Death of *Mycobacterium leprae* in Mice, and the Additional Effect of Dapsone Administration[1] (35134)

LOUIS LEVY
(Sponsored by A. Howard Fieldsteel)
Leprosy Research Unit, Public Health Service Hospital, San Francisco, California 94118

The effect of dapsone (4,4′-diaminodiphenylsulfone, DDS) on the multiplication of *Mycobacterium leprae* in the mouse foot pad has been studied primarily by means of experiments in which dapsone administration was begun at the time of inoculation or at a time when the organisms had entered the logarithmic phase of multiplication, well before maximal multiplication has been achieved (1). These experiments have yielded valuable information about the effect of the drug on the organism, permitting both the measurement of the minimal inhibitory concentrations of DDS for *M. leprae* (2) and also the demonstration of DDS-resistant strains of the organism (3, 4). But they have not permitted the observation of drug effects in established infection, the situation encountered in the treatment of the patient with lepromatous leprosy.

In mouse experiments, bacterial numbers are less than 10^5 per foot pad when drug administration is begun, whereas the untreated patient with lepromatous leprosy may harbor as many as 10^{11} *M. leprae* (5). Furthermore, because there are so few organisms, it is difficult to measure the rate of killing of *M. leprae* during DDS treatment of the mice, whereas the rate of killing of the organisms has been successfully measured in patients under treatment with one dosage regimen of DDS (6).

Shepard and Chang (7), in an effort to study the effect of DDS in established *M. leprae* infection of mice, began DDS treatment at about the time that bacterial numbers reached their maximum of approximately 10^6 per foot pad. Because bacterial killing occurred initially at the same rate in both treated and untreated mice, an effect of DDS could be demonstrated only by failure of a second growth phase of the *M. leprae* to occur in the treated mice as it did in untreated controls. It was not possible to measure the rate of bacterial killing during DDS treatment. We have undertaken to demonstrate an additional effect of dapsone by comparing the rate of bacterial killing in treated mice with that in the untreated animals. Measurement of the rate of killing has been attempted by a careful examination of the changes of bacterial morphology and of the ability of the *M. leprae* to multiply after passage to other mice.

Methods. The mouse foot pad technique for the cultivation of *M. leprae* developed by Shepard (8, 10) was used. The strain of *M. leprae* used in these experiments was originally isolated by C. C. Shepard, National Communicable Disease Center, Atlanta, Georgia, from a patient with lepromatous leprosy and has since been maintained in mouse passage in our laboratory. Locally bred BALB/c mice were used. DDS was added to the mouse meal as a solid, and mixing was accomplished in a liquid–solid twin-shell blender (Patterson-Kelly Co.). Measurement of the solid ratio—the proportion of brightly and solidly stained acid-fast bacilli (AFB) to the total number of AFB examined—was performed by Shepard's technique (11); the preparations were coded and examined in random order; each measurement was based on the examination of 500 AFB.

Results. In a preliminary experiment male mice were inoculated, each with 5×10^3 *M. leprae* into the right hind foot pad. The re-

[1] Supported by the US–Japan Cooperative Medical Science Program, National Institute for Allergy and Infectious Diseases, National Institutes of Health, Department of Health, Education, and Welfare (Grant R22-AI-07801).

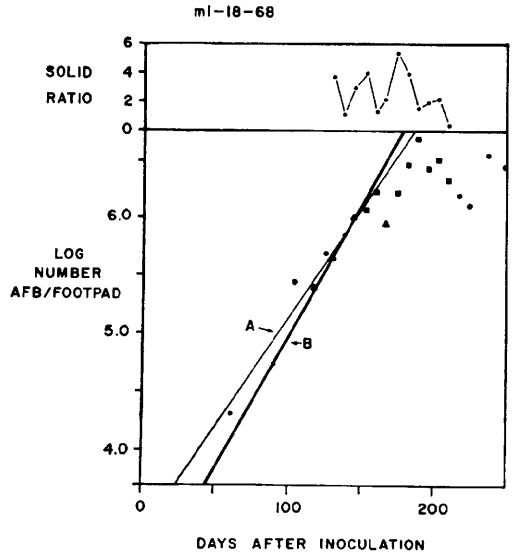

FIG. 1. Results of Experiment m1-18-68. Upper panel. The number of solid organisms per 100 AFB as a function of time. Lower panel. The common logarithm of the number of AFB harvested per foot pad as a function of time after inoculation. Harvest from pooled tissue of four mice (●); harvest from pool of six mice (■); harvest from pool of eight mice (▲); Line A: log number of AFB = 5.678 + 0.0188 (number of days—129.22); γ, the correlation coefficient, = 0.96; doubling time = 16 days. Line B: log number of AFB = 5.678 + 0.0232 (number of days—129.22); doubling time = 13 days.

