

The killing of *Mycobacterium leprae* in mice by various dietary concentrations of dapsone and rifampicin

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Accepted for publication 9 May 1986

Summary The killing of *Mycobacterium leprae* by various dietary concentrations of dapsone and rifampicin was assessed by the proportional bactericidal test. Dapsone 0·00001% and 0·0001% in mouse food were not bactericidal, while dapsone 0·001% and 0·01% were both found 87% ($\pm 22\%$) bactericidal. Concentrations of dapsone required for killing the strain of *M. leprae* were higher than had previously been found necessary for inhibition of bacterial multiplication (0·0001%). Rifampicin 0·01% and 0·005% in mouse food were found to be respectively 99·9% ($\pm 0\cdot 1\%$) and 90% ($\pm 6\%$) bactericidal. Rifampicin 0·003%, 0·001%, and 0·0003% did not result in significant killing. The strain studied was found to be killed by high dietary concentrations of rifampicin similar to that of previous studies but was far more resistant to killing by lower dietary concentrations of rifampicin than had been reported previously for other strains. The implications of these findings are discussed.

Introduction

It has been well established in bacterial endocarditis and a number of other infectious diseases where protective local or systemic host defences are inadequate (osteomyelitis, meningitis, and Gram-negative bacteremia in the neutropenic patient) that bactericidal therapy is crucial to a salutary outcome. In addition, the key to effective short-course chemotherapeutic regimens for pulmonary tuberculosis is the inclusion of two or more bactericidal agents.¹ Lepromatous leprosy is well known to be associated with a serious impairment of important protective cellular immune defence mechanisms against the causative bacterium. Although the requirement for bactericidal therapy for lepromatous leprosy has not been firmly established, the only drugs utilized significantly worldwide and advocated for combined therapy of lepromatous leprosy by the World Health Organization,² i.e. dapsone, rifampicin, clofazimine, and ethionamide, have been judged to have at least some bactericidal activity against *Mycobacterium leprae*.^{3,4}

Until recently, the conclusion that an antimicrobial was bactericidal for *M. leprae* depended entirely on finding that mice infected with *M. leprae*, and treated for a limited period during the bacterial growth phase, showed a significant delay in the resumption of bacterial multiplication that could not be accounted for solely by the accumulation of drug in the tissues,^{5,6} The bactericidal activity of drugs for *M. leprae* can now be experimentally assessed by the proportional bactericidal test,^{3,4} wherein mice infected with serial dilutions of an *M. leprae* suspension are treated for the initial 60 days and bacillary growth determined after a sufficient time has elapsed for the development of detectable growth arising from one or more surviving bacilli. This proportional bactericide technique is advantageous as it allows definite conclusions concerning bactericidal activity, while missing purely bacteriostatic or bacteriopausal drugs, and further permits comparison of various degrees of bactericide.⁷ Published studies using this technique to assess bactericidal activity for *M. leprae* of dapsone and rifampicin are limited and confined to constant and high dietary concentrations against only two strains of *M. leprae*. Colston *et al.*³ found dapsone 0.01% in mouse food to be 78% bactericidal for one strain and 72% bactericidal for the other, while rifampicin 0.003% and 0.01% were found to be 99.99% and 100% bactericidal, respectively, against these same two strains.

The antimicrobial therapy of leprosy in man, wherein drugs are administered no more than once daily, results in a range of bioavailability of active drug quite different from the relatively constant levels found in mice receiving fixed concentrations of drug distributed in the diet. In an attempt to provide further information to enable considered decisions concerning human dosage schedules, we initiated these studies to assess the killing potential for *M. leprae* by dapsone and rifampicin over a wide range of mouse dietary concentrations that result in the broad range of levels which may actually be experienced by leprosy patients in the course of therapy.

Methods

In the first experiment, groups of 10 mice were inoculated in both hind feet with 10, 100, and 1000 *M. leprae* and treated with no dapsone and dietary dapsone concentrations of 0.00001%, 0.0001%, 0.001%, and 0.01% for the next 60 days. In the second experiment, groups of 10 mice were inoculated in both hind feet with 10, 100, 1000, and 10,000 *M. leprae* and treated with no rifampicin and rifampicin concentrations in mouse food of 0.0003%, 0.001%, 0.003%, 0.005%, and 0.01% for the next 60 days. The strain of *M. leprae* used in both experiments was originally isolated from the skin of a patient with lepromatous leprosy and extensively studied and passaged in mice for the preceding 10 years at the Leprosy Research Unit of the United States Public Health Service Hospital, San Francisco.

