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Effectiveness of Various Antimicrobial Agents against *Mycobacterium avium* Complex in the Beige Mouse Model

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The results of five chemotherapeutic experiments in beige mice infected with organisms of the *Mycobacterium avium* complex are presented. After monotherapy with various antimicrobial agents for 4 weeks, only clarithromycin, amikacin, and ethambutol displayed definite bactericidal effects; sparfloxacin and clofazimine showed modest bacteriostatic effects; and rifampin and rifabutin were totally inactive against the isolate tested. After treatment for 4 weeks, the large quantities of clofazimine that had accumulated in the organs of mice seriously interfered with the enumeration of the CFU and assessment of the efficacy of the treatment. The in vitro synergistic effects of drug combinations against *M. avium* complex were not confirmed in beige mice. In combination with clarithromycin, amikacin could prevent the selection of clarithromycin-resistant mutants, whereas minocycline could not.

Mycobacterium avium complex (MAC) infection is the most common disseminated bacterial infection in patients during the later stages of AIDS. MAC organisms are far less susceptible than *Mycobacterium tuberculosis* to antimicrobial agents. The list of antimicrobial agents which have been reported to be active against MAC in vitro or in vivo is rather short, and information about the activities of most of the compounds is inconclusive or controversial. Therefore, effective drugs and regimens for the treatment of disseminated MAC infection are urgently needed.

In this report we present the results of five experiments performed in MAC-infected beige mice. Experiment I compared the anti-MAC activities of 4 weeks of monotherapy with various antimicrobial agents; experiment II compared the anti-MAC activities of 4 weeks of treatment with various combined therapies; and experiments III, IV, and V evaluated the effectiveness of a longer duration (12 weeks) of treatment with various monotherapies and combined therapies, with special emphasis on preventing the selection of clarithromycin (CLARI)-resistant mutants by the combined regimens. In addition, the results of these experiments revealed the carryover of clofazimine (CLO) in organ suspensions that seriously interfered with the assessment of the efficacy of the treatment.

MATERIALS AND METHODS

Mice. Four- to six-week-old female beige (C57BL/6J bg^j/bg^j) mice (3, 10) weighing 14 to 18 g were purchased from Jackson Laboratory, Bar Harbor, Maine. Before infection with MAC, the mice were housed in our animal facility for another 4 weeks until their body weights reached 18 to 20 g.

MAC. Strain 101 (3), obtained from L. S. Young's laboratory in San Francisco, was used in the first three experiments; strain Lpr (5), obtained from M. H. Cynamon's laboratory in Syracuse, N.Y., and strain MO1 (31, 32) from our laboratory were used in experiments IV and V, respectively. All three strains had been isolated from AIDS patients with disseminated MAC infection. In our laboratory, strain 101 was passaged regularly through beige mice; strains Lpr and MO1 were passaged on agar medium.

The MICs determined on 5% oleic acid-albumin-dextrose (OADC)-enriched Mueller-Hinton agar medium (33) for strains 101, Lpr, and MO1 are given in Table 1.

To prepare the inocula for experiments in mice, 300 to 400 transparent colonies of each strain were collected from 10% OADC-enriched 7H11 agar medium and were subcultured in 100 to 120 ml of Dubos broth (Diagnostic Pasteur, Paris, France) at 37°C for 7 days. The turbidity of the resulting suspension was adjusted with normal saline to match that of a standard suspension of *Mycobacterium bovis* BCG (1 mg/ml), and the suspension was further diluted fivefold. The number of CFU in the inoculum was determined by plating appropriate dilutions onto 5 to 10% OADC-enriched 7H11 or Mueller-Hinton (33) agar medium.

Antimicrobial agents. The following compounds were generously provided by the respective manufacturers: CLARI, Abbott Laboratories, Abbott Park Ill.; rifabutin (RBT), Farmitalia-Carlo Erba, Rueil, France; rifampin (RMP) and rifapentine (RPT), Marion Merrel-Dow, Neuilly, France; amikacin (AMIKA), Bristol-Myers Squibb, Paris, France; ethambutol (EMB) and minocycline (MINO), Lederle, Oullins, France; sparfloxacin (SPFX), Rhone D.P.C. Europe, Antony, France; and CLO, Ciba-Geigy, Basal, Switzerland. All agents except AMIKA were suspended in 0.05% agar in distilled water at the desired concentrations; the suspensions were prepared weekly and were stored at 4°C.

Inoculation of mice. Each mouse was inoculated intravenously with 0.5 ml of a bacterial suspension containing $10^{6.70}$ CFU in experiment I, $10^{7.91}$ CFU in experiment II, $10^{6.94}$ CFU in experiment III, $10^{7.55}$ CFU in experiment IV, and $10^{7.13}$ CFU in experiment V.

Chemotherapy. At day 1 (D1) or D14 after inoculation, 10 mice were sacrificed and the numbers of CFU in the individual spleens and lungs were enumerated. The remaining mice were allocated randomly to an untreated control group and various numbers of treated groups; each group initially had at least 10 mice. The drugs were administered six times weekly through an esophageal cannula; AMIKA, however, was injected subcutaneously. The following dosages were selected either to provide peak concentrations in serum or areas under the concentration-time curve in mice comparable to those achievable in

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TABLE 1. MICs of the tested antimicrobial agents for three different MAC strains

MAC strain	MIC (μg/ml)								
	CLARI	RMP	RBT	RPT	AMIKA	EMB	SPFX	MINO	CLO
101	1	16	2	4	16	16	2	32	0.03
Lpr	1	16	2	0.25	32	16	4	16	0.03
MO1	1	16	2	4	32	16	1	16	0.015

humans or because the dosages had been used by other investigators: CLARI, 50, 100, or 200 mg/kg of body weight (20); all rifamycin derivatives (RMP, RBT, and RPT), 10 mg/kg (21); AMIKA, 100 mg/kg (16); EMB, 125 mg/kg (34); SPFX, 50 or 100 mg/kg (28); MINO, 25 mg/kg (20); and CLO, 20 mg/kg (11). In experiments II to V, however, the dosage of CLARI, administered either alone or in combinations, was always 200 mg/kg. The duration of treatment was 4 weeks in experiments I and II and 12 weeks in experiments III, IV, and V. To avoid or reduce the carryover effects of the drugs in the organs, treated mice were sacrificed 48 to 144 h after administration of the last dose of the treatment.

