In Vitro and In Vivo Chemotherapeutic Activity of Rifabutin (LM 427) on *Mycobacterium avium-intracellulare* Complex

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SUMMARY

In vitro chemotherapeutic activity of rifabutin against *Mycobacterium avium* (*-intracellulare*) complex was studied with 20 Japanese disease-associated strains. *In vitro* superiority of rifabutin to rifampin (RMP) was clearly demonstrated in three different kinds of media. A tendency of parallelism in susceptibility of individual strains between rifabutin and RMP was confirmed in a correlation analysis of MICs of the both drugs in Kirchner's liquid medium, but not in a modified Dubos Tween® albumin liquid medium.

The murine experimental infection with a mouse-virulent strain 31F093T was used to evaluate *in vivo* activity of rifabutin in comparison with that of RMP with 3 treatment dosages. Rifabutin suppressed moderately the total number of grossly visible lung disease. In the evaluations of consecutive viable bacilli in the organs of mice, neither superiority of rifabutin to RMP, no dose responses of the both drugs were demonstrated.

This challenging results could be mostly due to a high resistance of 31F093T strain to rifabutin.

INTRODUCTION

Pulmonary and disseminated disease caused by *Mycobacterium avium* complex is of growing importance due to its high frequency in complicating locally as well as systemically compromised hosts (1-3). The organism is resistant to most of the available antimycobacterial drugs, and even chemotherapeutic trials of various multidrug regimens have been far from satisfactory (4-6).

Urgent need for better chemotherapeutic agents prompted us to evaluate especially *in vivo* activity of rifabutin (a spiro-piperidyl rifamycin, LM 427), which has been reported to have high *in vitro* activity against mycobacteria (7-12).

The present study was planned to assess, first, the *in vitro* activity of the drug against *M. avium* complex strains isolated in our laboratory, and then to evaluate *in vivo* activity in our experimental murine infection model (13-15).

METHODS

Rifabutin

Rifabutin (in pure powder) was obtained from Dr. A. Sanfilippo and Dr. C. Della Bruna of Microbiological Department, Farmitalia Carlo Erba C., Italy.

In vitro study

Mycobacterium avium complex strains used in the present *in vitro* study were all individually disease-associated, and the isolates before any treatment. Twenty strains were selected because of showing high purity of smooth thin-transparent colony form (16). The strains had been stored in a refrigerator at 4°C on 1% Ogawa medium until the experiments. Before the assessment of *in vitro* activity, their colony types were observed on cornmeal agar media with 1.5% glycerol, and the purification to smooth thin-transparent colony type was done by the method described beforehand (16). Inoculums were made by transferring to a modified Dubos Tween® albumin liquid medium (DTA), and the 2 week-old cultures were diluted with sterile normal saline so as to inoculate ca. 0.01 mg (0.05 ml) of bacilli into liquid media and also ca. 0.01 mg (0.1 ml) on Ogawa medium, respectively, with the aid of a comparable barium sulfate solution

Three kinds of medium, the modified Dubos Tween® albumin liquid medium with 0.05% Tween® 80 (DTA), Kirchner's liquid medium with 10% bovine serum (KB), and 1% Ogawa solid medium, were used for determination of MICs of rifabutin. The MICs of rifabutin were compared simultaneously with those of rifampin (RMP), Kanamycin (KM), and cefazolin (CEZ) (17) in the liquid media ; with only RMP on 1% Ogawa medium. Rifabutin and RMP were initially disolved in methanol (98%) and DMSO, respectively. In the liquid media, serially doubly diluted concentrations of the drugs (2.0 ml) were tested for determination of MICs, while the growths were observed on the Ogawa media containing 10, 25, 50, 100, 150, 200, 250, 300, and 400 µg/ml of the drugs. The MICs were determined after 2 wks incubation in the liquid media, 4 wks in Ogawa medium.

In vivo study

The mouse-virulent strain *M. avium* complex 31F093T (No. 6 in table 1) (15) was used to infect conventional ddY male mice weighing 20 to 25 g. The inoculum of the strain 31F093T was produced in the modified Dubos Tween® albumin liquid medium, and 0.2 ml of the 2-wk-old culture was used for intravenous injection into a tail vein in each mouse. Appropriate dilutions of the inoculum were inoculated onto Ogawa media to determine the number of colony forming units. The strain 31F093T was also included in the present *in vitro* study, and revealed to be partially resistant to 50 µg/ml of rifabutin on Ogawa medium (table 1).