Diets were prepared by first dissolving dapsone and rifampicin (both obtained from Sigma Chemical) in 95% ethanol and evenly distributing dilutions of these solutions in mouse food in a Patterson Kelly twin-shell diet-mixing apparatus. In order to limit deteriorating drug concentrations, drug-containing diets were prepared just prior to this study, refrigerated immediately after preparation, and mouse feeders replenished at least twice weekly. One year after the completion of therapy, five mice (ten feet) from each treatment group were sacrificed and *M. leprae* in each footpad counted individually. For purposes of this study footpads containing *M. leprae* per footpad greater than 5×10^4 were judged to harbour viable *M. leprae* after cessation of therapy. The percent bactericide was then calculated from the fraction of footpads containing viable bacilli in the graded inocula at each dosage level by both a most probable number calculation⁸ and the Spearman-Kärber method.⁷ The most probable number calculation has been criticized because it is based on the assumption of a Poisson distribution,⁷ which would require that *M. leprae* be randomly distributed on slides, which is demonstrably untrue.⁹

Results

The results of Experiment 1 are presented in Table 1. Neither 0.00001% nor 0.0001% dapsone in mouse food was found to have any bactericidal activity for *M. leprae*. Both 0.001% and 0.01% dapsone resulted in a modest equivalent bactericidal activity ($87 \pm 22\%$ bactericidal). This degree of killing by 0.01% dietary dapsone found in this study is quite similar to that previously reported by Colston *et al.*³

The results of Experiment 2 are presented in Table 2. The highest concentration of rifampicin studied, 0.01%, caused an impressive degree of *M. leprae* killing

Table 1. Dapsone killing by graded doses

	<i>M. leprae</i> /footpad inoculated			Percentage killed	
				Most probable number technique	Spearman Kärber
	10^3	10^2	10^1		
Control	10/0*	9/1	7/3		
Dapsone 0.00001%		10/0	9/1	0	0
Dapsone 0.0001%			8/0	0	0
Dapsone 0.001%	9/1	6/4	2/8	80	87 ± 22
Dapsone 0.01%	9/1	6/4	2/8	80	87 ± 22

* Number of footpads with $> 5 \times 10^4$ *M. leprae*
 Number of footpads with $< 5 \times 10^4$ *M. leprae*

Table 2. Rifampicin killing by graded doses

	<i>M. leprae</i> /footpad inoculated				Percentage killed		
	10 ⁴	10 ³	10 ²	10 ¹	Most probable number technique	Spearman Kärber	
Control			10/0*	9/1	4/6		
Rifampicin 0.0003%				10/0	2/8	0	20 ± 20
Rifampicin 0.001%				6/0	1/9	16	37 ± 16
Rifampicin 0.003%			4/0	7/1	1/9	41	53 ± 18
Rifampicin 0.005%			9/1	4/6		80	90 ± 6
Rifampicin 0.01%	1/9	0/10	0/10			99.9	99.9 ± 0.1

* See Table 1

(99.9 ± 0.1% bactericide). Lower concentrations resulted in progressively less bactericidal activity. The bactericidal activity of 0.005% rifampicin (90 ± 6%) was modest. In this study, 0.003% rifampicin's bactericidal activity was not found different from control ($p=0.19$) and quite different against the studied strain of *M. leprae* than previously found for another strain of *M. leprae* by Colston *et al.*³ (99.99% bactericidal). Rifampicin 0.001% and 0.0003% also produced no significant killing.

Discussion

These studies demonstrate that the killing of *M. leprae* in mice by both dapsone and rifampicin is dose dependent. In this respect the dose-response curves obtained for the two antimicrobials are quite different. The strain studied herein had been extensively studied previously in this laboratory and was known to be consistently inhibited by 0.0001% dapsone and 0.00003% dapsone, but not by 0.00001% dapsone.¹⁰ In the present study 0.00001% and 0.0001% dietary dapsone produced no measurable lethal consequences for the bacillus. Thus we observed a discordance between the minimal inhibitory concentration and minimal bactericidal concentration for this strain of *M. leprae*. The ability of higher dietary dapsone concentrations actually to kill *M. leprae* is modest and similar to that reported by others. It is noteworthy that increasing dietary concentration from 0.001% to 0.01% did not result in any increased bactericidal activity.

