Bactericidal Action of Dapsone Against *Mycobacterium leprae* in Mice

LOUIS LEVY

Leprosy Research Unit, Public Health Service Hospital, San Francisco, California 94118

Dapsone (4,4'-diaminodiphenylsulfone), incorporated into the mouse chow in a concentration of 0.1 g/100 g of diet, was administered for 1 week to mice in which Mycobacterium leprae had multiplied to the level of 10^6 organisms/footpad. M. leprae were harvested from these and also from control mice, diluted serially, and inoculated into additional mice. The organisms recovered from untreated mice multiplied in passage with a mean doubling time of 12.2 days, and 35% or more of the inoculated organisms were viable, i.e., capable of infecting mice. Growth curves of M. leprae recovered from dapsone-treated animals lagged behind those of organisms from control animals by an average of 78 days, equivalent to 98.8% killing. Foot-by-foot harvests showed that only 0.2% of the M. leprae recovered from treated mice were viable, suggesting that treatment of mice with dapsone had been accompanied by killing of 99.4% of the viable M. leprae.

Treatment of leprosy patients with dapsone (4,4'-diaminodiphenylsulfone [DDS]) is accompanied by killing of *Mycobacterium leprae* (9, 10); however, the action of the drug on *M. leprae* in mouse footpad infection has not been characterized. Previous studies in this laboratory (2, 3) suggested that treatment of mice with dapsone in a dosage that yielded plasma dapsone levels at least 10 times those produced by effective dosages in patients (5) and at least 1,000 times the minimal inhibitory concentration of dapsone for *M. leprae* (6) appeared to have resulted only in prolonged bacteriostasis. However, a recent experiment, somewhat modified in design, demonstrated unequivocally that *M. leprae* were indeed killed (that is, rendered noninfective for mice) during dapsone treatment of mice.

MATERIALS AND METHODS

The strain of *M. leprae* used in this experiment is the standard strain used for all studies of drug action in this laboratory. The dapsone was purchased from K & K Laboratories, Inc., Hollywood, Calif. Inocula were prepared, locally bred male Balb/c mice were inoculated, and harvests of *M. leprae* from footpad tissues were performed by published methods (7, 11).

Mice had been inoculated with $10^{3.70}$ *M. leprae*/footpad, and a harvest performed 121 days later yielded a mean value of $10^{6.12}$ organisms/footpad. At this time, administration of dapsone incorporated in the mouse chow in a concentration of 0.1 g/100 g was begun to one group of mice. Seven days later harvests of acid-fast bacilli (AFB) were performed from

the pooled tissues of eight footpads of both treated and untreated mice. The resulting bacterial suspensions were diluted serially so as to provide inocula of 10^4, 10^3, 10^2, 10, and 1 organism/footpad. Each of the 10 inocula was used to inoculate both hind feet of 15 passage mice. Harvests of *M. leprae* from pools of four footpads from all but one group were performed at intervals thereafter, and growth curves were constructed. When some of these growth curves were noted to be grossly irregular, the program of interval harvests was abandoned. Instead, foot-by-foot harvests were performed from all surviving passage mice between 361 and 364 days after passage.

RESULTS

Growth curves constructed from the results of harvests of *M. leprae* from the passage mice are shown in Fig. 1, and the time elasped from passage to multiplication to the level of 10^6 AFB/footpad for each growth curve is shown in Table 1. Multiplication of *M. leprae* in the mice inoculated with organisms recovered from untreated mice was quite uniform. The growth curves, except for that representing multiplication from the smallest inoculum, appear almost parallel, and the intervals between pairs of adjacent growth curves at their intersection with a line drawn parallel to the time axis through $\log_{10}$ number of AFB/footpad = 6.0 are virtually identical. The mean interval is 40.7 days; because each 10-fold increase in the number of bacilli represents 3.32 doublings, the doubling time for *M. leprae* recovered from untreated mice in this experiment was 12.2 days.

