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Studies of the Mouse Foot Pad Technique for Cultivation of *Mycobacterium leprae*

3. Doubling Time During Logarithmic Multiplication

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The doubling time of a strain of *Mycobacterium leprae* during logarithmic multiplication in the mouse foot pad was estimated by inoculating mice with serial dilutions of a bacterial suspension and measuring the time from inoculation to multiplication to 10^6 organisms per foot pad. The doubling time was found to be 11.1 ± 1.92 (mean ± 95% confidence limits) days, about 15% shorter than an earlier estimate based on measurements of the slopes of many single growth curves of *M. leprae*.

Introduction

Shepard has estimated that the doubling time of *Mycobacterium leprae* during logarithmic multiplication in the mouse foot pad infection is 12 to 13 days (Shepard and McRae, 1965). Work in this laboratory has yielded a similar estimate of 13 days (Levy, 1970). Both estimates, however, are imprecise because they depend upon measurements of the slopes of growth curves, which cannot be measured with precision.

A better approach for measuring the rate at which *M. leprae* multiply was suggested by the results of an experiment performed to ascertain the minimal number of *M. leprae* required to infect mice. Mice were inoculated with serial 10-fold dilutions of a bacterial suspension. The strikingly parallel character of the resulting growth curves suggested a more precise method that has subsequently been employed in a systematic study of the rate of doubling of *M. leprae* during logarithmic multiplication in the mouse foot pad.

Methods

The strain of *M. leprae* studied in these experiments is the standard strain that has been used for most of the studies performed in this laboratory. Inocula were

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prepared, locally-bred BALB/c mice inoculated, and harvests of *M. leprae* from mouse foot pad tissues performed by published methods (Shepard, 1960; Shepard and McRae, 1968). For these experiments, suspensions of *M. leprae* harvested from mouse foot pads were diluted serially to provide inocula of 5000, 500, 50 and, in most experiments, 5 organisms per foot pad. Each of the inocula was used to inoculate both hind feet of 10 or 15 mice. Harvests of *M. leprae* from pools of at least 4 foot pads were performed at intervals thereafter, and growth curves were constructed.

Results

The results of the first experiment are shown in Fig. 1, in which the $\log^{10}$ number of AFB per foot pad found at harvest is shown as a function both of the number of days elapsed from inoculation to harvest and the size of the inoculum. The time intervals between pairs of adjacent growth curves at $\log^{10}$ number AFB = 6.0 are 40 and 45 days for the 3 larger inocula and 72 days for the 2 smaller inocula. Because a 10-fold increase in the number of *M. leprae* represents 3.32 doublings, the mean number of days per doubling is 12.8 when only the

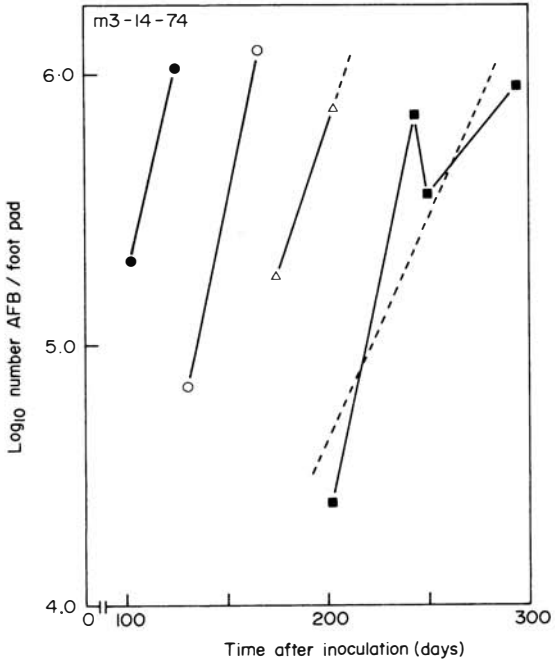

Fig. 1. The $\log^{10}$ number of acid-fast bacilli (AFB) per foot pad as a function of the number of days from from inoculation to harvest and the number of AFB inoculated. Points representing the harvests of *M. leprae* from each group of mice; the best-fitting straight line used to measure the time from inoculation to multiplication to the level of 10^6 organisms per foot pad. Mice were inoculated as follows: (●) 5000 organisms per foot pad; (○) 500 organisms per foot pad; (△) 50 organisms per foot pad; (■) 5 organisms per foot pad.

three largest inocula are considered. The irregularity of the growth curve representing the smaller inoculum and the large time interval between the growth curves derived from the 2 smaller inocula suggest that the smallest inoculum did not infect all of the foot pads.