Both rifabutin and RMP were orally administered of 2.0 ml of the drug emulsions, directly into the stomach through a small metal tube attached to a 1-ml syringe. Treatment dosages in milligrams per kilogram per day in both drugs were 25, 12.5, and 6.25. Treatment was begun immediately after infection, and was continued through 12 wk of infection, 6 days per week.

For evaluation of the effect of the chemotherapeutic regimens, 5 mice in each treatment group were killed at 1 wk and 3 wks of infection, and thereafter at 3 wks interval for observation of gross lesions, organ weights, and for counting viable bacilli in organs. With 3 of the 5 mice

killed, bacilli in organs were enumerated. Respective organs of 3 mice were combined and weighed, then ten fold organ homogenates with 2.0% NaOH were obtained, using an electric homogenizer. Counting of viable bacilli in organs was done as previously described (15).

RESULTS

In vitro study

Cumulative percentages of the 20 strains of *M. avium* complex inhibited by rifabutin in both DTA and KB were illustrated in figure 1 and 2, respectively. In DTA, rifabutin at concentration of 0.1 μg/ml inhibited all the 20 strains, whereas the same concentration of RMP inhibited only 4 strains. CEZ and KM were slightly less active than RMP. The concentrations which inhibited 80% of the strains tested, were 0.05 μg/ml in rifabutin and 1.56 μg/ml in RMP. Superiority of rifabutin to RMP was clearly demonstrated in DTA (figure 1). Although the absence of Tween® 80 and possibly the addition of bovine serum lowered considerably the activities of both rifabutin and RMP, the superiority of rifabutin to RMP was again demonstrated in KB. Rifabutin inhibited all the 20 strains at the concentration of 6.25 g/ml, and only 20% of the strains were inhibited at the same concentration of RMP (figure 2). Ten strains were randomly selected from the 20 strains, and the activity of rifabutin was studied on Ogawa medium (Table 1). The potent activity of rifabutin was also demonstrated on this medium. Rifabutin inhibited 9 out of the 10 strains at the concentration of 50 μg/ml, while 9 strains were resistant to 50 μg/ml of RMP. There existed two groups of strains, five strains demonstrated high degree of resistance to RMP, MICs of which were more than 400 μg/ml, while the remains showed lower resistance to RMP. Four strains of the latter were susceptible to less than 10 μg/ml of rifabutin, while the former were all resistant to 25 μg/ml of rifabutin.
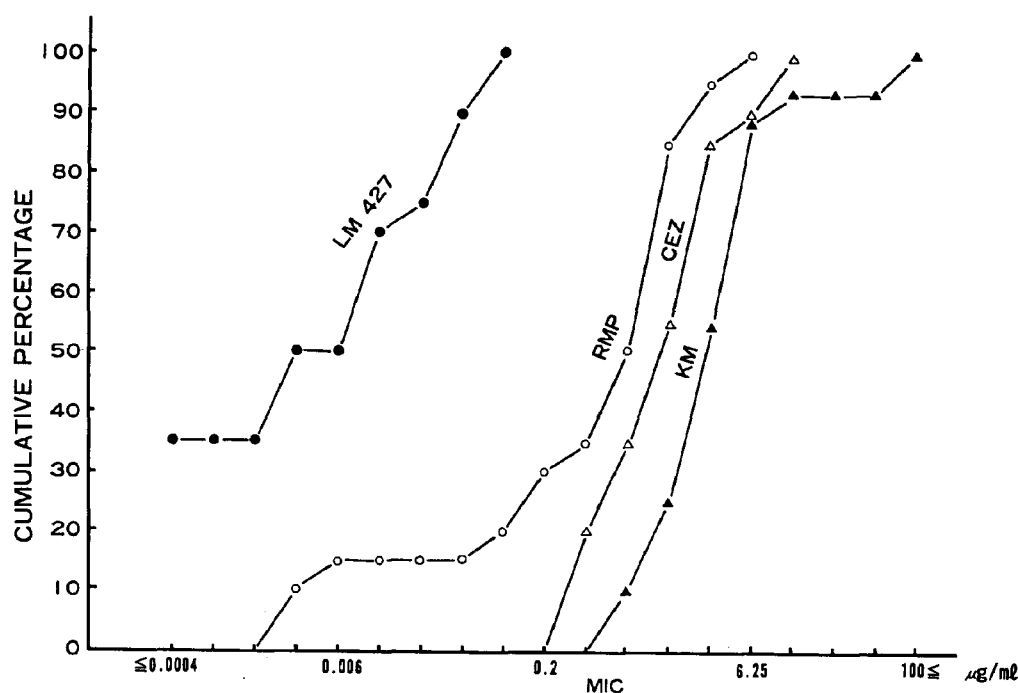