Assessment of results. Mortality; spleen weights; the numbers of CFU in the spleens, lungs, and livers (experiment II only); and the numbers of CFU of CLARI-resistant mutants in the spleens (experiments III, IV, and V only) were applied as parameters for assessing the severity of infection and the effectiveness of treatment.

Enumeration of total CFU. At the time of sacrifice or autopsy, the organs were removed aseptically and were homogenized by a standard procedure (13), and the suspensions were made up to 3.5 ml for each organ. At least four serial 10-fold dilutions of the suspensions were plated in duplicate onto 5 to 10% OADC-enriched 7H11 agar (experiments I and II) or Mueller-Hinton agar and Löwenstein-Jensen (L-J) medium (experiments III, IV, and V). The results of the cultures were recorded after incubation at 37°C for 2 weeks on agar medium and for 6 weeks on L-J medium. The lower limit of detectability of the CFU per organ was $10^{1.24}$, corresponding to a single colony appearing on two quadrants or tubes of medium which had been plated with 0.1 ml of undiluted suspension. The bactericidal effect of the treatment was defined as a significant decrease in the mean number of CFU in the treated group from the pretreatment value.

Enumeration of CLARI-resistant mutants. In experiments III, IV, and V, treatments were begun on D14 and were continued for 12 weeks. The CLARI-resistant mutants were enumerated among the untreated control groups on D1 (5 mice), D14 (10 mice), and D98 (8 to 20 mice) and among all surviving mice (8 to 10 mice per group) treated with CLARI alone, CLARI-AMIKA, and CLARI-MINO on D98. On each occasion, besides the enumeration of the total CFU in the organs, the undiluted suspensions were also plated onto 5% OADC-enriched Mueller-Hinton agar containing CLARI at a concentration of 16 μg/ml for measurement of the number of CFU of the CLARI-resistant mutants (19). The frequency of CLARI-resistant mutants in the bacterial population was defined as the ratio between the number of CFU of CLARI-resistant mutants and the total number of CFU.

Statistical analysis. Results were analyzed by the Student *t* test and Fisher's exact probability calculation; the correlations between spleen weights and spleen CFU counts, however, were analyzed by using JMP software (SAS Institute Inc., Cary, N.C.). Differences were considered significant at the 95% level of confidence.

TABLE 2. Mortalities of beige mice in untreated and treated groups of five experiments

Expt	MAC strain	Inoculum ($\log_{10}$)/ mouse	Duration (wk) of treatment[a]	Untreated control group		Treated group	
				No.[b]	Mortality (%)	No.[b]	Mortality (%)
I	101	6.70	4	84	7.1	120	0
II	101	7.91	4	49	32.7	90	18.9
III	101	6.94	12	13	53.9	90	14.4[c]
IV	Lpr	7.55	12	17	47.1	90	13.3[c]
V	MO1	7.13	12	30	33.3	100	7.0[c]

[a] Treatments in experiments II to V were begun on D14; treatments in experiment were begun on D1.
[b] Excluding the mice that were sacrificed.
[c] Mortalities in mice treated with CLO alone were 40, 30, and 0% in experiments III, IV, and V, respectively.

RESULTS

Mortality of mice in untreated control and treated groups. Table 2 shows the numbers of mice that died after infection with MAC. Among the untreated control mice, mortality varied from 7.1% in experiment I to 53.9% in experiment III and was correlated with the inoculum size and the duration of the experiment. The mortality rates among the treated mice were significantly lower than those among the untreated controls except in experiments III and IV, in which the mortality rate among mice treated with CLO alone did not differ significantly from that among untreated controls. The mortality data indicated that the nature of disseminated MAC infection in beige mice is chronic rather than acute, as was originally thought (10). From an experimental chemotherapy point of view, such a mortality rate was annoying, because it was not high enough to be capable of distinguishing an active treatment from an inactive one, whereas it interfered significantly with the enumeration of the CFU, by far the most important parameter in assessing the in vivo activity of the antimicrobial treatment.

Close correlation between spleen weight and spleen CFU counts. A variable degree of spleen enlargement was observed in mice after inoculation. As shown in Table 3, on D1 the mean spleen weight in inoculated mice was already significantly greater than that in noninfected mice ($P < 0.01$). Spleen weights and the numbers of CFU in the spleens of 150 mice in experiment I, including 30 infected but untreated and 120 treated mice, are presented in Fig. 1. Because there was a linear correlation between spleen weights and the spleen CFU ($\log_{10}$) ($r = 0.876$, $P < 0.0001$) (the greater the spleen weight, the higher the CFU [$\log_{10}$] count), spleen weight is therefore also useful as a parameter for assessing both the severity of infection and the effectiveness of treatment.