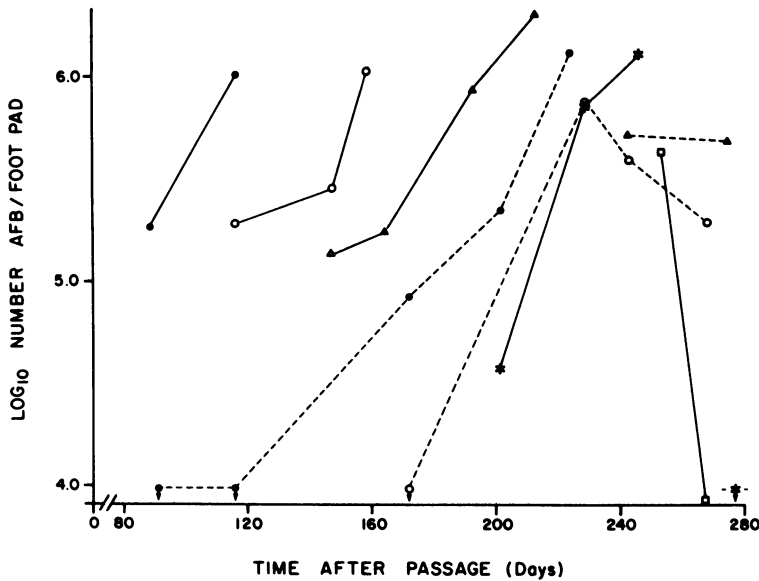Multiplication of the *M. leprae* recovered

FIG. 1. Log_{10} number of AFB per footpad as a function of the time after passage in days. The solid lines represent growth curves of M. leprae in passages from untreated control mice; the broken lines represent growth curves in passages from dapsone-treated mice. No organisms were found in the harvests represented by the points with downward extending arrows; these points were calculated as if one organism had been found in the counting procedure. Passage mice were inoculated as follows: (●) 10^4 organisms/footpad; (O) 10^3 organisms/footpad; (△) 10^2 organisms/footpad; (*) 10 organisms/footpad; (□) 1 organism/footpad.

from dapsone-treated mice was not so uniform. The mean number of organisms did not reach the level of 10^6/footpad in the passage mice inoculated with 10^3 or 10^2 M. leprae/footpad; no multiplication had occurred in the mice inoculated with 10 organisms/footpad that were harvested 277 days after passage; and no harvests were performed of the passage mice inoculated with 1 organism/footpad until 1 year after inoculation. The intervals between the pairs of adjacent growth curves (measured at the level of $10^{5.5}$ AFB/footpad) are not as uniform as for the passages of M. leprae recovered from untreated mice.

More pertinent to this presentation are the intervals between the pairs of growth curves derived from passages from treated and untreated mice at each level of inoculum, shown in the column of Table 1 headed "delay." This delay, averaging 78 days in duration, may have resulted from a prolonged bacteriostatic effect of dapsone. It appears more likely, however, that the delay represents killing of M. leprae that had occurred during dapsone treatment, with multiplication of the survivors after passage. Assuming that the surviving organisms multiply with a constant doubling time of 12.2 days, the 78-day delay, equivalent to 6.4 doublings, indicates that 98.8%, 100 × (1 − [1/2^6.4]),

TABLE 1. Calculations from growth curves of M. leprae in passage mice

No. of AFB inoculated/ footpad	Time from passage to multiplication to 10^6 AFB/footpad (days)		Delay[a] (days)	% Killing[b]
	Control	DDS		
10^4	116	220	104	99.7
10^3	157	231[c]	74	98.5
10^2	198	254[c]	56	95.8
10	238	NM[d]		

[a] The delay is the time in days between multiplication to 10^6 M. leprae/footpad passaged from control mice and multiplication to the same level of M. leprae passaged from dapsone-treated mice.
[b] Percentage of killing = 100 (1 − [1/2^x]), where x = the number of doublings calculated as the number of days delay/12.2 days per doubling.
[c] These values were determined by extrapolation.
[d] NM, Not measured.

of the organisms had been killed during treatment.

