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The "Proportional Bactericidal Test": A Method for Assessing Bactericidal Activity of Drugs Against *Mycobacterium leprae* in Mice

M. J. COLSTON, G. R. F. HILSON AND D. K. BANERJEE

St George's Hospital Medical School, London SW17 0QT, U.K.

A new method for assessing the bactericidal activity of antileprous drugs against *Mycobacterium leprae* using the mouse footpad technique is described. This approach, referred to as the "proportional bactericidal test", has been devised to overcome some of the problems of interpretation caused by drug persistence or prolonged bacteriostasis after drug administration has ended. The bactericidal activity of several drugs against *M. leprae* has been determined using this approach and the results obtained compared with those previously reported using alternative methods.

Introduction

The first attempt to detect and assess bactericidal activity of a drug against *Mycobacterium leprae* in the mouse footpad was made by Shepard and Chang (1967), who fed mice with an established *M. leprae* infection on 4,4′-diaminodiphenylsulphone (DDS, dapsone), and measured the viability of *M. leprae* by subinoculation at intervals into new groups of mice. Although this technique is considered reliable for detecting the presence of a bactericidal effect, it is tedious and time-consuming to perform, and the rate of bacterial killing by drug is difficult to distinguish from that due to the concurrent killing effect of the host's acquired immunity.

An alternative technique, the "kinetic method", was devised (Shepard, 1967), in which drug was given for a limited period of time early in the growth cycle, and the bactericidal activity of the drug assessed by comparing the delay in appearance of growth of *M. leprae* with that in untreated controls, with allowance for the delay due to inhibition during drug administration. The *absence* of bactericidal activity is reliably demonstrated by this approach; however, most bactericidal drugs are known to exert a "bacteriopausal" effect: i.e. a prolonged bacteriostasis after removal of drug due to the recovery of reversibly damaged cells (Dickinson and Mitchison, 1966)—while other drugs, such as clofazimine, persist in the host's tissues after drug administration has ended (Barry, 1958; Barry *et al.*, 1959, 1960; Conalty and Jackson,

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1962), making the presence and extent of bactericidal activity difficult to detect (Banerjee *et al.*, 1974; Levy, 1974).

A modified approach, devised to overcome these difficulties and referred to as the "proportional bactericidal test", was reported by Hilson and Banerjee (1974) using *Mycobacterium lepraemurium*. This involved the inoculation of groups of mice with serial dilutions of a bacillary suspension, treating them for a limited period, and then sampling for bacillary growth after sufficient time had elapsed for the development of detectable growth arising from one or more surviving bacilli. This approach has now been adapted for the assessment of bactericidal activity of a number of antileprous agents against *M. leprae*.

Materials and Methods

Two strains of *M. leprae* were used (SBL 16220 and TG) both of which were originally derived from untreated lepromatous leprosy patients and subsequently maintained in mouse passage in our laboratory. The inoculation of female ASH/CSI mice (Charles River U.K. Ltd), harvesting and counting of *M. leprae* from mouse footpads were performed according to previously reported methods (Hilson and Elek, 1957; Holmes and Hilson, 1972). Inocula were prepared and diluted in Bacto TB Broth (Difco).

Assessment of bacillary growth was carried out by "scoring" for the presence or absence of growth; growth was assumed to have occurred in mice in which the number of bacilli harvested was significantly greater than the number originally inoculated. In practice, scoring for the presence or absence of bacillary growth was straight-forward since growth was either undetectable (less than 10^4 *M. leprae* harvested), or had reached more than 10^5 *M. leprae* per footpad, with no equivocal results.

The drugs used were dapsone (DDS), rifampicin (RMP), clofazimine (B663), thiambutosine, thiacetazone, ethionamide, and prothionamide, and their incorporation into mouse diet was performed as described by Holmes and Hilson (1972).

Results

In the first experiment, *M. leprae* (TG) was inoculated into the footpads of groups of 10 mice using inocula of 10^4, 10^3, 10^2 and 10^1 AFB per footpad. Drug treatment with 0.003% B663, 0.003% RMP, 0.01% DDS, 0.1% thiambutosine and 0.1% thiacetazone was started on the day of inoculation and continued for 60 days, and mice were scored for the presence or absence of growth 12 to 18 months after inoculation. The results are shown in Table 1.

