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The Rate of Bactericidal Action of Rifampin on *Mycobacterium leprae* in the Mouse Footpad (38021)

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Continuous dietary administration of low dosages of 3-(4-methyl-1-piperazinyl-iminomethyl)rifamycin (rifampin; RMP) inhibits the multiplication of *Mycobacterium leprae* in the mouse footpad (1, 2). The drug is highly bactericidal against *M. leprae,* as estimated by inoculation into mice of bacilli from lepromatous leprosy patients at intervals during treatment with 600 mg RMP daily (2), and by the kinetic technique of Shepard (3) in treated experimental animals (1, 4). Moreover, the lethal action of RMP, which is observed after exposure to drug for only 2 days, is far quicker than that of dapsone (4,4′-diaminodiphenylsulphone; DDS) which in mice can be detected only after administration for periods of 60 days or longer (3, 5).

The present work was undertaken in an attempt to determine more precisely the rate of bactericidal action of RMP on *M. leprae* in the mouse footpad, by means of the kinetic technique and a modification of the subinoculation technique which was used by Shepard and Chang (6) in a study of the action of DDS on *M. leprae.*

Materials and Methods. The 3 strains of *M. leprae* used all derived from previously untreated lepromatous leprosy patients, had all been maintained in mouse passage and were sensitive to administration of 0.0001% DDS in the diet. The detailed methods of mouse inoculation, assessment of bacillary growth and drug diet preparation have been previously described (1). For estimation of drug action by the kinetic technique, treated groups (15 mice each) received 0.01% RMP in the diet for varying limited periods during the course of infection, either during early or middle logarithmic multiplication. The total amount of growth delay in treated groups was calculated by comparison of control and treated *M. leprae* growth curves for each strain. To prepare animals with an established footpad infection for estimation of drug action by a subinoculation technique, mice were inoculated with 10^4 bacilli per footpad and progress of the infection was monitored by acid-fast bacilli (A.F.B.) counts on homogenates of 3 pooled footpads at 2–4 week intervals. When bacillary numbers had reached 10^6 per footpad, the animals were divided into 2 groups: controls, and animals given 0.03% RMP continuously in the diet. At intervals during a 14 day period, numbers of A.F.B. in homogenates from 3 individual footpads per group were determined and the count in each adjusted to 1.5×10^6 per ml with Dubos tween albumin medium. Equal volumes of individual homogenates within a group were pooled, the pooled suspension diluted 3-fold with tween albumin medium and serial half-$\log_{10}$ dilutions in tween medium were made. Each dilution was inoculated into a group of 6 mice (0.02 ml per footpad). Bacillary growth was assessed after 8–11 months in mice inoculated with *M. leprae* from control animals, and after 13 mo in mice inoculated with bacilli from RMP-treated animals. This allowed sufficient time for any effects of persistent bacteriostasis (5) to disappear and for bacilli surviving exposure to RMP to grow to detectable numbers. Counts of 5.0×10^4 or greater were accepted as indicating growth of *M. leprae* (5-fold increase). From the pattern of growth obtained, the 50% infectious doses of *M. leprae* remaining after different periods of exposure to drug were calculated by the method of Reed and Muench (7) and hence the percentage survival values were obtained.

Results. Kinetic technique. Groups of mice inoculated with *M. leprae* strains SBL 16237 and 9593 were fed 0.01% RMP in the diet continuously for periods of 1, 2, 4 and 7 days respectively from day 33 post-inoculation for strain SBL 16237 and day 33 or 91 for strain 9593. These 2 starting times were chosen as representing the early and middle logarithmic *M. leprae* growth phases. Growth curves constructed from mean A.F.B. counts in control and treated animals are shown in Figs. 1 and 2. RMP treatment produced a delay in bacillary growth, the extent of which was dependent on the period of drug administration. The delay periods are quantitated in Table I by measurement of the displacement of the growth curves in treated groups from the control curve at a count of $10^{5.3}$ A.F.B./footpad.