Fig. 1. Cumulative percentage of 20 strains of *M. avium* complex inhibited by LM 427, RMP, KM, and CEZ in a modified Dubos Tween® albumin medium.
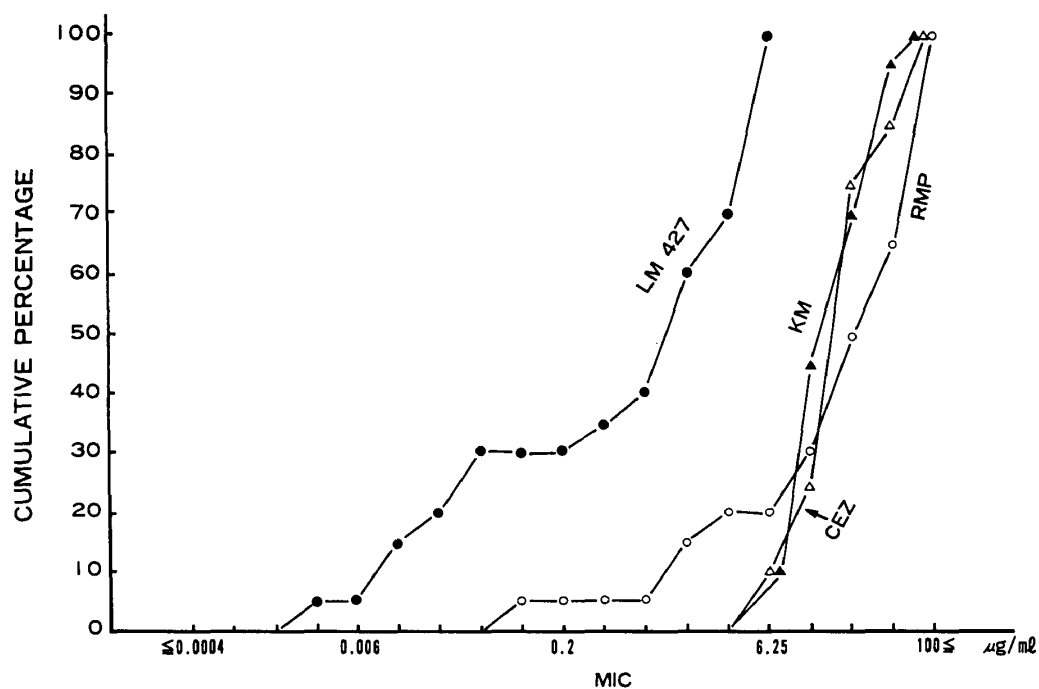
Fig. 2. Cumulative percentage of 20 strains of *M. avium* complex inhibited by LM 427, RMP, KM, and CEZ in Kirchner's medium.

Table 1 *In Vitro* Susceptibility of Rifabutin (LM427) and RMP on Ogawa Medium

Four weeks after inoculation, Inoculum 10^{-3}mg

Strain		Drug concentrations (μg/ml)									
		10	25	50	100	150	200	250	300	400	C
1	RMP	++	++	++	—	—	—	—	—	—	++++
	LM 427	—	—	—	—	—	—	—	—	—	++++
2	RMP	+++	+++	+	—	—	—	—	—	—	++++
	LM 427	—	—	—	—	—	—	—	—	—	++++
3	RMP	+++	++	+	+	—	—	—	—	—	++++
	LM 427	—	—	—	—	—	—	—	—	—	++++
4	RMP	++++	++++	++++	++++	+++	+++	+++	++	+	++++
	LM 427	++	+	—	—	—	—	—	—	—	++++
5	RMP	++++	++++	+++	+++	+++	++	++	+	+	++++
	LM 427	++	+	—	—	—	—	—	—	—	++++
6*	RMP	++++	++++	++++	++++	++++	++++	+++	++	+	++++
	LM 427	+++	++	+	—	—	—	—	—	—	++++
7	RMP	++++	++++	++++	++++	++++	+++	+++	+	+	++++
	LM 427	+++	+	—	—	—	—	—	—	—	++++
8	RMP	+++	+++	+++	+++	+++	++	++	+	+	++++
	LM 427	++	+	—	—	—	—	—	—	—	++++
9	RMP	+++	+	—	—	—	—	—	—	—	++++
	LM 427	—	—	—	—	—	—	—	—	—	++++
10	RMP	+++	+++	++	++	+	—	—	—	—	++++
	LM 427	++	—	—	—	—	—	—	—	—	++++

