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Multiplication of *Mycobacterium leprae* in Normal Mice

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In the absence of a method for cultivation of *Mycobacterium leprae in vitro,* its "cultivation" in the foot pad of the mouse must remain a laboratory technique of paramount importance to research in leprosy. As will be seen, multiplication of *M. leprae* in the mouse foot pad is the primary criterion of viability of the organism; when organisms have multiplied in mice, the inoculum must have included viable *M. leprae.* This paper, which has been derived largely from the laboratory manual prepared by the Leprosy Unit of the World Health Organization ([15]), discusses the characteristics of multiplication of *M. leprae* in the foot pads of immunologically normal mice, the theoretical basis for deciding that multiplication has occurred, and the statistical considerations to be taken into account in planning experiments based upon inoculation of *M. leprae* into the mouse foot pad.

In work based on the demonstration by Fenner ([2]) of multiplication in the hind foot pads of mice of *M. marinum* and *M. ulcerans,* for which the optimal temperature for growth is lower than 37°C, Shepard demonstrated ([8, 9]) that *M. leprae* multiply to a limited extent in the hind foot pads of immunologically intact mice. Following inoculation into a hind foot pad of fewer than 10^5 *M. leprae,* obtained from harvested mouse foot-pad tissues, or from skin biopsy specimens or sediments of nasal washings of patients with untreated lepromatous leprosy, there was very little change during the first several months. Histopathologically, granulomatous lesions consisting of large round cells, some containing AFB, appeared and gradually increased in size during the next several months, accompanied by an increase of the number of organisms. Grossly, a lesion was almost never evident. Inoculation of 10^5 or more *M. leprae* was followed by the early appearance of granulomas without evidence of bacterial multiplication. This most significant finding was subsequently confirmed and exploited by many workers.

Employing standard methods for inoculating, harvesting, and enumerating AFB, *M. leprae* may be demonstrated to multiply in foot pads of immunologically intact mice with the following characteristics: 1) the minimal infecting dose of *M. leprae* is of the order of five viable organisms ([6, 12, 14]). Because the inoculum is distributed to tissues, both contiguous and distant, not ordinarily encompassed in the harvest, a large fraction of the inoculum is effectively lost ([7]), suggesting that the minimal infecting dose may be as small as one or two viable *M. leprae*; 2) multiplication proceeds in a characteristic manner. Lag, logarithmic and stationary phases of multiplication may be readily identified (Fig. 1); 3) the doubling time** of *M. leprae,* measured during logarithmic multiplication, is 11 to 13 days ([6, 12]); 4) the stationary phase or "plateau" represents, in fact, the effect of the immune response of the mouse. Evidence for this is failure of multiplication when immunologically intact mice are inoculated with 10^5 or

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** The "doubling time" or "generation time" (the average time required for each twofold increase of the number of organisms),

$$G = \frac{\text{number of days between inoculation and harvest}}{\text{number of doublings between inoculation and harvest}}.$$

The number of doublings is the logarithm$_2$ of the fold-increase of the number of organisms. Thus, if 5000 *M. leprae* have been inoculated and, 100 days later, 10^6 AFB are harvested, then the number of organisms has increased 200-fold (10^6/5000) in the course of 100 days. $\text{Log}_2 200 = \frac{\log_{10} 200}{\log_{10} 2} = 2.301/0.301 = 7.644$; G = 100/7.644 = 13.08 days per doubling. The doubling time may be measured directly from the straight line representing the phase of logarithmic multiplication—i.e., from the straight line, calculated by the method of least squares, that best fits the experimentally observed values. Alternatively, the doubling time may be calculated from the average distance between the growth curves resulting in mice inoculated with serial dilutions of *M. leprae* ([6]).

more organisms; the resistance of *M. leprae*-infected mice to a second challenge with *M. leprae* ([3, 5, 13]); the activated appearance of the macrophages in which the *M. leprae* reside, beginning from the time at which multiplication of the organisms is maximal ([1]); and the higher ceiling to multiplication in immunosuppressed rodents.

The maximum of bacterial multiplication varies among strains of mice, and of *M. leprae*, and is approximately 10^6 to $10^{6.3}$ per foot pad in immunologically intact CFW, CBA, and BALB/c mice and those of several other inbred strains ([11]), and fails to reach this level in the mice of yet other strains. A later, secondary growth phase of *M. leprae* has been described in intact CFW mice.