The results were analysed by determining the "most probable number" of viable bacilli (MPN) (Halvorson and Ziegler, 1933; Taylor, 1962). This analysis indicates that the original inoculum contained at least 16% viable organisms, though this figure does not take into account the fact that more than one viable bacillus may be required to initiate an infection (Levy *et al.*, 1974). In the drug-treated mice, this analysis gives an estimate of the MPN of viable bacilli remaining after drug treatment. It can be seen from the table that treatment with thiambutosine produced no bacterial killing, whereas RMP,

TABLE 1

The proportional bactericidal test: the bactericidal activity of thiocarlide, thiambutosine, thiacetazone, DDS, RMP and B663 against M. leprae *(strain TG)*

Drug regimen	Inoculum size (*M. leprae* per footpad) 10^4	10^3	10^2	10^1	MPN* of viable *M. leprae*	Survival (%)	*P*§
Control	10/10†	10/10	9/9	6/8	1600	—	—
DDS (0.01%)‡	10/10	7/7	8/8	2/7	350	22	<0.01
RMP (0.003%)	2/8	1/9	0/8	0/9	0.2	0.01	<0.01
B663 (0.003%)	8/9	5/8	3/7	0/8	14	1	<0.01
Thiocarlide (0.1%)	9/9	8/8	10/10	8/10	1600	100	1
Thiambutosine (0.1%)	10/10	10/10	8/8	10/10	>1800	112	1
Thiacetazone (0.1%)	9/9	7/7	7/7	4/8	920	58	0.24

* Most probable number per 10^4 bacilli injected.

† Number of footpads showing growth of *M. leprae*/number of footpads harvested.

‡ All 6 drugs given for 60 days.

§ Probability of the difference in MPN in the drug-treated group and that in the control group having arisen by chance.

B663 and DDS all showed varying degrees of bactericidal activity, with 99.99%, 99% and 78% killing respectively. The analysis of the results with thiacetazone gives an MPN of 920 viable *M. leprae*, which is equivalent to a 42% killing effect; this reflects a "score" of 4/8 positive in the 10^1 group, compared with 6/8 in the control mice.

In the second experiment mice were inoculated with 10^4, 3×10^3, 10^3, 3×10^2, 10^2, 3×10^1, and 10^1 *M. leprae* per footpad, and B663 (0.01% and 0.003%), RMP (0.01%) and DDS (0.01%) administered for 30 days. Harvests of *M. leprae* were made 12 to 18 months after inoculation. The results are shown in Table 2.

The viability of the original inoculum was rather low, with only about 2.5% of the bacilli viable. All 4 drug regimens produced some bactericidal effect, with 0.01% B663 producing a 98% kill, 0.003% B663 a 96% kill, 0.01% DDS a 72% kill, whilst 0.01% RMP totally eliminated the infection in all the mice.

In the final experiment, ethionamide and prothionamide were assessed for bactericidal activity against *M. leprae*. Treatment with 0.1% ethionamide and prothionamide was started on the day of inoculation and continued for 45 days; treatment with 0.2% ethionamide was stopped after 30 days. Mice were sacrificed and *M. leprae* harvested from footpads 12 to 14 months after inoculation. The results are shown in Table 3.

Treatment with either 0.1% ethionamide or 0.1% prothionamide for 45 days produced identical bactericidal activity with survival of 1.4% of the inoculum, while 30 days' administration of 0.2% ethionamide resulted in survival of 2.6% of the inoculum.

Discussion

The events which are assumed to take place in the proportional bactericidal test are shown in Fig. 1. The assumption is made that the rate of fall in viable

TABLE 2

The proportional bactericidal test: the bactericidal activity of B663, RMP and DDS against M. leprae *(SBL 16220)*

Drug regimen	Inoculum sizes (*M. leprae* per footpad)							MPN* of viable *M. leprae*	Survival (%)	*P*§
	10^4	3×10^3	10^3	3×10^2	10^2	3×10^1	10^1			
Control	5/5†	5/5	5/5	5/5	5/5	2/5	0/5	250	—	—
B663 (0.01%)‡	6/9	5/8	4/8	1/10	0/7	0/8	0/8	4	2	<0.01
B663 (0.003%)	8/8	7/7	5/8	2/9	0/10	0/8	0/9	10	4	<0.01
RMP (0.01%)	0/10	0/7	0/9	0/10	0/8	0/9	0/9	0	0	<0.01
DDS (0.01%)	9/9	7/7	9/9	8/10	6/10	1/9	0/10	70	28	<0.01

* Most probable number per 10^4 bacilli injected.

† Number of footpads showing growth of *M. leprae*/number of footpads harvested.