The table also records the estimates of "delay due to bactericidal action" and "% survival" for the 2 strains of *M. leprae* studied. The considerations on which such determinations are based have been previously described (1, 5). From the data in

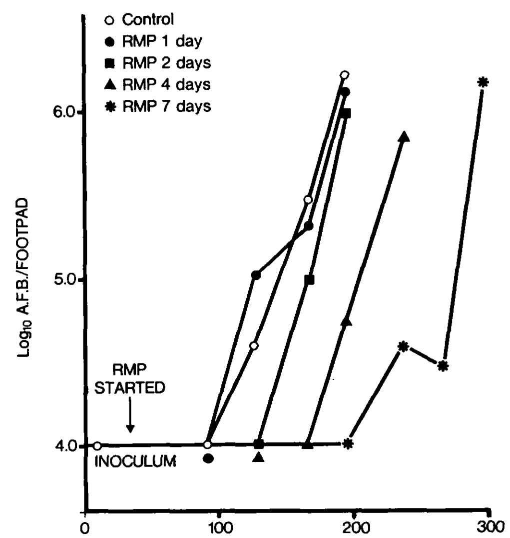

FIG. 1. The effect of temporary administration of rifampin (RMP) on the growth of *M. leprae* (strain SBL 16237). RMP 0.01% was administered in the diet for the time periods indicated. Values for treated mice represent the mean of A.F.B. counts from 2–3 individual footpad homogenates; control values are from 3 pooled footpads.

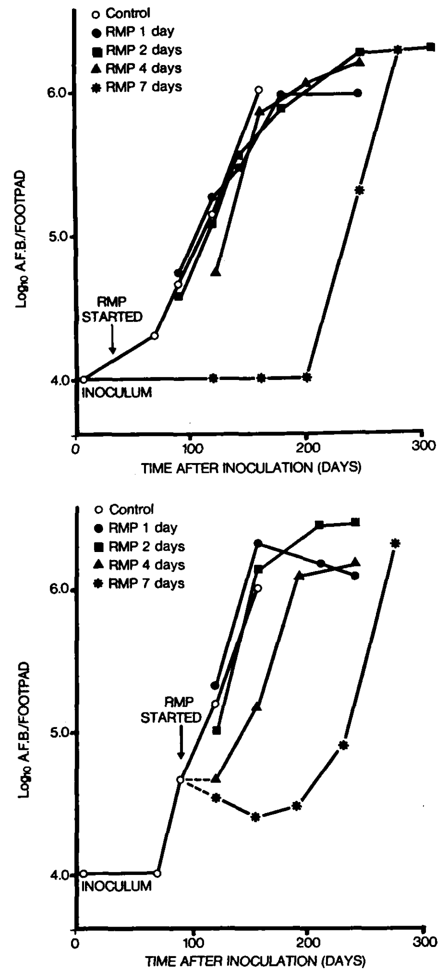

FIG. 2. The effect of temporary administration of rifampin (RMP) to mice on the growth of *M. leprae* (strain 9593). Upper and lower graphs record results obtained when 0.01% RMP was administered in the diet from day 33 and 91 post-inoculation respectively. Values for treated mice represent the mean of A.F.B. counts from 2–3 individual footpad homogenates; control values are from 3 pooled footpads.

TABLE I. Delayed Growth of *M. leprae* Following Temporary Administration of Rifampin, and Its Interpretation in Terms of Bactericidal Action.

M. leprae strain	Estimated exposure to RMP[a] (days)	Bacterial growth delay (days)	Delay due to bactericidal action[b] (days)	% survival[c]
	3	9	6	64.3
SBL 16237	4	18	14	33.0
(day 33)	6	58	52	1.8
	9	123	114	0.01
	3	3	0	100.0
9593	4	3	0	100.0
(day 33)	6	14	8	67.3
	9	112	103	0.6
	3	0	0	100.0
9593	4	3	0	100.0
(day 91)	6	38	32	20.3
	9	117	108	0.5

[a] Administration time plus estimated excretion time of 2 days (1).
[b] Column 3 minus column 2.
[c] Shepard (5).