The results of this experiment and of 6 subsequent experiments are summarized in Table 1, in which the numbers of days elapsed from inoculation to multiplication of *M. leprae* to the level of 10^6 per foot pad in mice inoculated with 5000, 500, 50 and, in some experiments, 5 organisms per foot pad are listed. In addition, the doubling times, calculated from the linear regression of the time to 10^6 organisms per foot pad on the $\log^{10}$ number of *M. leprae* inoculated, are shown. The mean doubling time for the seven experiments is 11.1 days; the 95% confidence limits are 9.2 and 13 days.

TABLE 1

Doubling time of M. leprae *during logarithmic multiplication in the mouse foot pad*

Experiment No.	Time from inoculation to multiplication to 10^6 AFB per foot pad (days)				Doubling time (days)
	Number *M. leprae* inoculated per foot pad				
	5000	500	50	5	
m 3-14-74	123	163	208	280[a]	12.8
m 9-19-74	126	177	208	248	12.0
m 10-16-74	144	186	199	ND[b]	8.28
m 12-10-74	123	159	207	261	13.9
m 12-23-74	134	140	191	ND	8.58
m 1-14-75	121	159	190	236	11.3
m 4- 3-75	125	178	198	ND	11.0
Mean ± 95% confidence limits					11.1 ± 1.92

[a] This value was not used for calculation because the growth curve suggested irregularity of infection.
[b] Not done.

Discussion

The purpose of this study was to measure more precisely the rate at which *M. leprae* multiply during the logarithmic phase in the mouse foot pad. Previous estimates of this rate were derived from measurements of the slopes of growth curves of *M. leprae* in mice. Although the typical growth curve represents at least 200-fold multiplication (7.3 doublings) from the inoculum of 5000 *M. leprae* per foot pad to the ceiling of somewhat more than 10^6 organisms per foot pad, the entire length of the growth curve cannot be used for calculating the slope. A harvest of 5000 *M. leprae* per foot pad from a pool of 4 foot pads results when only a single organism has been counted in the examination of 60 microscopic fields with a magnification of x 1250, and a harvest of 100,000 organisms per foot pad results when only 20 organisms have been counted. Assuming that the distribution of the organisms is random and described by the Poisson distribution (Goldstein, 1964), the 95% confidence limits around a count of 20 are 11 and 39 organisms. This broad confidence band implies that one may overestimate the

number of organisms by two-fold (one doubling), or underestimate it by one-half. The confidence band may actually be somewhat broader, because *M. leprae* tend to clump and probably are not distributed randomly. Thus, only the upper portion of the growth curve—from 1 or 2×10^5 to 10^6 organisms per foot pad, equivalent to only 2.3 to 3.3 doublings and constructed from only 2 or 3 harvests—can be used to measure the slope with confidence.

The upper end of the growth curves also poses problems. At some time after multiplication passes the level of 10^6 organisms per foot pad, the rate slows, and one cannot be certain whether a harvest of 2×10^6 *M. leprae* per foot pad, for example, represents the peak of logarithmic multiplication or the stationary phase of bacterial growth.

An alternative method for measuring the doubling time of microorganisms was suggested by Youmans and Youmans (1949). These investigators inoculated liquid media with serially diluted suspensions of *M. tuberculosis* and took as the end point the first appearance of subsurface growth in the cultures. A plot of the logarithm of the inoculum against the time from inoculation to visible subsurface growth yielded a straight line; the doubling time was readily calculated from the slope of the line. This method, which depends simply on the appearance or non-appearance of an event, yields a better estimate of the doubling time than does a method that depends upon the measurement of some variable quantity that cannot be measured with great precision. In growth curves of *M. leprae*, we can determine with much greater precision that time at which multiplication reaches the level of 10^6 per foot pad than we can the slopes of growth curves.

The doubling time of the strain of *M. leprae* studied in these experiments, 11.1 days, is 15% shorter than our previous estimate of 13 days, based on repeated studies of the same strain (Levy, 1970). More importantly, it is a better estimate, because each of the seven experimentally-derived values shown in the sixth column of Table 1 was based on from 8 to 12 harvests.

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