Multiplication of an organism. As long as they may be assumed to be distributed both randomly and independently, one from another, the distribution of particles suspended in a volume is described by the Poisson* distribution. This assumption is probably valid for the distribution of red blood cells in a blood-cell counting chamber. However, although red blood cells are distributed randomly, they are not distributed uniformly. Thus, when one counts only a sample of the total population of red blood cells (the number seen to overlie a given area of the counting chamber), and wishes to generalize to the entire population of cells, he must estimate the counting error—i.e., the error that results because the sample counted is not identical with every other possible sample of the population of red blood cells. The estimate of the counting error is then used to adjust the observed value, in order to provide an estimate of the range of values within which the "true" value lies.

An important property of the Poisson distribution is that the standard deviation of the mean, λ, the number counted in the sample, and taken to represent all possible samples, is simply $\lambda^{1/2}$. Thus, if one counts λ red blood cells per mm^3, the 95% confidence limits around the value of λ—i.e., the range that may be expected to include the value obtained from 95% of all possible samples—is simply: $\lambda \pm t_{0.95} \times \lambda^{1/2}$, where $t_{0.95}$ is the familiar "Student's t" value. This is approximately 2.0 for any value of $\lambda \geq 30$; therefore, the 95% confidence limits are: $\lambda + 2 \times \lambda^{1/2}$ and $\lambda - 2 \times \lambda^{1/2}$.

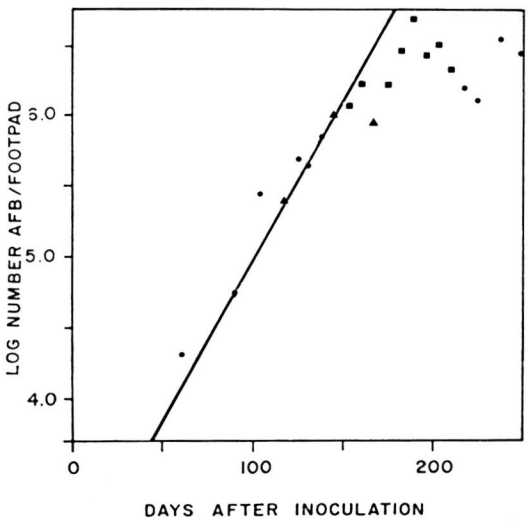

FIG. 1. Growth curve of *M. leprae* in the hind foot pad of the immunologically normal BALB/c mouse. Harvests were made from pools of 4 (●), 6 (▲) or 8 (■) foot pads.

The second important property of the Poisson distribution is a direct consequence of the first: the larger the number of particles actually counted, the smaller the counting error and the narrower the 95% confidence limits, in relation to the value of λ. Thus, if in a given area of the counting chamber one counted 100 red blood cells, then the standard deviation is $100^{1/2} = 10$, and the 95% confidence limits are $100 - 2 \times 10 = 80$ and $100 + 2 \times 10 = 120$—i.e., $\lambda \pm 20\%\ \lambda$. If, on the other hand, one were willing to continue counting until he had counted 10,000 cells, the 95% confidence limits would be much narrower—$10{,}000 \pm 2 \times 10{,}000^{1/2} = 10{,}000 - 200$ and $10{,}000 + 200$—i.e., $\pm$ 2%—and one could have much greater security that the observed value represented the "true" value. Thus, one may increase greatly the precision of the estimate of the number of red blood cells by counting a larger fraction of the ruled surface of the counting chamber. The same considerations apply to the counting of radioactive disintegrations—events that occur randomly and independently in time.

Of course, because of their tendency to occur in "clumps," *M. leprae* are not randomly distributed in a bacterial suspension or on the surface of a smear, and their dis-

* S. D. Poisson, 1781–1840, French mathematician.

tribution is not that of the Poisson distribution ([4]). Nevertheless, it is useful to apply the properties of the Poisson distribution to the counting of *M. leprae,* and then to make some adjustment for non-randomness.

If, in a smear prepared according to Shepard's technique, one counted a single AFB, the total number of organisms per foot pad would be calculated as $10^{3.7}$–10^4—about the same as that inoculated. A count of 10 organisms would be calculated as $10^{4.7}$–10^5 AFB per foot pad—the result of 10-fold multiplication. But the confidence limits would be at least: $10 \pm t_{0.95}$ for $n = 10 \times 10^{1/2} = 10 \pm 2.63 \times 3.16 = 10 \pm 8.3$. Thus, the range of values within which the true value should lie 95% of the time includes 2, a number of organisms that might have been counted if there had been no multiplication. If, on the other hand, one had counted 100 organisms, then the confidence limits will probably be farther apart than 100 ± 20% × 100, because of the non-random distribution, but one could conclude with some security that the *M. leprae* had multiplied.

To summarize briefly a complex subject: a) "multiplication" means that the number of AFB harvested is at least 10-fold greater than that inoculated. This is the criterion of multiplication generally accepted for work in the mouse foot-pad system; and b) at least as important as the "fold-multiplication" are the numbers of AFB actually counted. The larger these are, the greater the confidence one may have in the values calculated from them, and in the conclusion that *M. leprae* have truly multiplied.