sults of harvests of the pooled foot pad tissue of four to eight mice performed 61 days after inoculation, and at intervals of 1 or 2 weeks from 91 until 248 days after inoculation are presented in Fig. 1. The number of AFB per foot pad increased, reaching 10^6 organisms per foot pad at about 150 days; shortly thereafter, multiplication ceased. Line A, the regression line through the points representing the harvests between 91 and 161 days, yields a doubling time of 16 days; line B has been drawn with a doubling time of 13 days, the usual doubling time of this strain of *M. leprae* in these mice measured during logarithmic multiplication in our laboratory. The solid ratio varied between 1.2 and 5.4/100 during logarithmic multiplication, and fell below 1/100 only for the 210-day harvest.

Another group of male mice was inoculated with *M. leprae* as was the first, and harvests were performed with the results shown in Fig. 2. In order to conserve mice, fewer harvests were performed to define the growth curve. One hundred thirty-eight days after inoculation, a harvest was performed, and organisms were passed to another group of mice. The remaining mice were randomly divided between a control group and a group to which was administered DDS, 0.1%, in the mouse diet. At weekly intervals thereafter, harvests were performed of the pooled foot pad tissue of four mice from each group, and organisms from each harvest were passed to other groups of mice. DDS was administered continuously until 194 days after inoculation, at which time the last harvests and passages were performed. Line A, the regression line through the points representing the harvests from untreated mice between 91 and 152

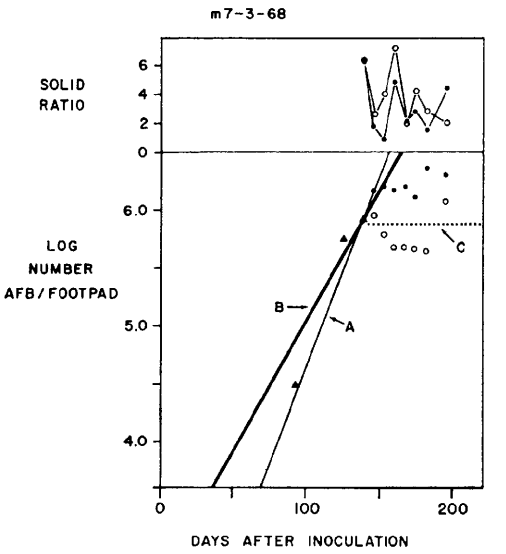

FIG. 2. Upper panel. The number of solid organisms per 100 AFB as a function of time. Lower panel. The common logarithm of the number of AFB harvested per foot pad as a function of time after inoculation. Harvest from pooled tissue of four mice (○ ●); harvest from pooled tissue of eight mice (▲). Harvests from untreated mice are denoted by solid symbols; harvests from mice treated with 0.1% dapsone are denoted by open circles. Line A: log number of AFB = 5.688 = 0.0305 (number of days — 130.00); γ = 0.98; doubling time = 9.9 days. Line B: log number of AFB = 5.688 + 0.0232 (number of days — 130.00); doubling time = 13 days. Line C: log number of AFB = 5.73 + 0.0005 (number of days — 163.50); γ = 0.05.

TABLE I. Harvests from Passage Mice.

Time from inoculation of m7-3-68 to harvest and passage (days)	Time after passage (days)	Number AFB[a] recovered per foot pad ($\times 10^5$)	Time to 10^6 AFB per foot pad (days)
138	103	0.6	
	148	32	136
	156	15	
145 (Control)	150	10	149
145 (Treated)	150	2.4	
	177	4.8	184
	205	22	
152 (Control)	149	8.9	151
152 (Treated)	149	0.4	
	189	7.5	193
	206	21	
159 (Control)	149	6.5	154
	157	13	
159 (Treated)	182	3.9	196
	200	13	
166 (Control)	143	3.0	162
	168	15	
166 (Treated)	183	5.0	202
	211	7.2	
173 (Control)	183	14	168
	185	31	
173 (Treated)	163	2.7	
	184	6.5	195
	185	4.7	
	218	13.7	
181 (Control)	158	0.94	
	180	9.2	188
	194	10.7	
181 (Treated)	193	6.0	
	214	5.8	216
	233	3.0	
194 (Control)	197	18.9	184
194 (Treated)	182	1.1	
	210	2.8	222
	240	34	

[a] AFB = acid-fast bacilli.

days, yields a doubling time of 10 days; line B has been drawn with a doubling time of 13 days. Line C is the regression line through the points representing the harvest after 138 days and the subsequent harvests from the treated mice. Multiplication of *M. leprae* in the untreated mice ceased shortly after the mice were divided into two groups and DDS treatment started; DDS treatment caused immediate inhibition of multiplication of the organisms. There was no consistent trend of the solid ratio during logarithmic multiplication, after cessation of multiplication of *M. leprae* in the untreated mice, or during DDS administration.