That killing of M. leprae rather than prolonged bacteriostasis is the explanation for the delay is confirmed by the results of the foot-by-foot harvests shown in Table 2. These demonstrate that fewer than 25% of the footpads inoculated with 1 organism from untreated mice

were infected. Although foot-by-foot harvests were not performed from footpads inoculated with larger numbers of organisms recovered from untreated mice, the constant time interval between pairs of adjacent growth curves representing passages from untreated mice suggests that all of the footpads inoculated with 10 or more organisms obtained from untreated mice were infected. The inocula prepared from the untreated mice may be shown by the most-probable-number technique (12) to have contained at least 35% viable organisms. Actually, these inocula may well have contained 100% viable *M. leprae*, because as many as five viable organisms may be required for infection (4).

It is clear from the results shown in Table 2 that even the inoculum of 10^3 organisms prepared from the dapsone-treated mice failed to infect all of the footpads, and the inocula of 1 and 10 organisms failed to infect any of the footpads harvested. Calculation by the most-probable-number technique shows that only about 0.2% of the organisms harvested from dapsone-treated mice were capable of infecting passage mice. Thus, dapsone treatment of the mice was accompanied by the killing of 99.4% of the viable *M. leprae*. This is consistent with the calculation of 98.8% killing based on the mean duration of the delay shown in Table 1.

DISCUSSION

The purpose of this experiment was to study in a more direct manner than we had employed in our previous studies (2, 3) whether killing of *M. leprae* occurred during the administration to mice of 0.1 g of dapsone per 100 g of diet for 1 week. In the first study of dapsone administration during "established infection" of *M. leprae* in mice, Shepard and Chang (8) showed that killing of the organisms had occurred, but it was not possible to estimate the rate of killing from their data.

We subsequently performed a similar study designed to permit measurement of this rate (2). Half of a group of mice in which *M. leprae*

TABLE 2. *Results of foot-by-foot harvests*

No. of AFB inoculated/footpad	Proportion showing multiplication[a]	
	Control	DDS
10^4	ND[b]	ND
10^3	ND	3/4
10^2	ND	5/18
10	ND	0/24
1	3/16	0/30

[a] Number of footpads showing multiplication of *M. leprae*/number of footpads harvested.
[b] ND, Not done.

had multiplied almost to 10^6/footpad was treated with 0.1 g of dapsone per 100 g of diet for 8 weeks, during which passages were made of *M. leprae* recovered from treated and untreated control mice at intervals of 1 or 2 weeks. The time from passage to multiplication to 10^6 organisms/footpad increased in a linear fashion for passages of *M. leprae* from untreated mice. The time required for multiplication to 10^6 AFB/footpad increased at the same rate for passages of *M. leprae* from dapsone-treated mice, but was longer by an average of 34 days than that of corresponding passages from untreated mice. Because these passages were performed during continued dapsone administration, these results were interpreted to indicate prolongation of the lag phase of passages from treated mice (i.e., prolonged bacteriostasis).

To establish more firmly that the action of dapsone was that of prolonged bacteriostasis rather than bactericide, we carried out a second study (3). Dapsone was administered for 1, 3, or 7 days to mice in which *M. leprae* had multiplied almost to 10^6/footpad. Passages of *M. leprae* were made from the several groups of treated mice and from untreated mice at intervals of 1 or 2 weeks for 10 weeks, and the time from passage to multiplication to 10^6 AFB/footpad was measured for each passage. Soon after the period of treatment, the time from passage to multiplication to 10^6 AFB/footpad was considerably longer for passages from treated mice than for those from untreated mice. However, the passages made 8 and 10 weeks after the period of treatment showed no difference of the time required for multiplication to 10^6/footpad between passages from treated and untreated mice. The transitory effect of dapsone that was evident soon after the period of dapsone administration but had waned 8 to 10 weeks later was felt to be consistent with prolongation of the lag phase, but not with killing of *M. leprae*.