Table I, survival curves of *M. leprae* exposed to RMP have been constructed (Fig. 3). The extent of exposure to RMP has been taken as the period of drug administration plus the estimated time of 2 days for excretion of RMP from mice after cessation of continuous dietary administration of 0.01% RMP (1, 8). The half-life of *M. leprae* during the exponential phase of lethal action of RMP was of the order of 0.5 days for both bacterial strains.

Subinoculation technique. Table II shows the pattern of growth of *M. leprae* (strain SBL 16220) subinoculated from control and RMP-treated animals into new groups of mice. The fraction of mice in each subinoculum group showing positive A.F.B. counts and the percentage survival values calculated by the method of Reed and Muench are recorded. The loss of infectivity (viability) of *M. leprae* due to host immunity in the untreated animals was very

TABLE II. Growth in Mice of *M. leprae* Subinoculated from Control and Rifampin-Treated Animals with an Established Footpad Infection (10^6 A.F.B. Per Footpad).

Treatment	Time[a] (days)	No. of mice/6 with positive A.F.B. counts at the given reciprocal inoculum dilution: 1	10	30	100	300	1000	3000	Survival[b] (%)
Control	0	6	6	ND	6	6	2	2	100.0
	14	6	6	ND	5	4	1	0	26.0
RMP 0.03%	3	6	6	6	4	4	0	0	21.4
	5	6	6	5	0	0	0	0	3.9
	7	6	0	0	0	0	0	0	0.3
	14	0	0	0	0	0	0	0	<0.03

[a] Days after start of drug administration.
[b] Results based on calculation of I.D. 50 [Reed and Muench (7)]. ND = not determined. The undiluted subinoculum contained 1.0×10^4 *M. leprae*.

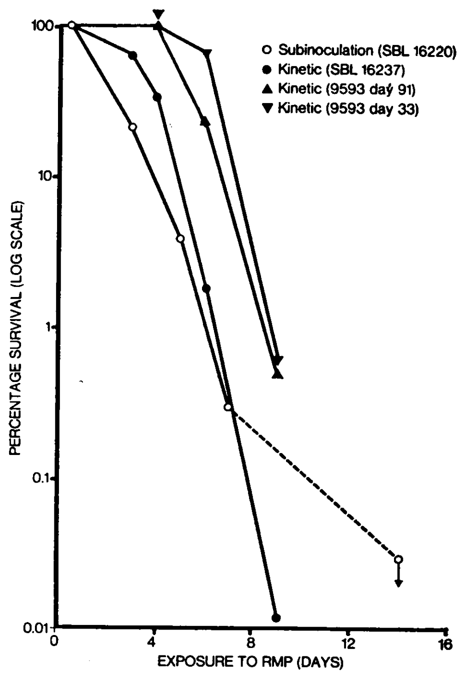

FIG. 3. Survival curves of *M. leprae* exposed to rifampin (RMP) in the mouse footpad as estimated by the kinetic (strains SBL 16237 and 9593) and subinoculation (strain SBL 16220) techniques. The 2 curves for strain 9593 represent 2 times post-inoculation at which drug administration was started (day 33 and 91). Survival half-life values in days for the 4 groups were: ○ = 0.6; ● = 0.4; ▲ and ▼ = 0.45.

slight after 14 days. The effect of RMP has therefore been calculated without taking the host immunity into account, since its even smaller effects after 1–7 days would change only marginally the calculations of RMP bactericidal effect. Figure 3 records the survival curve of strain SBL 16220 in the presence of RMP.

A significant bactericidal action was observed after RMP administration for 5 days. The relationship between percentage survival and period of exposure to RMP indicates a survival half-life of 0.6 day. Administration of the drug for 14 days was sufficient to reduce the number of viable bacilli to such an extent that a subinoculum containing 1.0×10^4 bacilli (undiluted) was completely noninfective.