Anti-MAC activities of 4 weeks of monotherapy with various antimicrobial agents (experiment I). Mean spleen weights and the mean numbers of CFU in the spleens and lungs of each group of mice after treatment with various antimicrobial agents for 4 weeks (Table 3) indicate that (i) CLARI was active, as shown by the significantly lower spleen weights and the numbers of CFU in the organs of all three treated groups (50, 100, and 200 mg/kg) compared with those for untreated control mice sacrificed simultaneously ($P < 0.01$). The activity of CLARI was dose related because the values decreased progressively when the dosages of CLARI increased: at a dose of 50 mg/kg, CLARI displayed only a bacteriostatic effect, whereas a dose of 200 mg/kg was bactericidal, and 4 weeks of treatment reduced the number of CFU by more than 1 $\log_{10}$

TABLE 3. Mean spleen weights and organ CFU counts in control groups and mice treated with various antimicrobial agents for four weeks (experiment 1)[a]

Group, day (dose [mg/kg])	Spleen wt (mg)	CFU (log_{10})/organ[b]	
		Spleen	Lung
Noninfected, D1	80 ± 12	ND[c]	ND
Infected and untreated (control), D1	111 ± 16	5.39 ± 0.29	4.12 ± 0.23
Control, D14	649 ± 188	7.04 ± 0.33	5.42 ± 0.87
CLARI (200), D14	137 ± 40	4.00 ± 0.52	2.75 ± 0.43
Noninfected, D28	75 ± 18	ND	
Control, D28	740 ± 230	7.57 ± 0.59	6.46 ± 0.62
CLARI (50)	340 ± 115	5.52 ± 0.51	3.78 ± 0.69
CLARI (100)	275 ± 102	5.17 ± 0.27	3.09 ± 0.89
CLARI (200)	137 ± 60	4.00 ± 0.54	2.48 ± 0.57
RBT (10)	748 ± 294	7.63 ± 0.37	6.15 ± 0.23
RMP (10)	782 ± 179	7.62 ± 0.24	6.51 ± 0.53
AMIKA (100)	221 ± 56	5.17 ± 0.17	2.15 ± 0.63
EMB (125)	294 ± 61	5.27 ± 0.43	3.33 ± 0.37
RMB-RBT	375 ± 97	5.53 ± 0.50	3.53 ± 0.48
SPFX (50)	472 ± 137	6.50 ± 0.42	4.89 ± 0.30
SPFX (100)	411 ± 189	5.89 ± 0.48	3.71 ± 0.43
CLO (20)	553 ± 102	6.52 ± 0.22	5.88 ± 0.25

[a] Treatment was begun on D1 and was continued to D28. Mice were sacrificed 48 h after administration of the last dose of the treatment. Each value represents the mean for 10 mice.
[b] The CFU was enumerated on 10% OADC-enriched 7H11 agar medium.
[c] ND, not determined.

unit from the pretreatment values. (ii) Both RBT and RMP were totally inactive. (iii) AMIKA and EMB displayed modest but significant degrees of bactericidal activity that were similar to that of CLARI at 100 mg/kg. (iv) SPFX showed a dose-related bacteriostatic effect. The activity of SPFX at 100 mg/kg was similar to that of CLARI at 50 mg/kg. (v) CLO displayed a very weak bacteriostatic effect that was similar to or weaker than that of SPFX at 50 mg/kg. (vi) The values for mice treated with EMB-RBT, the only combined regimen in experiment I, did not differ significantly from those for mice treated with EMB alone, indicating that the combination did not show a synergistic effect.

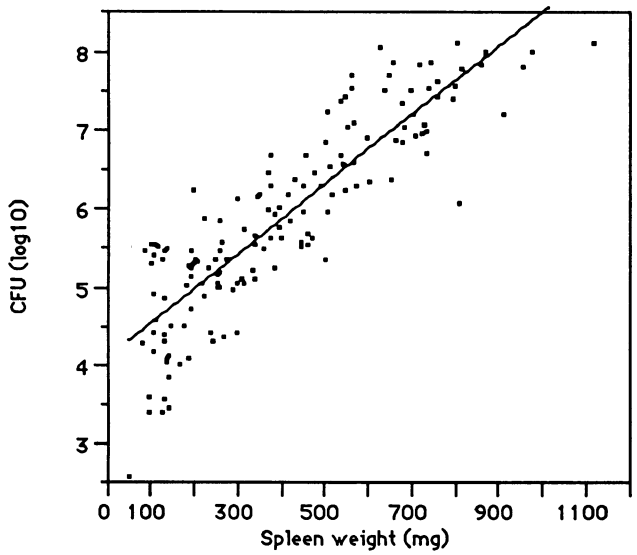

FIG. 1. Scattergram of log_{10} CFU in spleens against spleen weight for 150 mice in experiment I. After inoculation with $10^{6.70}$ CFU of MAC strain 101 per mouse, treatment was begun on D1 at a frequency of six times weekly and was continued for 4 weeks. All mice were sacrificed 48 h after administration of the last dose of the treatment.

Anti-MAC activities of 4 weeks of treatment by multidrug regimens (experiment II). After 4 weeks of treatment, the mean spleen weights and numbers of CFU per organ for all treated groups were significantly lower than those for the corresponding control mice (Table 4). When compared with the pretreatment values, CLARI at 200 mg/kg alone was active; the activity of the combination CLARI-EMB-SPFX did not differ significantly from or was only slightly better than that of CLARI alone; the activities of the three combinations consisting of CLARI, EMB, and RPT, RMP, or RBT were virtually the same and were slightly greater than that of CLARI alone. Therefore, the combination of EMB with either SPFX, RMP, RPT, or RBT only marginally increased the activity of CLARI. Thus, the in vitro synergistic effects of triple drug combinations containing both CLARI and EMB (18) were not confirmed in beige mice.