Definition of abbreviations: — =no growth; + =1 to 200 colonies; ++ =200 to 500 colonies; +++ =500 to 2,000 colonies; ++++ =more than 2, 000 colonies. The growths designated as +++ and ++++ are confluent abundant growths. The number of colonies are the estimates by experimental data.; C =control medium.
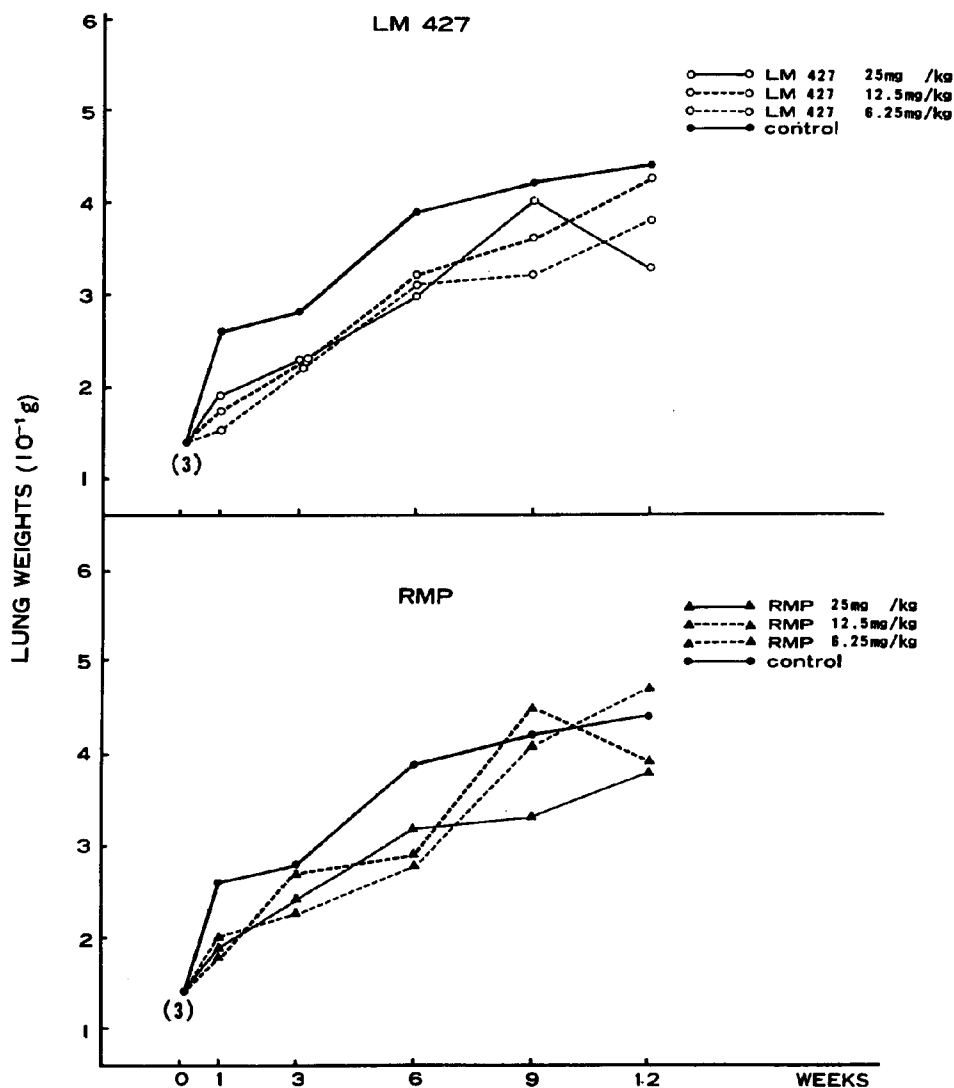* 31T093T strain

Fig. 3. Average lung weights of mice infected with *M. avium* complex 31F093 T. Treatment with LM 427 demonstrated moderate suppressions of lung weights in all 3 treatment dosages. Each point represents the average value for 5 mice except indicated by a number in parentheses.

In vivo study

In view of a high *in vitro* susceptibility of *M. avium* complex to rifabutin, a trial of experimental chemotherapy with our murine model (13, 15) was warranted to evaluate *in vivo* therapeutic efficacies of rifabutin in comparison with those of RMP.

Oral treatment with all three treatment dosages of rifabutin demonstrated moderate suppressions of lung weights during the entire period of experiment (figure 3). No dose responses of rifabutin were clearly discernible, although the highest dosage (25 mg/kg) appeared only the regimen which showed unequivocal suppresion of lung weights in RMP.