Fisher's exact probability calculation. This extraordinarily useful statistical technique involves calculation of the exact probability with which a given arrangement of two sets of results may occur. The probability of any particular arrangement of data in the "2 × 2 contingency" table,

a	b
c	d

is given by the equation:

$$P = \frac{(a + b)! \times (c + d)! \times (a + c)! \times (b + d)!}{(a + b + c + d)! \times a! \times b! \times c! \times d!}$$

in which the symbol, "!", following a number indicates the "factorial" of the number—i.e., $n! = n \times (n - 1) \times (n - 2) \times (n - 3) \times \ldots \times [n - (n - 1)]$. Thus, in the case of sets of three harvests from each of two groups of mice, one sets up the following 2 × 2 table, choosing some value, x, intermediate between the two sets of harvests results:

	Result	
	>x	<x
Group 1	0	3
Group 2	3	0

Employing the conventions that both 1! and 0! = 1,

$$P = \frac{3! \times 3! \times 3! \times 3!}{6! \times 3! \times 3! \times 0! \times 0!}$$

$$= \frac{(3!)^2}{6!} = 0.05.$$

In the case of sets of two harvests, the following 2 × 2 table is appropriate:

	Result	
	>x	<x
Group 1	0	2
Group 2	2	0

$$P = \frac{(2!)^4}{4! \times (2!)^2} = 0.167.$$

Of course, one cannot say, on the basis of these results, that the two sets of harvest results are different. However, should the same distribution of results be obtained from a second set of harvests, then P = 0.167 also for the second set of results, and P for the two sets of harvests, taken together, = 0.167 × 0.167 = 0.0278, and one may say with confidence that the two groups of mice are different.

The difference between the two sets of results being compared may not always be so obvious. Suppose that, among eight patients being treated by regimen A, all but one shows decided improvement; whereas, among an equal number of patients under treatment by regimen B, only two show improvement. To determine the likelihood that the two regimens are equally effective (the "null" hypothesis, that the difference between the two treatments is not significant, and that two such different results are not unlikely when successive eight-patient samples are selected from among a much larger number of patients treated by either of the

two regimens), one analyzes the following 2 × 2 contingency table:

	Improvement	
	+	0
Regimen A	7	1
Regimen B	2	6

For this distribution of 16 results,

$$P = \frac{(8!)^2 \times 9! \times 7!}{16! \times 7! \times 6! \times 2!} = 0.0196.$$

To this value of P must be added other values, the probabilities that even more favorable distributions of two sets of eight results, yielding the same over-all totals of improved and unimproved patients, could have occurred. In this case, there is only one more favorable distribution possible:

	Improvement	
	+	0
Regimen A	8	0
Regimen B	1	7

For this distribution,

$$P = \frac{(8!)^2 \times 9! \times 7!}{16! \times 8! \times 7!} = 0.0007.$$

The sum of these two probabilities, 0.0203, is smaller than 0.05. Therefore, one should reject the null hypothesis that both groups of patients were drawn from the same population—i.e., that the observed difference could have occurred by chance—and conclude that regimen A was more effective than regimen B.

A problem sometimes encountered in performing Fisher's exact probability calculation is that calculation of the factorials of numbers larger than those employed in these two examples can be very tedious. Calculation of factorials as large as that of 69 ($69! = 1.71 \times 10^{98}$) is possible on many electronic calculators, including those of pocket size. Moreover, one may find tables of factorials and (even more valuable) of $\log_{10}$ factorials in many books of mathematical tables (one must remember that logarithms are to be added and subtracted, rather than multiplied and divided).

Results of multiple harvests of *M. leprae.** In comparing the results of harvests of *M. leprae* from the foot pads of experimental mice—e.g., mice treated with a drug or vaccine—with those from the foot pads of untreated control mice, one wishes to learn if the observed differences are statistically significant. To phrase the question more precisely, one wishes to learn the likelihood (probability) that the observed difference might have arisen by change from the same group of mice—i.e., from the same population of harvest results.