Carryover of CLO in the organ suspensions. As is also shown in Table 4, the mean numbers of CFU in the spleens and livers of mice treated with the two CLO-containing combinations, CLARI-MINO-CLO or CLARI-EMB-RMP-CLO, were significantly smaller than the pretreatment values and the values for the other treated groups ($P < 0.01$). However, enumeration of the CFU in mice treated with the two CLO-containing regimens is problematic. As shown in Table 5, either no colony (group F) or a few colonies (group D) were observed on agar medium plated with undiluted suspensions, which showed significantly fewer colonies than those plated with 10^{-1} diluted suspensions, and there were disproportionately fewer numbers of colonies on agar medium plated with the suspension diluted 10^{-1} than on those plated with 10^{-2} diluted suspensions. The most likely explanation of the phenomenon is drug carryover from the organ suspensions. There was a very significant difference in the CFU counts between mice treated with CLARI-EMB-RMP and those treated with CLARI-EMB-RMP-CLO (Table 4), it has been reported that CLO accumulates to high levels in the organs of mice treated with CLO at 50 mg/kg for only 12 days (1), and the MIC of CLO for 90% of MAC strains tested on Mueller-Hinton agar medium was 0.06 μg/ml (unpublished data). If any drug is carried over, it should be CLO. Because of the carryover, the small numbers of CFU in mice treated with the two CLO-containing combined regimens must be interpreted with great caution.

Carryover of CLO was more evident in experiments III, IV, and V, in which the duration of treatment was 12 weeks and the CFU were enumerated simultaneously on 5% OADC-enriched Mueller-Hinton agar and L-J medium. Almost all of the cultures of organs from mice treated with CLO-containing combined regimens were negative on both media. The mean weights of the spleens of mice treated with these CLO-containing combined regimens (Table 6) did not differ significantly from those of the spleens of mice treated with CLARI alone; cultures of organs from the latter group were all positive, indicating that use of L-J medium does not solve the problem caused by the carryover of CLO. The only significant difference between the two media was that the cultures of both spleens and lungs from mice treated with CLO alone were almost all positive on L-J medium (Fig. 2). Because the mean numbers of CFU in the lungs of mice treated with CLO alone were significantly greater than those in the lungs of mice treated with CLARI alone ($P < 0.05$), it can be concluded that after 12 weeks of treatment, CLO continued to be less effective

TABLE 4. Mean spleen weights and organ CFU in control groups and mice treated with various combined regimens for 4 weeks (experiment II)[a]

Group	Mean spleen wt (mg)	Mean CFU (log_{10}) per[b]:		
		Spleen	Liver	Lung
Control (before treatment)	519 ± 86	7.93 ± 0.55	7.91 ± 0.55	6.33 ± 0.33
Control (end of treatment)	960 ± 166	8.75 ± 0.52	8.93 ± 0.37	8.63 ± 0.28
CLARI alone	736 ± 134	7.34 ± 0.90	7.42 ± 0.50	6.70 ± 0.37
CLARI-EMB-RPT	536 ± 112	6.58 ± 0.71	6.90 ± 0.25	5.14 ± 0.37
CLARI-EMB-RMP	556 ± 89	6.73 ± 0.57	6.96 ± 0.71	5.24 ± 0.19
CLARI-EMB-RBT	463 ± 102	6.87 ± 0.45	7.20 ± 0.46	5.01 ± 0.53
CLARI-MINO-CLO	443 ± 122	4.94 ± 0.76 (?)[c]	4.82 ± 0.44 (?)	5.04 ± 0.17
CLARI-EMB-SPFX	717 ± 192	7.14 ± 0.22	7.17 ± 0.23	5.49 ± 0.33
CLARI-EMB-RMP-CLO	391 ± 79	<3.94 ± 0.77 (?)	<3.90 ± 0.57 (?)	<4.08 ± 0.75 (?)

[a] Mice were sacrificed 48 h after administration of the last dose of the treatment. Each value represents the mean for 7 to 10 mice.
[b] The CFU was enumerated on 5% OADC-enriched Mueller-Hinton agar medium.
[c] (?), uncertainty of the enumeration.

than CLARI, as it was after 4 weeks of treatment in experiment I. In sharp contrast to the fact that the numbers of CFU in the spleens of non-CLO-treated mice were always significantly greater than the corresponding values in the lungs (Tables 3 and 4 and Fig. 2), the mean number of CFU in the spleens of mice treated with CLO alone was significantly smaller than that in lungs of the same mice and was also significantly smaller than that in the spleens of mice treated with CLARI alone (Fig. 2). These results suggest that, even in the spleens of mice treated with CLO alone, the number of CFU determined on L-J medium was still partially suppressed by carried over CLO.

TABLE 5. Enumeration of CFU in spleens of controls and mice treated with CLARI-MINO-CLO (group D) and CLARI-RMP-EMB-CLO (group F) for 4 weeks

Group and mouse no.	No. of colonies per quadrant plated with the dilutions[a]:								CFU/spleen
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Control									
1					∞,∞	∞, ∞	35, 37[b]	3, 5	9.10
2					∞, ∞	83, 90[b]	7, 7	1, 1	8.48
3					∞, ∞	26, 35[b]	3, 10	0, 1	8.03
4					∞, ∞	26, 42[b]	6, 6	0, 0	8.07
5					∞, ∞	157, 162[b]	7, 15	0, 0	8.75
6					∞, ∞	∞, ∞	21, 27[b]	4, 5	8.92
7					∞, ∞	∞, ∞	38, 48[b]	3, 7	9.17
8					∞, ∞	∞, ∞	64, 93[b]	7, 9	9.44
Mean ± SD					∞, ∞	∞, ∞	64, 93[b]	7, 9	9.44
Group D									
1	0, 0	0, 0	0, 0	0, 2[b]	0, 0				4.54 (?)
2	0, 1	2, 4	0, 2	1, 1[b]	0, 0				4.54 (?)
3	0, 0	1, 3	0, 0	1, 1[b]	0, 0				4.54 (?)
4	Cont., Cont.	46, Cont.	3, 10	0, 1[b]	0, 0				4.24 (?)
5	9, 10	15, 30	5, 7	3, 3[b]	0, 0				5.02 (?)
6	12, 23	35, 64	18, 24	4, 5	1, 1[b]				5.54 (?)
7	0, 0	43, 58	7, 10	4, 5	1, 1[b]				5.54 (?)
8	0, 0	8, 24	10, 11[b]	0, 1	0, 0				4.57 (?)
Mean ± SD									4.94 ± 0.76 (?)
Group F									
1	0, 0	0, 1	0, 1[b]	0, 0	0, 0				3.24 (?)
2	0, 0	0, 0	0, 1[b]	0, 0	0, 0				3.24 (?)
3	0, 0	1, 2[b]	0, 0	0, 0	0, 0				2.72 (?)
4	0, 0	0, 0	0, 0	0, 1*	0, 0				4.24 (?)
5	0, 0	0, 0	0, 0	0, 0	0, 0				(?)
6	0, 0	1, 1	0, 0	2, 2	0, 1*				5.24 (?)
7	0, 0	0, 0	0, 0	0, 0	0, 0				(?)
8	0, 0	1, 3	3, 4[b]	0, 0	0, 0				4.09 (?)
9	0, 0	8, 10	5, 5	1, 1[b]	0, 0				4.54 (?)
10	0, 0	5, 5	1, 1	0, 1[b]	0, 0				4.24 (?)
Mean ± SD									<3.94 ± 0.77 (?)