Low level sulphone therapy, including 1 mg dapsone/day, sulphetrone,¹¹ and DADDS,¹² maintains plasma levels consistently above *M. leprae*'s minimal inhibitory concentration, 3 ng/ml,^{13,14} but levels near those required for bactericidal activity for the strain herein studied. Such therapy has resulted in treatment failure: low dosage dapsone¹⁵ and sulphetrone¹⁶ therapy have resulted in an increased incidence of dapsone-resistant relapse, and DADDS monotherapy has resulted in a substantial 'persister' population.¹⁷ These examples suggest the possible importance of bactericidal and not just bacteriostatic therapy in the successful therapy of lepromatous leprosy. Conversely, the significant killing of *M. leprae* by both 0.001% and 0.01% dapsone in mouse food might also serve to explain why patients resistant to 0.0001% dapsone but not to higher dietary levels when treated with full dosage (100 mg) dapsone daily improve clinically and bacteriologically.¹⁸

The highest studied dietary concentration of rifampicin (0.01%) resulted in considerable bactericidal activity, again in agreement with previous studies of Colston *et al.*³ using the same methods. On the other hand, lower levels of dietary rifampicin showed a considerably and progressively diminished ability to kill *M. leprae*. While Colston found 0.003% dietary rifampicin to be 99.99% bactericidal, we found rifampicin 0.005% to be $90 \pm 6\%$ bactericidal and rifampicin 0.003% to be only $50 \pm 18\%$ bactericidal (not significantly different from untreated controls). Such major differences between these two studies suggest that differing strains of *M. leprae* vary considerably in their susceptibility to the lethal effects of rifampicin. This is not surprising as previously Holmes¹⁹ had demonstrated, amongst different strains of *M. leprae*, a range of minimum inhibitory dietary concentrations of rifampicin from 0.0003% to 0.003%. Furthermore, where Rees²⁰ found the minimal inhibitory dietary concentration of rifampicin for *M. leprae* to be 0.0025%, Shepard⁵ required 0.01% for his strain. Thus *M. leprae* strains appear to exhibit a range of susceptibility both to the inhibitory and bactericidal activity of rifampicin.

We found a rapid fall-off in the concentration-dependent killing of *M. leprae* in mice by rifampicin. A decrease in rifampicin's activity for *M. leprae* by dosage reduction was found previously by Shepard *et al.*²¹ in experiments in mice wherein single doses of between 10 and 40 mg/kg of rifampicin were given to mice 70 days following footpad infection. In this study 10 mg/kg was found inactive, 15 and 25 mg/kg demonstrated minimal but progressively increased activity, while 40 mg/kg was found to inhibit the growth of *M. leprae* impressively. Also, in clinical trials utilizing single-dose rifampicin therapy in untreated lepromatous leprosy patients 1500 mg and 1200 mg were demonstrably more effective than 900 mg and 600 mg, while daily 600 mg rifampicin was similarly more effective than 300 mg rifampicin.²² Though it is not statistically significant, we found in a clinical trial in lepromatous leprosy of 1 month's duration monitored by neonatally thymectomized Lewis rat inoculation that daily 100-mg dapsone plus a single 1500 mg dose of rifampicin resulted in a decreased percentage of viable *M. leprae* in the skin at

each of the four sampling intervals as compared to therapy with daily 100 mg dapsone plus weekly 900 mg rifampicin.²³ Thus dosage reduction in both rodents and man may result in discernible differences in rifampicin's efficacy. It then appears that rifampicin levels above some critical concentration for significant periods are important for optimal bacterial killing. Especially for certain relatively insensitive strains of *M. leprae*, such as that studied in this report, it is of some concern if monthly 600 mg rifampicin as advocated by the WHO results in an optimal concentration of drug at its active site for a sufficient period of time.

Acknowledgments

This work was supported by a grant from the USPHS Central Clinical Investigations Committee and the National Hansen's Disease Center, Carville, Louisiana. We wish to thank Mrs Lydia P Murray and Mr P R Henika for their excellent technical assistance, Mr G A Gibson for preparation of this manuscript, and Dr D Heilbron of the Scientific Computing Services, University of California, San Francisco, for his assistance with the statistical analysis of these studies.

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