Highest dosages of both rifabutin and RMP (25 mg/kg) lowered considerably spleen weights (figure 4). With two lower dosages of both drugs, no dose responses were demonstrated, although slight to moderate suppressions of spleen weights were undoubtedly discernible around 9 wks of infection.

Total number of mice which showed macroscopic lung lesions (+, ++, +++, ++++ in table 2)

Table 2 Chemotherapeutic Effects of Rifabutin (LM427) and Rifampin on *Mycobacterium avium* complex
Infection in Mice   Macroscopic Lesion

Lapse of Time after Infection	Untreated			LM427 25mg/kg			LM427 12.5mg/kg			LM427 6.25mg/kg		
	Lung	Liver (4 to 5 mice)*	Kidney	Lung	Liver (5 mice)	Kidney	Lung	Liver (4 to 5 mice)	Kidney	Lung	Liver (5 mice)	Kidney
1 week	₩‡ + + −	0/4	0/4	− − + − −	0/5	0/5	− + − − ± −	0/5	0/5	− − − − − −	0/5	0/5
3 weeks	++ ₩ ++ ++ +	1†/5	2/5	+ − − − ₩	0/5	0/5	₩ − − − ± +	0/5	2/5	− − − ₩ +	1/5	0/5
6 weeks	₩ ₩ ++ ₩ ₩	3/5	1/5	++ ₩ ₩ + ++	3/5	4/5	++ ₩ ₩ ++ ₩	4/5	1/5	++ + ++ ++ ₩	1/5	2/5
9 weeks	₩ ₩ ₩ ₩ ₩	5/5	2/5	₩ ₩ ₩ + ₩	2/5	1/5	+ ++ ₩ ++ ₩	4/5	2/5	₩ + ₩ ₩ ++	5/5	1/5
12 weeks	₩ ₩ ₩ ₩ ₩	4/5	2/5	+ ₩ ++ ₩ ₩	2/5	2/5	₩ + ₩ ++	4/5	0/5	++ ++ ₩ ₩ ₩	5/5	2/5

Lapse of Time after Infection	RMP 25mg/kg			RMP 12.5mg/kg			RMP 6.25mg/kg		
	Lung	Liver (4 to 5 mice)	Kidney	Lung	Liver (5 mice)	Kidney	Lung	Liver (5 mice)	Kidney
1 week	− − − −	0/4	0/4	− + ++ + −	0/5	0/5	− + − + −	0/5	0/5
3 weeks	− + + − +	0/5	0/5	+ + ++ − ₩	1/5	2/5	− ₩ ++ + ₩	0/5	2/5
6 weeks	₩ − ₩ + ₩	2/5	0/5	₩ ++ ++ − ₩	2/5	2/5	++ ++ ₩ ++ +	1/5	1/5
9 weeks	++ ₩ ₩ ₩ ++	2/5	1/5	₩ ₩ ₩ ₩ ₩	4/5	2/5	₩ ₩ ₩ ₩ ₩	4/5	2/5
12 weeks	₩ ₩ ++ ₩	3/4	2/4	++ ₩ ++ ₩ ₩	4/5	1/5	₩ ₩ ₩ ₩ ₩	4/5	1/5

* Number of mice sacrificed at each interval.

† Number of mice with gross lesions.