[a] ∞, >200 colonies; (?), uncertainty of the calculation; Cont., contamination.
[b] The numbers of colonies taken for calculation of CFU.

TABLE 6. Mean spleen weights in mice in experiments III, IV, and V

Day and group[a]	Mean spleen wt (mg)		
	Expt III	Expt IV	Expt V
D14 control (Pretreatment)	395 ± 50	364 ± 65	334 ± 104
D98 control (end of treatment)	714 ± 68	484 ± 115	829 ± 227
CLARI alone	372 ± 99	471 ± 76	276 ± 79
CLO alone	520 ± 108	456 ± 121	309 ± 104
AMIKA alone	441 ± 58	482 ± 114	293 ± 70
CLARI-CLO	323 ± 117	438 ± 110	242 ± 59
CLARI-AMIKA	319 ± 111	483 ± 53	265 ± 85
CLARI-MINO	471 ± 63	424 ± 89	286 ± 69
EMB-RMP	663 ± 282		
CLARI-MINO-CLO	318 ± 169	433 ± 72	271 ± 58
CLARI-EMB-RMP-CLO	396 ± 109	450 ± 95	230 ± 56
CLARI-AMIKA-CLO		426 ± 89	231 ± 54
CLO-AMIKA			263 ± 51

[a] Treatment was begun on D14 and was continued to D98 after inoculation. Drugs were administered six times weekly at the following dosages: CLARI at 200 mg/kg, CLO at 20 mg/kg, AMIKA at 100 mg/kg, MINO at 25 mg/kg, RMP at 10 mg/kg, and EMB at 125 mg/kg.

The accumulation of CLO in the organ and carryover of the drug in organ suspensions were confirmed by demonstration of high concentrations of CLO in the organs of beige mice treated with CLO at 20 mg/kg six times weekly for 12 weeks in experiment IV. The mean concentrations of CLO, as determined by high-pressure liquid chromatography (30), were 4.9 ± 1.1, 20.8 ± 18.9, and 1.2 ± 0.45 μg/ml in the "undiluted" spleen, liver, and lung suspensions, which respectively, was equivalent, to 39.3 ± 12.3, 41.3 ± 39.6, and 19.3 ± 6.5 μg/g in the spleens, livers, and lungs, respectively.

Anti-MAC activities of 12 weeks of treatment with various regimens that did not include CLO. In experiments III, IV, and V, the CFU were enumerated in parallel on 5% OADC-enriched Mueller-Hinton agar and L-J medium. Because the results indicated that L-J medium supported the growth of MAC at least as well as Mueller-Hinton agar medium did (data not shown) and because the organisms from mice treated with CLO alone multiplied better on L-J medium than on Mueller-Hinton agar, only the results obtained on L-J medium are presented in Fig. 2. After 12 weeks of treatment, regardless of the strain of MAC used, the mean numbers of CFU in mice treated with CLARI were always significantly smaller than pretreatment values (Fig. 2), although the reductions in the numbers of CFU varied from 1 to 2 $\log_{10}$ units in experiment V to more than 4 $\log_{10}$ units in experiment IV; the mean spleen weights of this group remained at the same level as that before treatment (Table 6). Both parameters in mice treated with AMIKA alone were, in general, comparable to those in mice treated with CLARI alone (Table 6 and Fig. 2), indicating that 12 weeks of treatment with AMIKA displayed bactericidal activity similar to that of the same duration of treatment with CLARI. The mean spleen weight and the number of CFU in mice treated with CLARI-AMIKA did not differ significantly from those in mice treated with monotherapy of the stronger component in the combination, suggesting that the combination did not enhance the bactericidal activity. The numbers of CFU in mice treated with CLARI-MINO were virtually the same as those in mice treated with CLARI alone. After 12 weeks of treatment, the combination EMB-RMP merely displayed a bacteriostatic effect, because the number of CFU remained at the same level as before treatment (Fig. 2, experiment III).

Selection of CLARI-resistant mutants in the spleens of untreated controls and treated mice. CLARI-resistant mutants, defined as the organisms that multiplied on Mueller-Hinton agar containing CLARI at 16 μg/ml, were isolated from a majority of the spleens of untreated control mice on D98 (Table 7). The frequencies of CLARI-resistant mutants in untreated bacterial populations of different strains were between 10^{-7} and 10^{-8}. CLARI-resistant mutants were also isolated on D98 from mice treated with CLARI alone or CLARI-MINO, but not from mice treated with CLARI-AMIKA (Table 7). The proportions of spleens containing resistant mutants among the former two treated groups varied from experiment to experiment (range, 1 of 10 to 7 of 9); such variation was most likely caused by the differences in the bacterial populations in the spleens when the treatment was begun. The smallest proportion, 1 of 10, was encountered in experiment IV, in which the bacterial population was only $10^{5.74}$ per spleen when the treatment was begun; because the frequency of CLARI-resistant mutants in an untreated bacterial population was between 10^{-7} and 10^{-8}, such a small population was very unlikely to include a resistant mutant. Even though the total numbers of CFU in treated mice were far smaller than those in untreated controls (Fig. 2), the frequencies of resistant mutants in mice treated with CLARI alone or CLARI-MINO were very significantly greater than those in controls ($P < 0.001$), indicating that the CLARI-resistant mutants multiplied selectively during the course of treatment with the two regimens. The proportions of spleens harboring CLARI-resistant mutants and the frequencies of CLARI-resistant mutants in mice treated with CLARI-MINO were virtually the same as those in mice treated with CLARI alone, whereas no CLARI-resistant mutants were isolated from mice treated with CLARI-AMIKA (Table 7), indicating that the combination of CLAR-MINO could not prevent the selection of mutants resistant to CLARI, whereas CLARI-AMIKA could.