The most direct approach is to perform multiple harvests from one of the two groups of mice, or to perform harvests from multiple groups of mice treated (or untreated) in the same way. In the usual experiment performed for the purpose of screening drugs, one should include four groups of untreated controls among 8–12 groups of drug-treated mice. Harvests of *M. leprae* from pools of four mice from each of the four groups of control mice are begun between 100 and 130 days after inoculation (one learns by trial and error how soon after passage *M. leprae* of a particular strain are likely to have multiplied to a readily detectable level), and are repeated at intervals of 28–30 days. Simultaneous harvests, one harvest per group, from treated mice are begun as soon as multiplication has been detected in all four control groups, and are repeated at the same 28–30 day intervals. The likelihood that the numbers of *M. leprae* harvested from all four control groups will exceed the number of harvested from a given experimental group is 1/5 (0.20) (there are five possible arrangements of the five results, and any given arrangement may be encountered with equal probability). That the same arrangement may be encountered at 100 and again at 130 days is less likely; in fact, the probability of such an occurrence is simply the product of the two individual probabilities ($0.20 \times 0.20 = 0.04$). At this point, one may conclude that the drug is active, and has produced a delay in multiplication of *M. leprae* of at least 30 days. Should the same arrangement of five results be encountered on yet a third occasion, 151–160 days after inoculation, the probability that such an arrangement of three sets of harvests could have occurred by chance after repeated harvests from the same (or identical) group(s) of mice is even smaller ($0.2^3 = 0.008$), and one may conclude that

* Based on reference no. 10.

the drug has produced a delay of multiplication of *M. leprae* of at least 51–60 days.

If one wishes also to examine the difference between two treatments, one must perform more than a single harvest from each group at each interval. As shown by the Fisher exact probability calculation, no overlapping of values between two sets of three harvests yields a probability of 0.05, and no overlapping of values between two sets of two harvests on each of two occasions yields a probability of 0.028. In order to examine the difference between two effective treatments, it may be necessary to perform additional harvests after an interval shorter than 28–30 days, so that the *M. leprae* will not have multiplied to the maximal level of approximately 10^6 organisms per foot pad in both groups before all the required harvests had been performed.

REFERENCES

1. EVANS, M. J. and LEVY, L. Ultrastructural changes in cells of the mouse foot pad infected with *Mycobacterium leprae.* Infect. Immun. **5** (1972) 238.
2. FENNER, F. The pathogenic behaviour of *Mycobacterium ulcerans* and *Mycobacterium balnei* in the mouse and developing chick embryo. Am. Rev. Tuberc. **73** (1956) 650.
3. KAWAGUCHI, Y. Superinfection with leprosy bacilli in mice. Int. J. Lepr. **40** (1972) 91.
4. KRUSHAT, W. M., SCHILLING, K. E., EDLAVITCH, S. A. and LEVY, L. Studies of the mouse foot pad technique for cultivation of *Mycobacterium leprae.* 4. Statistical analysis of harvest data. Lepr. Rev. **47** (1976) 275.
5. LEVY, L. Superinfection in mice previously infected with *Mycobacterium leprae.* Infect. Immun. **11** (1975) 1094.
6. LEVY, L. Studies of the mouse foot pad technique for cultivation of *Mycobacterium leprae.* 3. Doubling time during logarithmic multiplication. Lepr. Rev. **47** (1976) 103.
7. LEVY, L., MOON, N., MURRAY, L. P., O'NEILL, S. M., GUSTAFSON, L. E. and EVANS, M. J. Studies of the mouse foot pad technic for cultivation of *Mycobacterium leprae.* 1. Fate of inoculated organisms. Int. J. Lepr. **42** (1974) 165.
8. SHEPARD, C. C. Acid fast bacilli in nasal excretions in leprosy, and results of inoculation of mice. Am. J. Hyg. **71** (1960) 147.
9. SHEPARD, C. C. The experimental disease that follows the injection of human leprosy bacilli into foot pads of mice. J. Exp. Med. **112** (1960) 445.
10. SHEPARD, C. C. Statistical analysis of results obtained by two methods for testing drug activity against *Mycobacterium leprae.* Int. J. Lepr. **50** (1982) 96.
11. SHEPARD, C. C. and HABAS, J. A. Relation of infection to tissue temperature in mice infected with *Mycobacterium marinum* and *Mycobacterium leprae.* J. Bact. **93** (1967) 790.
12. SHEPARD, C. C. and MCRAE, D. H. *Mycobacterium leprae* in mice: minimal infectious dose, relationship between staining quality and infectivity, and effect of cortisone. J. Bact. **89** (1965) 365.
13. SHEPARD, C. C., VAN LANDINGHAM, R. and WALKER, L. L. Immunity to *Mycobacterium leprae* infections in mice stimulated by *M. leprae,* BCG and graft-versus-host reactions. Infect. Immun. **14** (1976) 919.
14. WELCH, T. M., GELBER, R. H., MURRAY, L. P., NG, H., O'NEILL, S. M. and LEVY, L. Viability of *Mycobacterium leprae* after multiplication in mice. Infect. Immun. **30** (1980) 325.
15. WHO LEPROSY UNIT. Laboratory techniques for leprosy. WHO, Geneva, in press.