The *M. leprae* recovered in each of the harvests from both treated and untreated mice were diluted to provide an inoculum of 5×10^3 organisms per foot pad and each inoculum was passed to a group of 20 mice, hereafter designated as "passage" mice. Harvests of the pooled foot pad tissue from four mice were performed at intervals from each group of passage mice (Table I). The time elapsed between inoculation of the passage mice and multiplication to a level of 10^6 AFB per foot pad was calculated for each passage, assuming that those *M. leprae* capable of multiplying in the mice do so with a doubling time of 13 days. The time required for bacterial numbers to reach 10^6 per foot pad in the passage mice, termed the "time to plateau," has been plotted as a function of the time from inoculation to passage for each group of treated or untreated mice (Fig. 3). Line A, the regression line through the points calculated from the results of the passage from the untreated mice, is described by the expression

$$D = 161.5 + 0.908\ (d - 63.5)$$

where D is the time to plateau for each passage in days and d the time in days from inoculation to passage. The 95% confidence interval for the slope of the regression line is 0.637 to 1.179. Line B, the regression line through the points from the passages from the treated mice, has the equation

$$D = 201.14 + 0.734\ (d - 167.14).$$

The 95% confidence interval for its slope is 0.401 to 1.067. Because the slope of each line lies within the confidence interval of the other, Lines A and B may be considered parallel. That the two lines are not identical is demonstrated by the confidence interval around the value for D on each line at the

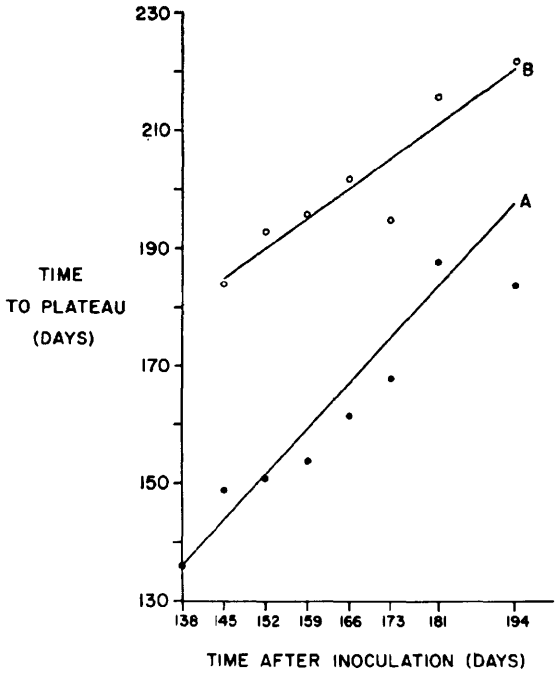

FIG. 3. Results of passages from Experiment m7-3-68. The time required for the multiplication of *M. leprae* to the plateau value of 10^6 AFB per foot pad is plotted for each passage as a function of the time of passage. Untreated mice (●); treated mice (○).

same d. At $d = 165$ days, D has the 95% confidence interval 158.15 to 167.71 for Line A and the interval 194.23 to 204.81 for Line B. Thus, the two lines are distinct, and the points through which each is drawn come from two different populations. These plots suggest that the time to plateau of each successive passage increased in proportion to the length of time from inoculation to passage, and that the time to plateau is always longer for the passages from treated mice than for those from the untreated mice by an average of about 36 days.

Discussion. The number of organisms in each passage was standardized. If one assumes the doubling time of a given strain of *M. leprae* to be constant in the same strain of inbred mice, and if the duration of the lag phase is assumed to be constant for each passage from the untreated mice, then the time to plateau of each passage is a function of the proportion of organisms in each passage inoculum capable of multiplication in the mouse foot pad. The increase of the time to plateau in the passage mice may then be interpreted to represent a decrease of the proportion of infective *M. leprae*. In fact, for a given duration of the lag phase, the proportion of infective organisms in each passage inoculum may be readily computed. When this proportion is plotted as a function of the time from inoculation to passage, the loss of infective *M. leprae* may be shown to have occurred exponentially, with a half-time of 14 days. The loss of *M. leprae* infective for the mouse foot pad may result from death of the organisms.