DISCUSSION

The antimicrobial agents reported to be active against MAC in vitro or in vivo include the macrolides (CLARI and azithromycin), the aminoglycosides (AMIKA and streptomycin), the rifamycins (RMP, RBT, and RPT), the fluoroquinolones (SPFX and ciprofloxacin), an ethylenediamine (EMB), and a rimino phenazine (CLO). Therefore, the antimicrobial agents tested in experiment I, CLARI, RMP, RBT, AMIKA, EMB, SPFX, and CLO, represented almost all of the classes of compounds with potential activity against MAC. This appears to be the first comparison of all of these compounds in a single experiment.

The results of all five experiments demonstrated that CLARI is active against MAC in beige mice in a dose-dependent fashion: at a dosage of 50 mg/kg daily, the drug displayed a bacteriostatic effect, whereas a four-times-greater dosage displayed a bactericidal effect. These results are consistent with those of earlier studies both in vitro (8, 15, 33) and in vivo (6, 8, 17, 19, 25). Because CLARI at a dosage of 200 mg/kg daily displayed similar bactericidal activity against all three strains of MAC tested in our laboratory, because the same dosage of CLARI was active against all four other strains of MAC tested by other investigators (25), and because all AIDS patients with disseminated MAC infection responded favorably to treatment with CLARI (6), it appears reasonable to assume that if a drug was truely active against MAC it should be expected to be active against the majority of the