Discussion. The assumptions on which estimation of the bactericidal action of compounds by the kinetic technique are based and the significance of such findings have been previously discussed in detail. The present results of an experiment using a serial dilution subinoculation technique, in which the loss of mouse infectivity of *M. leprae* was determined, support the contention that the kinetic technique does indeed provide a valid measure of drug bactericidal effect. The *M. leprae* strain and RMP dosage in the present work were different for the 2 techniques. Moreover, in the subinoculation experiment, the number of bacilli exposed to RMP was greater (10^6 versus 10^4) and these bacilli were probably approaching the stationary phase of growth (9). Notwithstanding these differences in conditions, the results from the 2 techniques are comparable in that both indicate rapid bactericidal action of RMP on *M. leprae.* The difference in bacillary strain may probably be disregarded, as unpublished observations from kinetic experiments using the *M. leprae* strain employed in the subinoculation experiment (SBL 16220) showed that exposure to RMP for 12 days rendered the bacilli noninfective, so confirming the kinetic results reported in this paper.

The bactericidal action of RMP is extraordinarily rapid, with a half-life of viable (infective) bacilli of 0.5–1.0 day. An inoculum of 10^4 *M. leprae* is rendered noninfective after administration for as little as 14 days of 0.01–0.03% RMP in the diet (10–30 × minimum inhibitory dosage: Holmes and Hilson (1)). The rapid bactericidal action of RMP on *M. leprae* is similar to that observed with other mycobacteria *in vitro* and *in vivo* (10, 11), and with fast-growing bacteria (12). When related to mean generation time of the organism concerned, however, the lethal action of RMP appears to be inherently stronger against *M. leprae.* The rapidity of action is vastly superior to that of DDS, for which administration to mice for 60 days or longer is required for significant bactericidal activity to be observed (3, 5, 6). With strain 9593, the degree and rate of bactericidal action was similar whether drug administration was started on day 33 or day 91 post-inoculation. This may be explained by the fact that bacilli were already in the logarithmic phase of growth by day 33. However, experimental results to be reported later, in which RMP was administered for 5 days during different bacillary growth phases between day 0 and 125 post-inoculation, suggest that the drug is equally effective against bacilli in lag, logarithmic and stationary phases of growth.

The present findings confirm earlier results of a direct comparison of the effects of DDS and RMP in the kinetic model, in which administration of 0.01% RMP for 30 days resulted in the absence of subsequent growth of *M. leprae* (1). Shepard *et al.* (4) observed that administration of 0.03% RMP for 2 days resulted in a very low percentage survival; treatment for 86 days resulted in the death of all bacilli. The apparently greater lethal action after 2 days may have been due to differences in drug dosage or bacillary strain.

Mouse serum RMP concentrations attained during administration of 0.01–0.03% RMP in the diet are of the order of 2–8 μg/ml (1, 4, 8). Such serum levels are attained in patients receiving standard therapy with 600 mg RMP daily (13). Moreover, Rees, Pearson and Waters (2) demonstrated that the bacilli of lepromatous leprosy patients receiving such treatment became noninfective for mice (10^4 A.F.B. per inoculum) after 17–24 days. The present experimental data thus confirm previous data from animal and clinical investigations.

The main drawback to both the kinetic and subinoculation techniques relates to the sensitivity of the methods. In order that growth of surviving organisms may be detected, the total number of bacilli which can be investigated after drug action is 10^5 per footpad or fewer. This allows a 10-fold increase in numbers to be observed before host immunity prevents further multiplication. The methods are therefore not strictly analogous to conditions to be expected in lepromatous leprosy, in which tissue bacillary concentrations may be as high as 10^8 per g (14). The use in chemotherapeutic trials of animals immunosuppressed by thymectomy and total body irradiation (15), thymectomy and antithymocyte globulin (16), or by antithymocyte globulin and steroids (8) may provide conditions in which considerably greater populations of *M. leprae* are exposed to drug action.

Summary. The rate of bactericidal action of rifampin on *Mycobacterium leprae* in the mouse footpad was determined by the kinetic technique of Shepard and a serial dilution subinoculation technique. In kinetic experiments, rifampin (0.01%) in continuous dietary administration, produced a delay in bacillary growth, the extent of which was dependent on the period of drug administration. The estimated survival half-life of *M. leprae* was 0.5 day. This was confirmed by the subinoculation technique, by which a survival half-life of 0.6 day was obtained. Drug administration for 14 days rendered an inoculum of 10^4 *M. leprae* noninfective for mice.

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