These earlier experiments suffered because the distinction between prolonged bacteriostasis and bacterial killing could be only inferential. The description by Hilson of his technique of "proportional bactericide" (1) suggested a more direct approach to the distinction between bacteriostatic and bactericidal effects of a drug. Therefore, the experiment was repeated with two modifications. Passage inocula were serially diluted to permit "titration" of the proportion of viable organisms in the inocula, and foot-by-foot harvests were performed in addition to those needed for the construction of growth curves of *M. leprae*. The results of the experiment as modified, described in this report, demonstrate that the administration of 0.1 g of dapsone per 100 g of diet for 1 week was

accompanied by killing of about 99% of the viable organisms.

Remaining to be considered is whether the technique of proportional bactericide permitted the demonstration of bacterial killing that was obscured in the earlier experiments by the use of a less direct and less precise technique, or whether bacterial killing occurred in this experiment but not in the two earlier experiments.

Colston and Hilson have recently reported that treatment of mice with 0.01 g of dapsone per 100 g of diet for 30 days was accompanied by 41% killing, and treatment with the same dosage for 60 days by 82% killing (M. J. Colston et al., personal communication; M. J. Colston and G. R. F. Hilson, Int. J. Leprosy, in press). Thus, killing of *M. leprae* during dapsone treatment has been demonstrated by other workers. The results of our earlier experiments cannot easily be reinterpreted to show killing of *M. leprae;* however, it would appear that treatment of mice with dapsone may be accompanied sometimes by bactericidal and at other times by prolonged bacteriostatic effects. Further study is needed to bring to light the factors determining the character of dapsone action from experiment to experiment.

LITERATURE CITED

1. Hilson, G. R. F., and D. K. Banerjee. 1974. The proportional bactericidal test: a method for testing *in vivo* bactericidal action of a persistent drug. I.R.C.S. Med. Sci. 2:1037.
2. Levy, L. 1970. Death of *Mycobacterium leprae* in mice, and the additional effect of dapsone administration. Proc. Soc. Exp. Biol. Med. 135:745-749.
3. Levy, L. 1972. Prolongation of the lag phase of *Mycobacterium leprae* by dapsone. Proc. Soc. Exp. Biol. Med. 139:263-266.
4. Levy, L., N. Moon, L. P. Murray, S. M. O'Neill, L. E. Gustafson, and M. J. Evans. 1974. Studies of the mouse footpad technic for cultivation of *Mycobacterium leprae*. 1. Fate of inoculated organisms. Int. J. Lepr. 42:165-173.
5. Ozawa, T., C. C. Shepard, and A. B. A. Karat. 1971. Application of spectrophotofluorometric procedures to some problems in *Mycobacterium leprae* infections in mice and man treated with dapsone (DDS), diacetyl-DDS (DADDS), and di-formyl-DDS (DFD). Am. J. Trop. Med. Hyg. 20:274-281.
6. Peters, J. H., G. R. Gordon, J. F. Murray, Jr., A. H. Fieldsteel, and L. Levy. 1975. Minimal inhibitory concentration of dapsone for *Mycobacterium leprae* in rats. Antimicrob. Agents Chemother. 8:551-563.
7. Shepard, C. C. 1960. The experimental disease that follows the injection of human leprosy bacilli into footpads of mice. J. Exp. Med. 112:445-454.
8. Shepard, C. C., and Y. T. Chang. 1967. Effect of DDS on established infections with *Mycobacterium leprae* in mice. Int. J. Lepr. 35:52-57.
9. Shepard, C. C., L. Levy, and P. Fasal. 1968. The death of *Mycobacterium leprae* during treatment with 4,4'-diaminodiphenylsulfone, (DDS). Initial rate in patients. Am. J. Trop. Med. Hyg. 17:769-775.
10. Shepard, C. C., L. Levy, and P. Fasal. 1972. The death rate of *Mycobacterium leprae* during treatment of lepromatous leprosy with acedapsone (DADDS). Am. J. Trop. Med. Hyg. 21:440-445.
11. Shepard, C. C., and D. H. McRae. 1968. A method for counting acid-fast bacteria. Int. J. Lepr. 36:78-82.
12. Taylor, J. 1962. The estimation of numbers of bacteria by tenfold dilution series. J. Appl. Bacteriol. 25:54-61.