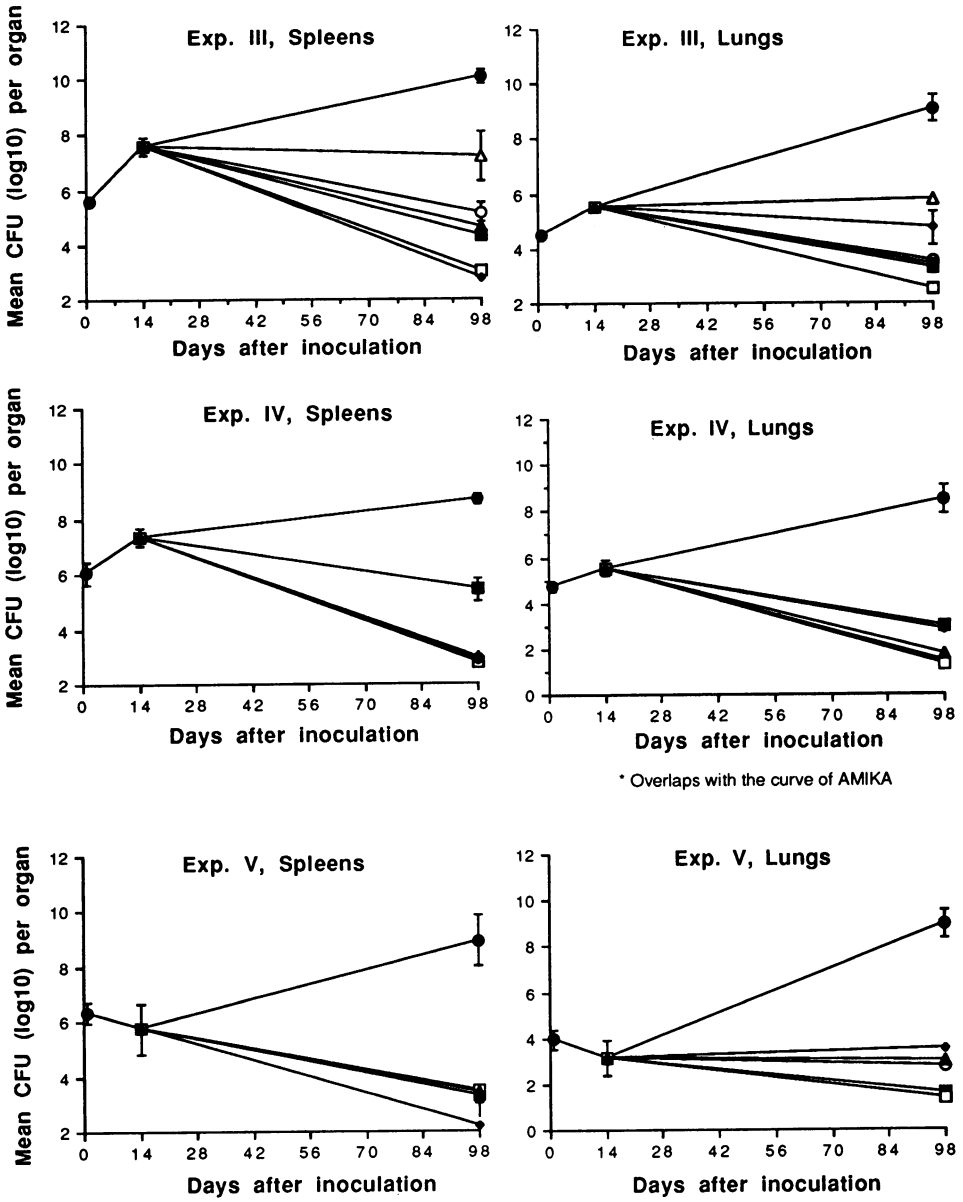

FIG. 2. Mean CFU counts of MAC in experiments III, IV, and V. Mice were inoculated intravenously with about 10^7 CFU of different MAC strains; treatments at a frequency of six times weekly were begun 14 days later and were continued for 12 weeks. The CFU were enumerated on L-J medium. Each points represents the mean number of CFU from 7 to 10 mice. Error bars represent standard deviations. Symbols for experiment III: ●, control; ○, CLARI; ■, AMIKA; □, CLARI-AMIKA; ▲, CLARI-MINO; △, EMB-RMP; ◆, CLO. Symbols for experiments IV and V: ●, control; ○, CLARI; ■, AMIKA; □, CLARI-AMIKA; ▲, CLARI-MINO; ◆, CLO. The results for CLO in experiment IV (lungs) overlap with those for AMIKA.

MAC strains in beige mice as well as in patients with disseminated MAC infection.

CLARI is one of the few antimicrobial agents that has shown unequivocal activity against MAC in vitro (8, 15, 33), in vivo (8, 17, 19, 25), and in patients (6, 7). However, because of the enormous bacterial population in AIDS patients with disseminated MAC infection, patients frequently have relapses with CLARI-resistant organisms after monotherapy with CLARI for more than 3 months (6); in addition, the selection of CLARI-resistant mutants by CLARI monotherapy in beige mice has been well documented (19). Therefore, monotherapy with any antimicrobial agent is not an appropriate therapy for disseminated MAC infection, and it is important to identify companion drugs that might prevent the emergence of organisms resistant to CLARI or other active drugs. The fact that CLARI-resistant mutants were isolated from mice treated with CLARI alone or CLARI-MINO but not from mice treated with CLARI-AMIKA are encouraging; this is the first demonstration that a companion drug has successfully prevented the selection of CLARI-resistant mutants. The failure of the combination CLARI-MINO in preventing the selection of CLARI-resistant mutants suggests that an inactive compound such as MINO (35) could not serve as a companion drug with CLARI. The potential contribution of EMB or SPFX as a

TABLE 7. CLARI-resistant mutants in spleens of untreated control and treated mice[a]

Expt. no. (MAC strain)	Total $\log_{10}$ CFU (day 14[b])	Proportion of spleens with mutants on D98[c]				Frequency of mutants in bacterial population on D98[d]			
		Untreated control	CLARI alone	CLARI-MINO	CLARI-AMIKA	Untreated control	CLARI alone	CLARI-MINO	CLARI-AMIKA
III (101)	07.57 ± 0.30	8/8	5/8	6/8	0/9	$10^{-7.87} \pm 10^{-0.36}$	$10^{-3.53} \pm 10^{-0.26}$	$10^{-2.55} \pm 10^{-0.70}$	$10^{-1.80} \pm 10^{-1.09}$
IV (Lpr)	7.34 ± 0.32	6/8	5/10	7/9	0/8	$10^{-6.74} \pm 10^{-0.79}$	$10^{-1.09} \pm 10^{-0.34}$	$10^{-1.24} \pm 10^{-0.94}$	$10^{-1.54} \pm 10^{-0.70}$
V (MO1)	5.74 ± 0.94	15/19	1/10	1/10	0/8	$10^{-7.45} \pm 10^{-0.44}$	$10^{-1.40}$	$10^{-0.34}$	$10^{-2.23} \pm 10^{-0.45}$

[a] CLARI-resistant mutants were defined as organisms that multiplied on Mueller-Hinton agar containing CLARI at 16 μg/ml.
[b] Treatment was begun on day 14.
[c] Data are numbers of spleens with mutants/total number of spleens tested.
[d] Defined as the ratio between the number of CFU of CLARI-resistant mutants and the number of total CFU in the individual spleen. The mean values were calculated by using data for spleens from which CLARI-resistant mutants were isolated; the values for mice treated with CLARI-AMIKA, however, were estimated from all by using data for all of the spleens.

companion drug should be tested in future experiments; although CLO may also be considered, the experiments will face the difficulties caused by the carryover of CLO, because it will be necessary to administer the drug for a long time.

The activity of monotherapy with AMIKA at a dosage of 100 mg/kg daily was tested against three different strains of MAC, against all of which it demonstrated a bactericidal effect, as has been observed by other investigators (5, 12, 16). Depending on the duration of treatment, its activity was either slightly weaker than or similar to that of CLARI given at a dosage of 200 mg/kg daily.

Because AMIKA must be administered by injection and because its use has been associated with potential ototoxicity and nephrotoxicity, it has not been widely used for the treatment of disseminated MAC infection, and its duration of administration is usually limited to 8 weeks. However, because it is the only one of the drugs tested that might prevent the selection of mutants resistant to CLARI, its importance in the treatment of MAC infection may gradually increase until other effective companion drugs are identified. Because AMIKA displayed a significant postantibiotic effect against MAC (2), it may be possible to administer the drug intermittently, thus permitting a substantial increase in the duration of treatment. In order to use AMIKA in the most effective way, additional studies in beige mice are required to compare the activities of different dosages, frequencies, and durations of AMIKA administration in preventing the selection of CLARI-resistant mutants.

