

REVIEW

## The mouse foot-pad technique for cultivation of *Mycobacterium leprae*

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*Summary* Although multiplication of *Mycobacterium leprae* in the foot pads of immune-competent mice is limited, and no leprosy-like lesions are produced in these animals, the mouse foot-pad system represents the first truly useful and reproducible animal model of *M. leprae* infection. Its employment has enabled research into basic questions with respect to the microbiology of *M. leprae*, and the epidemiology, treatment and control of leprosy. The mouse foot-pad technique is labour-intensive and time-consuming, and is expensive in terms of the costs of animal purchase and maintenance. In addition, the technique appears to be rather imprecise and insensitive, compared with the techniques employed in working with cultivable micro-organisms. For these reasons, and also as a by-product of the success of multi-drug therapy, the technique has been abandoned in many research centres. Nevertheless, until a more simple and sensitive technique for demonstrating the viability of *M. leprae* is developed, the mouse foot-pad system remains an essential tool for leprosy research. In this review, we discuss the mouse foot-pad technique in detail, analyse its precision, point out its shortcomings, describe its most important applications, and prescribe a method by which to assess the ability of an alternative technique to serve in place of this established technique.

### Introduction

Despite decades of effort by many workers, *Mycobacterium leprae*, the causative organism of human leprosy, has not been cultivated in cell-free medium, although some metabolic activity appears to have been preserved in several systems *in vitro*.<sup>1</sup> Moreover, analysis of the genome of *M. leprae* suggests that cultivation of the organism in cell-free medium may not be possible: less than half of the genome contains functional genes, whereas inactivated or pseudogenes are abundant; the genome appears to have undergone reductive evolution,

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accompanied by the decay of genes and downsizing. These evolutionary changes have eliminated important metabolic pathways, together with their regulatory circuits and accessory functions, particularly those involved in catabolism.<sup>2,3</sup>

The inability to cultivate *M. leprae* either in cell-free medium or in cell culture led the late C.C. Shepard to attempt cultivation in the hind foot pad of the mouse. As he described in two seminal publications,<sup>4,5</sup> the technique involves inoculation of small numbers ( $\leq 10^4$ ) of the organism into the hind foot pad of the immune-competent mouse. The organisms multiply very slowly *in situ*, with a doubling time of approximately 2 weeks. In the course of a number of months, the *M. leprae* undergo a maximum of six to eight doublings, their multiplication apparently being limited by an immune response on the part of the mouse-host. Larger inocula ( $\geq 10^5$  organisms) appear to immunize the mouse before discernible multiplication can occur. As a result, inocula containing only minute proportions ( $< 1:10^4$ ) of viable organisms do not regularly give rise to multiplication, rendering the technique insensitive. Moreover, the immune response of the mouse is quite efficient; once the maximum of multiplication has been achieved, almost all of the organisms are killed,<sup>6</sup> so that it is difficult to obtain from the foot pad bacterial suspensions that contain large proportions of viable organisms.

Despite these limitations, there is currently no generally accepted alternative to the mouse foot-pad technique as a means of cultivating *M. leprae*. However, despite the historical importance of the mouse foot-pad technique in acquiring knowledge of *M. leprae* and leprosy, and its continuing importance for the foreseeable future, the technique has been abandoned in many laboratories, and investigators now entering the field of leprosy research are impatient with it, whereas the generation of scientists who employed the technique successfully is being depleted.

This review is not intended as a 'do it yourself' manual for the guidance of those who would establish the technique in their own laboratories, nor is it intended to provide an encyclopaedic review of the vast numbers of scientific articles in which the results of employing the technique have been reported. Rather, the purposes of this paper are to elaborate the scientific basis of the mouse foot-pad technique, to provide examples of several applications, to propose standards by which investigators should report the results of studies involving the technique, and to suggest a procedure by which to prove that a more satisfactory alternative technique may be deemed an adequate substitute.