‡ grades of lung lesion: − =no gross lesion; + =scattered small nodules; ++ =many small nodules; ₩ =innumerable small nodules; =innumerable small nodules with scattered big nodules.
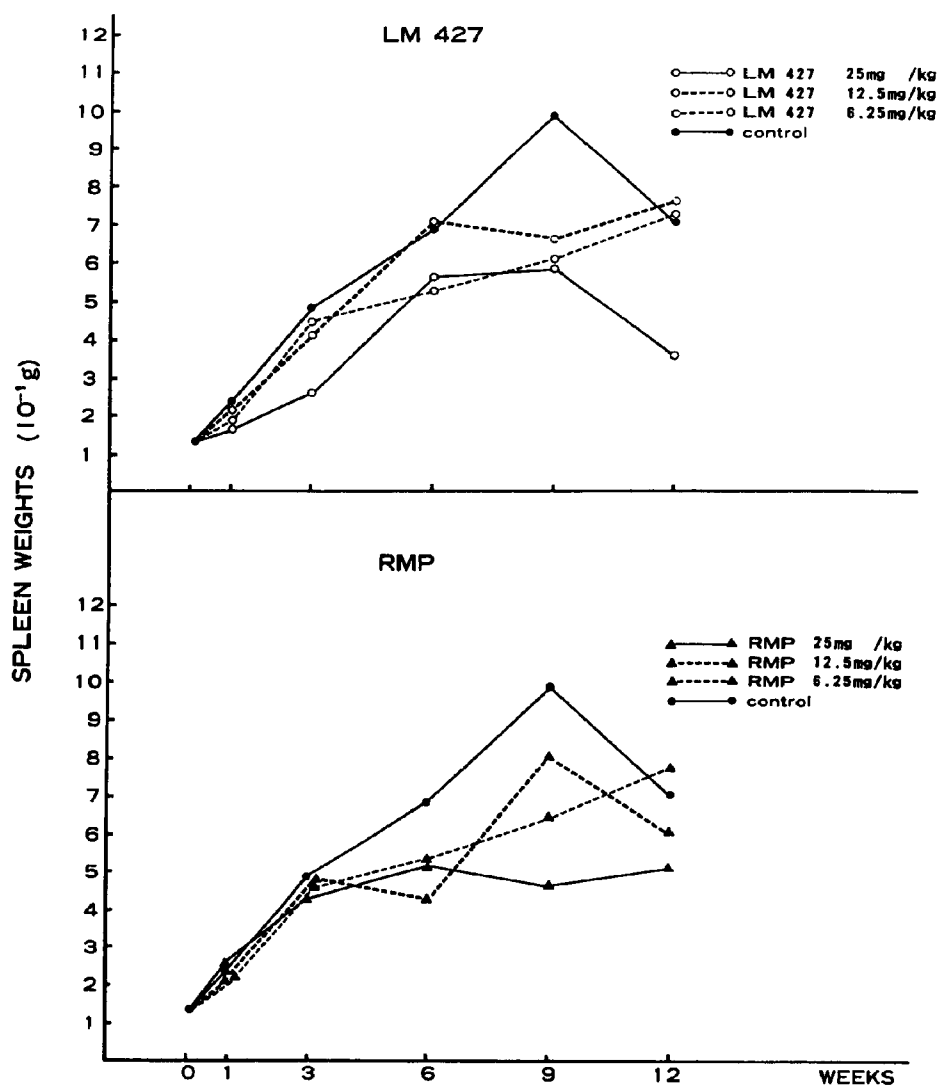
Fig. 4· Average spleen weights of mice infected with *M. avium* complex 31F093T. Highest dosages of both LM 427 and RMP lowered considerably spleen weights. Each point represents the average value for 5 mice.

during the entire period of experimentation were 23 out of 24 (96%) in the untreated mice, while 70% (52/74) and 79% (58/73) of the mice treated with rifabutin and RMP, respectively, demonstrated grossly visible lung diseases (table 2). Concerning advanced diseases (卌 to 卌 in table 2), rifabutin allowed them to develop in 31% (23/74) of mice, while 42% (31/73) demonstrated advanced diseases in the mice treated with RMP. The percentage in untreated mice was 67% (16/24) (Table 2).

The above scores of both the total gross diseases and advanced diseases confirmed therapeutic efficacy of rifabutin (P < 0.05, P < 0.01, X^2-test), although the drug was not potent enough to cure the diseases in single usage.

Total number of gross disease as well as advanced disease appeared lower in the mice treated with RMP than in untreated mice. The differences were, however, not significant in total gross diseases, but significant (P < 0.05) in advanced diseases. No dose responses of both rifabutin and RMP were demonstrated.

