–530.
45. **Shafran, S. D., J. Singer, D. P. Zarowny, P. Phillips, I. Salit, S. L. Walmsley, I. W. Fong, M. J. Gill, A. R. Rachlis, R. G. Lalonde, M. M. Fanning, and C. M. Tsoukas.** 1996. A comparison of two regimens for the treatment of *Mycobacterium avium* complex bacteremia in AIDS: rifabutin, ethambutol, and clarithromycin versus rifampin, ethambutol, clofazimine, and ciprofloxacin. N. Engl. J. Med. **335:**377–383.
46. **Shafran, S. D., J. A. Talbot, S. Chomye, E. Davison, J. Singer, P. Phillips, I. Salit, S. L. Walmsley, I. W. Fong, M. J. Gill, A. R. Rachlis, and R. G. Lalonde for the Canadian HIV Trials Network Protocol 010 Study Group.** 1998. Does in vitro susceptibility to rifabutin and ethambutol predict the response to treatment of *Mycobacterium avium* complex bacteremia with rifabutin, ethambutol, and clarithromycin? Clin. Infect. Dis. **27:**1401–1405.
47. **Stauffer, F., O. Dörtbudak, and E. Lahonik.** 1991. In vitro testing of clarithromycin in combination with ethambutol and rifampin against *Mycobacterium avium* complex. Infection **19:**343–345.
48. **Tomioka, H.** 2000. Prospects for development of new antimycobacterial drugs. J. Infect. Chemother. **6:**8–20.
49. **Tomioka, H., C. Sano, K. Sato, and T. Shimizu.** 2002. Antimicrobial activities of clarithromycin, gatifloxacin and sitafloxacin, in combination with various antimycobacterial drugs against extracellular and intramacrophage *Mycobacterium avium* complex. Int. J. Antimicrob. Agents. **19:**139–145.
50. **Tomioka, H., K. Sato, H. Kajitani, T. Akaki, and S. Shishido.** 2000. Comparative antimicrobial activities of the newly synthesized quinolone WQ-3034, levofloxacin, sparfloxacin, and ciprofloxacin against *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. Antimicrob. Agents Chemother. **44:**283–286.
51. **Wallace, R. J. Jr.** 2000. Antimycobacterial agents, p. 436–447. *In* G. L. Mandell, J. E. Bennet, and R. Dolin (ed.), Principles and practice of infectious diseases, 5th ed. Churchill Livingstone, Philadelphia, PA.
52. **Wallace, R. J. Jr, B. A. Brown, D. E. Griffith, W. Girard, and K. Tanaka.** 1995. Reduced serum levels of clarithromycin in patients treated with multidrug regimens including rifampin or rifabutin for *Mycobacterium avium-Mycobacterium intracellulare* infection. J. Infect. Dis. **171:**747–750.
53. **Woods, G. L., N. Williams-Bouyer, R. J. Wallace, B. A. Brown-Elliott, F. G. Witebsky, P. S. Conville, M. Plaunt, G. Hall, P. Aralar, and C. Inderlied.** 2003. Multisite reproducibility of results obtained by two broth dilution methods for susceptibility testing of *Mycobacterium avium* complex. J. Clin. Microbiol. **41:**627–631.
54. **Young, L. S., and L. E. Bermudez.** 2001. Perspective on animal models: chronic intracellular infections. Clin. Infect. Dis. **33**(Suppl. 3)**:**S221–S226.
55. **Zimmer, B., D. R. DeYoung, and G. D. Roberts.** 1982. In vitro synergistic activity of ethambutol, isoniazid, kanamycin, rifampin, and streptomycin against *Mycobacterium avium-intracellulare* complex. Antimicrob. Agents Chemother. **22:**148–150.