‡ All 4 drugs given for 30 days.

§ Probability of the difference in MPN in the drug treated group and that in the control group having arisen by chance.

TABLE 3

The proportional bactericidal test: the bactericidal activity of ethionamide and prothionamide against M. leprae *(strain TG)*

Drug regimen	Inoculum size (*M. leprae* per footpad) 10^4	10^3	10^2	10^1	MPN* of viable *M. leprae*	Survival (%)	*P*§
Control	5/5†	5/5	5/5	3/5	920		
Prothionamide (0.1% for 45 days)	5/5	4/5	0/5	0/5	13	1.4	<0.01
Ethionamide (0.1% for 45 days)	5/5	4/5	0/5	0/5	13	1.4	<0.01
Ethionamide (0.2% for 30 days)	5/5	5/5	0/5	0/5	24	2.6	<0.01

* Most probable number per 10^4 bacilli injected.

† Number of footpads showing growth of *M. leprae*/number of footpads harvested.

§ Probability of the difference in MPN in the drug-treated group and that in the control group having arisen by chance.

numbers of bacilli under the action of a bactericidal drug is independent of inoculum size; though it is not possible to demonstrate that this is so for *M. leprae*, it has been shown to be true for cultivable organisms *in vitro* (Garrett, 1971). On withdrawal of drug, this period of "proportional" killing may be followed by a prolonged bacteriostatic action in some cases, or a slow

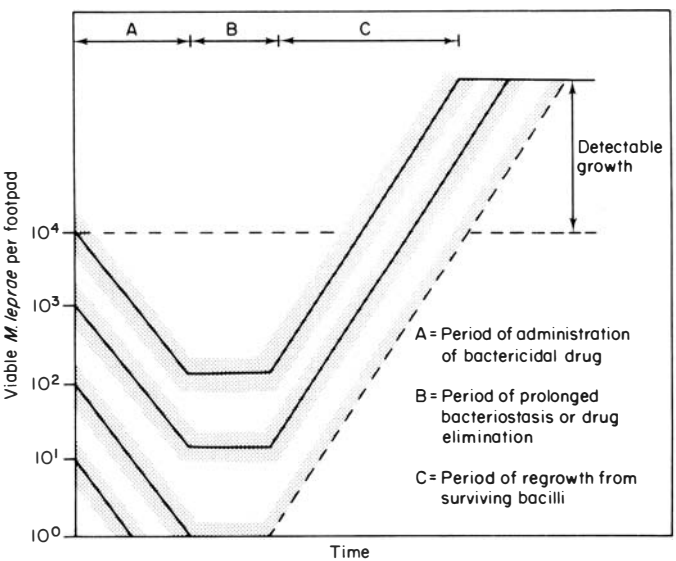

Fig. 1. Schematic representation of events occurring in the proportional bactericidal test. Inoculum sizes are 10^4, 10^3, 10^2, and 10^1 *M. leprae* per footpad. The shaded areas represent the variability inherent in the inoculum size and in the response to drug. The observed results in this example are: 10^4 group—all footpads positive, 10^3 group—all footpads positive, 10^2 group—some footpads positive, 10^1 group—all footpads negative.

elimination of drug in others. In those footpads in which viable bacilli still remain (10^4, 10^3 and in some 10^2 inoculated mice in the example shown in the figure), growth of the survivors then occurs at a rate independent of the numbers of remaining viable bacilli; the independence of growth rate and size of bacillary population has been demonstrated for cultivable organisms *in vitro* (Youmans and Youmans, 1949) and for *M. leprae* in the mouse footpad (Levy, 1976). The mice are assessed for the presence or absence of growth at the end of the regrowth period, and the proportion of mice which are positive in a particular group (i.e. the number of mice which still had viable bacilli remaining after drug treatment) is a reflection of the amount of bacterial killing which occurred during drug administration and hence of the rate of bactericidal action of the drug.

In all 3 experiments described here the distribution of results in each of the groups is statistically acceptable ($P' > 0.05$) using the analysis recommended by Taylor (1962).

Thiambutosine showed no bactericidal activity at all, confirming previous findings (Shepard, 1967; Colston and Hilson, 1976). RMP proved to be the most rapidly bactericidal of the drugs tested, with 0.003% for 60 days producing 99.99% killing (Table 1) and 0.01% for 30 days producing 100% killing (Table 2). If the assumption is made that the bactericidal effect is exponential in character, the bacterial survival half-life ($T_{\frac{1}{2}}$) for 0.003% RMP was 4 days, and for 0.01% less than 2 days. The rapid bactericidal effect of RMP against *M. leprae* in mice has previously been reported (Shepard *et al.*, 1971; Holmes and Hilson, 1972; Shepard, Levy and Fasal, 1972), with an estimated $T_{\frac{1}{2}}$ of 0.6 days at a dosage of 0.01% (Holmes and Hilson, 1974).