Slight reductions of the viable counts of mycobacteria in lung were observed in mice
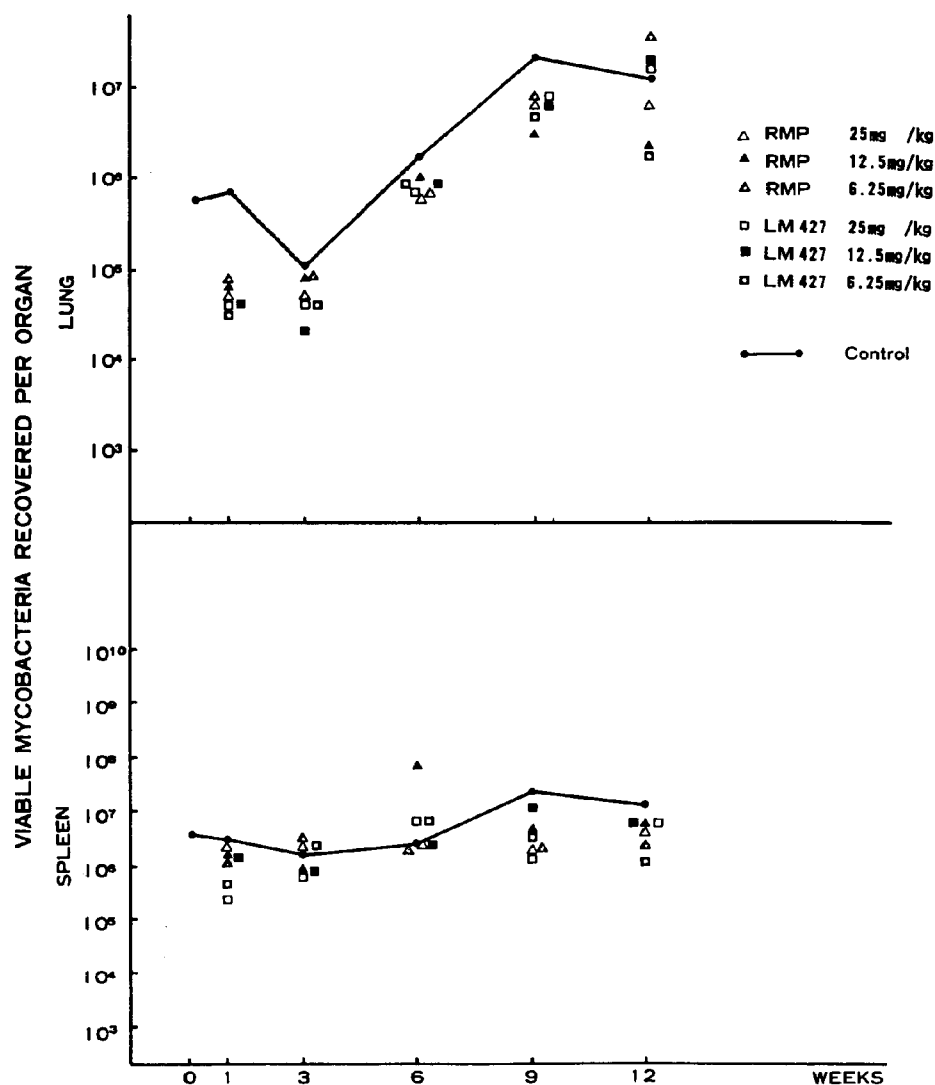
Fig. 5. Average viable counts of mycobacteria from lungs and spleens of mice infected with *M. avium* complex 31F093T. Each point represents the average value for 3 mice. Slight reductions of the viable counts of mycobacteria were observed in mice treated with both LM427 and RMP.

treated with both rifabutin and RMP, while the trend was not demonstrated in the counts of spleens (figure 5). No dose responses were discernible in both drugs. It is worthy to note that rifabutin and RMP showed almost the same *in vivo* activity in our murine model in the evaluations of consecutive viable bacilli in the organs.

DISCUSSION

Rifabutin has already been reported to have high *in vitro* activity against mycobacteria (7-12). Preliminary report showed the drug to be comparable or even slightly better than RMP in its *in vitro* and *in vivo* chemotherapeutic activities against *M. tuberculosis* (7, 9), although undisputable *in vivo* experimental chemotherapeutic trials have not appeared in literature as far as the authors concern. A certain degree of cross-resistance between rifabutin and RMP against *M. tuberculosis* may indicate limited usefulness in the treatment of RMP-resistant tuberculosis (10). Enthusiasm has been prevailing, however, in the studies on rifabutin regarding *M. avium* com-

plex, in the hope that the treatment regimens may be improved by this agent. *In vitro* superior antimycobacterial activity of rifabutin to RMP has already been clearly demonstrated, although a kind of cross-resistance between rifabutin and RMP exists as well for *M. avium* complex (11).

Twenty disease-associated strains of *M. avium* complex isolated in our laboratory were demonstrated again to be highly susceptible *in vitro* to rifabutin in the present study. Superiority of rifabutin to RMP was undisputably confirmed with the three kinds of media. On Ogawa medium, however, the strains with higher MICs of RMP were also the strains with higher MICs of rifabutin. This tendency of parallelism in susceptibility between rifabutin and RMP was confirmed in a correlation analysis of MICs of both drugs in KB but not in DTA, since the correlations of MICs were 0.97 (p<0.01, t-test) and 0.27 (N.S.), in KB and DTA, respectively. It is likely that the relationship between varying levels of resistance to RMP and a concentration-dependent cross-resistance to rifabutin in KB was disarranged in DTA due to unknown factors, which probably existed in some ingredients in DTA.