The loss of infective *M. leprae* appears to have occurred at the same rate in treated as in untreated mice after 1 week of DDS administration. But there is a marked lengthening of the time to plateau during the first week of DDS, which is equivalent to a loss of 90% of the infective organisms present before treatment. This interpretation suggests that 90% of the *M. leprae* are susceptible to DDS and die rapidly during treatment, whereas the remaining 10% are DDS-resistant and survive DDS administration in high dosage, only to be killed more slowly by the mice. But what we know about DDS-resistant *M. leprae* is entirely inconsistent with so large a proportion of DDS-resistant organisms. Tests of the susceptibility of this strain of *M. leprae* in our laboratory have demonstrated that it is inhibited from multiplying by a dose of DDS 1/1000 of that used in this experiment. Moreover, disease caused by DDS-resistant *M. leprae* occurs only rarely (3, 4).

A more tenable explanation, which requires a homogenous population with respect to DDS susceptibility, is that this DDS treatment uniformly lengthened the lag phase of multiplication of *M. leprae* after as little as 1 week's exposure to the drug. The lag phase of multiplication of *M. leprae* cannot be readily measured in mice. When an inoculum of usual size (5×10^3 AFB per foot pad) is used, the organisms are too few for accurate counting. In order to provide bacterial numbers sufficiently large to permit accurate counting, inocula no smaller than 5×10^4 AFB per foot pad might be employed. Multiplication might then be followed during the

four or five doublings which could occur before logarithmic multiplication ceased. If all of the *M. leprae* in the inoculum were capable of multiplication in the mouse foot pad, one might readily recognize the end of the lag phase and the beginning of logarithmic multiplication. But only a fraction of the total number of *M. leprae* in an inoculum are infective for the mouse foot pad (12); if this fraction were relatively small, the larger number of noninfective organisms would obscure the first doublings of the infective organisms, and the duration of the lag phase would be overestimated.

In an analogous experiment, Dickinson and Mitchison (13) have shown that *M. tuberculosis* subcultured after varying periods of exposure *in vitro* to isoniazid demonstrate prolongation of the lag phase, the duration of which is proportional to the duration of contact with the drug. The results of Shepard's "kinetic" experiments (1), in which the resumption of multiplication of *M. leprae* after cessation of DDS administration to the mice followed a delay which could not be attributed to continued presence of inhibitory concentrations of the drug, may be interpreted to show "persisting bacteriostasis" or bacterial death. The results of our experiments suggest that bacteriostasis persisting after removal of the organisms from contact with the drug may be the more correct explanation of Shepard's findings.

Summary. If the assumptions be valid that the lag phase of bacterial multiplication is constant when *M. leprae* are repeatedly harvested from untreated mice and passed to other mice of the same inbred strain, and that those *M. leprae* capable of multiplying in the mouse foot pad do so always at the same rate, then the results of these experiments may be interpreted to show that once the peak of bacterial multiplication has been reached, death of *M. leprae* ensues. Death of *M. leprae* appears to have occurred in mice during DDS treatment at the same rate as in the untreated mice, but the lag phase of bacterial growth was uniformly prolonged as a result of treatment.

1. Shepard, C. C., Int. J. Lepr. **35**, 429 (1967).

2. Shepard, C. C., McRae, D. H., and Habas, J. A., Proc. Soc. Exp. Biol. Med. **122**, 893 (1966).

3. Rees, R. J. W., Bull. World Health Organization **40**, 785 (1969).

4. Shepard, C. C., Levy, L., and Fasal, P., Amer. J. Trop. Med. Hyg. **18**, 258 (1969).

5. Shepard, C. C., Discussion, Trans. Leonard Wood Memorial–Johns Hopkins University Symposium on Research in Leprosy, Baltimore, May, 1961, p. 230.

6. Shepard, C. C., Levy, L., and Fasal, P., Amer. J. Trop. Med. Hyg. **17**, 769 (1968).

7. Shepard, C. C., and Chang, Y. T., Int. J. Lepr. **35**, 52 (1967).

8. Shepard, C. C., J. Exp. Med. **112**, 445 (1960).

9. Shepard, C. C., Int. J. Lepr. **30**, 10 (1962).

10. Shepard, C. C., and McRae, D. H., Int. J. Lepr. **36**, 78 (1968).

11. McRae, D. H., and Shepard, C. C., Int. J. Lepr. **36**, 588 (1968).

12. Shepard, C. C., and McRae, D. H., J. Bacteriol. **89**, 365 (1965).

13. Dickinson, J. M., and Mitchison, D. A., Tubercle 47, 370 (1966).

Received June 29, 1970. P.S.E.B.M., 1970, Vol. 135.