With respect to the rifamycin derivatives, we always treat the mice with no more than 10 mg/kg per dose because this is equivalent to the clinically tolerated dosage of all major rifamycin derivatives, and the pharmacokinetic properties of rifamycin derivatives in mice are similar to those in humans (21). After 4 weeks of treatment with 10 mg/kg daily, both RMP and RBT were totally inactive in beige mice, a result similar to those of Fernandes et al. (8) and Gangadharam et al. (11). In fact, at a dosage of 10 mg/kg daily, neither RBT nor any other rifamycin derivative has demonstrated unequivocal bactericidal activity against MAC in beige mice (8, 11, 23, 24, 26), suggesting that, at a clinically tolerated dosage, RBT and other rifamycin derivatives can have only a very limited role in the treatment of MAC infection, notwithstanding the report that RBT at 300 mg (about 5 mg/kg) daily is the only therapy that is effective in preventing MAC infection in humans (29). Although the MICs of RBT for MAC were only 1/4th those of RMP (4), this advantage was counterbalanced by its relatively low maximum concentration of drug in serum and area under the concentration-time curve, which were 1/5th and 1/12th those of RMP in mice, respectively (21). It is therefore understandable that RBT was not more active than RMP against MAC at the same dosage.

The MIC of EMB for 90% of MAC isolates tested ranged from 20 (38) to 32 μg/ml (unpublished data), which is much greater than the peak level achievable in the sera of humans (38). However, 4 weeks of treatment with EMB alone displayed a weak bactericidal effect, similar to that of CLARI at 100 mg/kg, against MAC in beige mice; the same duration of treatment with EMB alone also significantly reduced the numbers of CFU in the blood cultures of AIDS patients with disseminated MAC infection (22). Therefore, EMB is one of the very few drugs that displays a degree of bactericidal effect against MAC, although it is less potent than CLARI and is probably also less potent than AMIKA.

In vitro synergism against MAC has frequently been observed in various EMB-containing combinations (14, 18, 36–38), especially when EMB was combined with either RMP (37, 38), ciprofloxacin (37), or SPFX (36), or in triple-drug combinations containing both CLARI and EMB (18). In our experiments in beige mice, we tested the bactericidal activities of a number of combinations that did not include CLO; these included EMB-RBT, CLARI-EMB-RMP, CLARI-EMB-RBT, CLARI-EMB-RPT, and CLARI-EMB-SPFX for 4 weeks and EMB-RMP, CLARI-AMIKA, and CLARI-MINO for 12 weeks. In terms of significantly reducing the CFU counts from that in mice treated with monotherapy with the most potent component (usually CLARI) in the combination, no real synergism was demonstrated in beige mice treated with any of these combinations. Because other investigators have also failed to demonstrate synergism in regimens without CLO in beige mice (11, 23, 27), it appears that the in vitro synergistic effects of the drug combinations against MAC are irrelevant to their in vivo activities.

The MICs and MBCs of SPFX for MAC are significantly lower than those of ciprofloxacin (36), suggesting that, on a weight-to-weight basis, SPFX is more active in vitro than the other fluoroquinlones against MAC. However, SPFX displayed only a weak bacteriostatic effect in beige mice, and the activity of 100 mg/kg daily, which corresponded to 400 mg daily or the highest recommended dosage in humans (28), was similar to that of CLARI at 50 mg/kg daily. Therefore, although the latest developments with fluoroquinolones have led to considerable progress in the treatment of infectious diseases including tuberculosis (28) and leprosy (9), the potential contribution of the available fluoroquinolones to the treatment of disseminated MAC infection is very limited.

Rather limited information on the activity of CLO against MAC infection in beige mice is available. The results of treatment with this drug have characteristically been highly

dependent on the duration of treatment; the numbers of CFU in the organs were only slightly reduced during the initial 10 days to 4 weeks of treatment with CLO at 20 mg/kg daily alone (11, 12, 23) and declined steeply when the duration of treatment was longer than 4 weeks (11, 12). More importantly, the numbers of CFU were very small or the results of culture were negative for mice after 4 to 6 weeks of treatment with several CLO-containing combined regimens, giving the impression that such combined regimens displayed "remarkable activity" or even "complete sterilization" (11, 12). We observed similar phenomena in our experiments. When the drug was administered alone for 4 weeks, CLO demonstrated only a very weak bacteriostatic effect. Cultures on agar media (commonly used by most investigators) of the organs from mice that had been treated with CLO alone for 12 weeks were all negative, whereas the numbers of CFU in the lungs determined on L-J medium were significantly greater than those in the lungs of mice treated with CLARI alone for 12 weeks. After carefully reviewing all of the available data, we believe that the in vivo activity of CLO against MAC is very weak and in definitely weaker than that of CLARI; the very low CFU counts or negative cultures for mice treated with regimens containing CLO either alone or in combination were due to the carryover of CLO in the organ suspensions.

To overcome the difficulties caused by the carryover of CLO, we tried to enumerate the CFU on L-J medium. It was thought that L-J medium was an appropriate medium because it supports the growth of MAC at least as well as agar medium and because the MIC of CLO for 90% of organisms tested on L-J medium was very high (64 μg/ml [unpublished data]), which is significantly greater than the concentration of CLO (1.2 to 20.0 μg/ml) carried over in the organ suspensions. However, the use of L-J medium failed to solve the problem of carryover because we ignored the fact that CLO is lipophilic and failed to dilute it appropriately while preparing the tissue suspensions for CFU counts. It is very likely that in the tissue suspensions CLO remained undiluted in the immediate vicinity of the bacilli. The only way of diluting CLO properly might be to use a strong water-soluble organic solvent that will not damage the viability of MAC. Such a solvent remains to be identified.

In summary, the results of the current experiments indicated that, among the drugs tested, only CLARI, AMIKA, and EMB displayed definite bactericidal activities against MAC in beige mice; SPFX and CLO showed some bacteriostatic effects, whereas RBT, RMP, and possibly other rifamycin derivatives were inactive. Because the bactericidal activities of CLARI, AMIKA, and EMB are modest to moderate, it is unlikely that combinations of the drugs are capable of sterilizing the disseminated MAC infections in AIDS patients. Therefore, additional drugs, especially with bactericidal mechanisms entirely different from those of the available agents, are needed; and the most realistic and economic approach is to continue the screening of anti-MAC activities among antimicrobial agents that display potent activities against gram-positive microorganisms.

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