The bactericidal effects of DDS and B663 have proved difficult to demonstrate. In Shepard and Chang's original work (Shepard and Chang, 1967), DDS was shown to produce killing in the mouse footpad, but in subsequent studies it has been suggested that administration of DDS produced prolonged bacteriostasis rather than bactericidal activity (Levy, 1970, 1972). DDS showed a slow bactericidal activity, with 78% killing at a dosage of 0.01% for 60 days against strain TG ($T_{\frac{1}{2}} = 27$ days), and 72% at 0.01% for 30 days against strain SBL 16220 ($T_{\frac{1}{2}} = 18$ days).

The bactericidal effect of B663 has proved even more difficult to estimate due to its marked tissue accumulation in both the mouse and in man (Barry, 1958; Barry *et al.*, 1959, 1960; Conalty and Jackson, 1962; Banerjee *et al.*, 1974; Levy, 1974). In combined bacteriological and pharmacological studies, Levy (1974) suggested that its action was probably bactericidal and Holmes, Banerjee and Hilson (1976) have confirmed this by studying its effect on the solid ratio of an established infection. The results reported here confirm the bactericidal activity of B663, with a dosage of 0.003% for 60 days killing 98% of the inoculum (strain TG), and the same dosage for 30 days producing 96% killing (strain SBL 16220). The difference between the bactericidal activity of 0.01% and 0.003% B663 was not significant, but in each case B663 was significantly more bactericidal than DDS ($P' < 0.01$). Clinical experience with B663 suggests that during continuous treatment, its bactericidal activity in man is similar to that of DDS (Pettit and Rees, 1966; Pettit, Rees and Ridley, 1967; Levy, Shepard and Fasal, 1972). The relationship between the degree of

tissue accumulation of B663 and its bactericidal activity against *M. leprae* is unknown; different accumulation properties in man and in the mouse could account for its different bactericidal properties in the 2 species. Alternatively, the significantly greater bactericidal effect of B663 as compared with DDS reported here could be due to drug accumulation and persistence, resulting in leprosy bacilli being killed after drug administration has stopped. Thus although in this experiment the extent of bacterial killing was markedly different, the rate of killing may well have been similar.

The killing effect seen with thiacetazone suggests that 60 days' treatment with 0.1% produced 42% killing of the inoculum with a $T_{\frac{1}{2}}$ of 68 days. However, the difference between the MPN of the thiacetazone-treated group (930 viable bacilli) and the control group (1600 viable bacilli) is not significant at the 95% level.

The bactericidal effect seen with ethionamide and prothionamide is supported by further findings (Colston *et al.*, 1978) and the results with ethionamide confirm previous findings using the kinetic technique (Shepard, 1969). The results obtained when both drugs were administered at 0.1% were identical, with 98.6% killing in 45 days, representing a $T_{\frac{1}{2}}$ of 7.5 days. The amount of bacterial killing produced by 30 days' administration of 0.2% ethionamide was less than that produced by 45 days of 0.1% though the difference was not significant. However, the *rate* of bactericidal action was greater with 0.2% ethionamide ($T_{\frac{1}{2}} = 6.0$ days) than with 0.1% ($T_{\frac{1}{2}} = 7.5$ days). This value of $T_{\frac{1}{2}}$ is twice that obtained by the kinetic approach (Colston, unpublished data) and perhaps demonstrates the way in which the kinetic technique may exaggerate bactericidal activity by not differentiating between prolonged bacteriostasis and bactericidal action.

The results suggest that the proportional bactericidal test is a reliable method for assessing the bactericidal activity of antileprous drugs. All the results were statistically acceptable ($P' > 0.05$) and there is agreement with other techniques in which the purely bacteriostatic action of thiambutosine, the rapid bactericidal effect of RMP and the intermediate bactericidal effect of ethionamide, have been demonstrated. The method of bacillary growth assessment (simply scoring for the presence or absence of growth) has obvious advantages over the kinetic technique in which bacillary counts are made, and the problems of interpretation due to prolonged bacteriostasis or slow drug elimination are overcome.

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