In *in vivo* study, the total numbers of grossly visible disease in lung of mice demonstrated marginal superiority of rifabutin to RMP. This superiority, however, was not like the one which had been expected from *in vitro* activities of rifabutin. The discrepancy between *in vitro* and *in vivo* studies may be explained from several points of view, although our results are not completely conclusive and much study should be done in the future.

First, the strain 31F093T (No. 6 in table 1) used in *vivo* study was partially resistant ot 50 μg/ml of rifabutin on Ogawa medium. Provided the bioavailability of rifabutin is same as RMP, this rather high degree of resistance does not appear to allow us to expect potent activity against human as well as animal infections. In chemotherapy of human *M. tuberculosis* infection with RMP, the resistance to 50 μg/ml on Ogawa medium usually inidcates no or low chemotherapeutic activity. Four of 10 strains tested on Ogawa medium (table 1) were susceptible to 10 μg/ml of rifabutin. Therefore, *in vivo* activity of rifabutin is still to be expected against these strains. Unfortunately, in animal study (15), high virulence *in vivo* and high degrees of resistance *in vitro* to drugs including RMP and possibly rifabutin coexist, and, moreover, 31F093T strain has been only the mouse-virulent strain thus far selected in our laboratory for experimental chemotherapy of *M. avium* complex infection.

Second, in a correlation analysis of MICs between rifabutin and RMP in DTA, the correlation coefficient was 0.27 with no significance, although a high correlation (0.97, P<0.01) was observed in KB. It is probable that Tween 80® in DTA influenced the permeability of individual strains with varying degrees, and *in vitro* activity of rifabutin may depend more on these changes of permeability than RMP. In the present study with DTA, *in vitro* activity of rifabutin could have been unduly enhanced in some strains. In this sense, *in vitro* susceptibility test could be more reliable on KB or Ogawa medium for correlating *in vitro* and *in vivo* activities of rifabutin.

Third, in view of the *in vitro* superiority of rifabutin to RMP even on Ogawa medium, apparently same potency of rifabutin and RMP in the evaluations of consecutive viable bacilli in mouse organs is still to be explained. One explanation, although by pure speculation, may be that : a part of rifabutin could be metabolized to less active metabolites possibly in mouse macrophages, and the difference of potency between rifabutin and RMP could be much smaller in

experimental chemotherapy.

Finally, no dose responses in the present *in vivo* study of both rifabutin and RMP were beyond explanation. Although all the parameters at 12 wks of infection (figure 3, 4, 5) appeared to demonstrate some favorite responses in the mice treated with highest dose (25 mg/Kg) of rifabutin, the authors thought it as negligible.

One thing is certain that rifabutin suppressed moderately the grossly visible lung diseases of mice infected with our resistant strain. It is still encouraging that the addition of rifabutin to the treatment regimens of *M. avium* complex infections may prove synergism which could be valuable for clinical use.

ACKNOWLEDGMENTS

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Minimal Inhibitory Concentration of LM 427 and Rifampin against
20 *Mycobacterium avium* complex strains

Strain No.	0.05% Tween albumin liquid medium		Kirchner's liquid medium with 10% bovine serum	
	LM 427	RFP	LM 427	RFP
11	≦0. 00039	0. 00313	0. 00156	0. 1
12	≦0. 00039	0. 00313	0. 0125	3. 13
13	≦0. 00039	0. 1	0. 05	25. 0
15	≦0. 00039	0. 78	6. 25	100. 0
17	≦0. 00039	1. 56	6. 25	100. 0<
14	≦0. 00039	1. 56	6. 25	100. 0
1	0. 00313	0. 2	1. 56	50. 0
6	0. 00313	0. 2	0. 05	1. 56
18	0. 00313	3. 13	6. 25	100. 0
19	0. 00625	0. 00625	0. 025	1. 56
3	0. 0125	0. 78	3. 13	50. 0
8	0. 0125	1. 56	1. 56	50. 0
16	0. 0125	1. 56	6. 25	100. 0
9	0. 0125	6. 25	1. 56	50. 0
4	0. 025	0. 39	0. 39	12. 5
5	0. 05	0. 78	0. 78	12. 5
2	0. 05	1. 56	6. 25	100. 0
7	0. 05	3. 13	6. 25	100. 0
10	0. 1	1. 56	0. 0125	25. 0
20	0. 1	1. 56	1. 56	25. 0

$r = 0. 27$ (N.S.) $r = 0. 97$ ($p < 0. 0001$)