## Background

Shepard chose the hind foot pad of the mouse as the site in which to attempt cultivation of *M. leprae* for two reasons. Immunologists had often used the mouse foot pad to measure responses to antigens. More important, Fenner<sup>7</sup> had described multiplication in the mouse foot pad of *M. marinum* and *M. ulcerans*, both of which organisms exhibit temperature optima lower than 37°C. The temperature optimum of *M. leprae* had long been suspected to be lower than 37°C because of its apparent predilection for the cooler areas of the skin and the peripheral nerves. Shepard demonstrated<sup>8</sup> that the foot pad is several degrees cooler than the core temperature of the mouse (although his data showed that the tail of the mouse is even cooler than the foot pad, it is a much larger organ, so that a small volume of inoculum does not remain localized; also, the tail is more difficult to inject).

Shepard found<sup>4,5</sup> that *M. leprae*, recovered from skin-biopsy specimens or nasal washings obtained from patients with lepromatous leprosy and inoculated into the foot pads of mice,

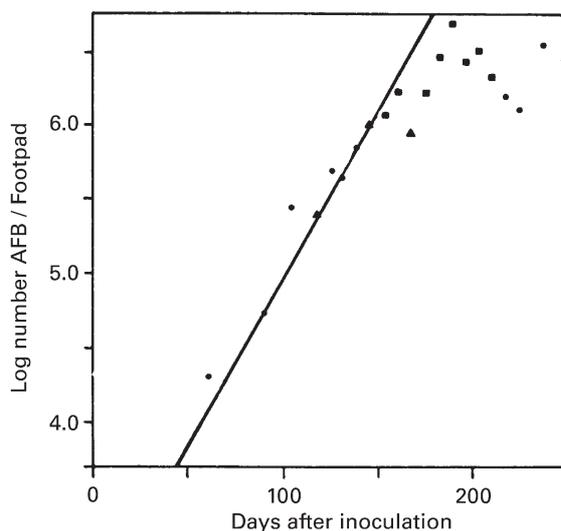
multiplied locally if the inoculum were appropriately diluted. Moreover, the organisms harvested from the inoculated feet consistently multiplied upon passage into the foot pads of uninfected mice. If the inocula delivered  $\leq 10^4$  acid-fast bacilli (AFB) per foot, an increase of 50- to 1000-fold was observed in each passage. One strain of *M. leprae* multiplied  $6 \times 10^9$ -fold in the course of five passages, and 17 strains demonstrated increases of  $10^4$ - to  $10^7$ -fold in the course of three passages. Shepard's findings were subsequently confirmed by Rees<sup>9</sup> and Pattyn<sup>10</sup> among others, and the mouse foot-pad system became an important research tool in leprosy.

Before Shepard's work, many workers had inoculated *M. leprae* into mice and other small mammals by a variety of routes. However, these workers sought evidence of a leprosy-like disease, whereas Shepard simply enumerated the AFB in the inocula and, after multiplication, in the inoculated tissues. Although similar results were obtained in other rodents, including the rat, gerbil, hamster, and *Mystromys*,<sup>4,11</sup> and although leprosy-like disease has been produced in immune-compromised mice<sup>12,13</sup> and rats,<sup>14</sup> the armadillo<sup>15</sup> and various primates,<sup>16</sup> the immune-competent laboratory mouse offered the great advantages of economy and the availability of a large number of genetically well-defined inbred strains. Therefore, most of the work employing this technique has been carried out in the mouse.

### Multiplication of *M. leprae* in the mouse

#### MULTIPLICATION IN THE IMMUNE-COMPETENT MOUSE

Multiplication of *M. leprae* in the hind foot pad of the immunologically intact BALB/c mouse is shown by the typical growth curve presented in Figure 1. In this example,<sup>17</sup> mice were inoculated in the hind foot pad with  $5 \times 10^3$  *M. leprae*, and, beginning 60 days later, harvests



**Figure 1.** Growth curve of *M. leprae* in the hind foot pad of the immune-competent BALB/c mouse. Mice were inoculated in a hind foot pad with  $5 \times 10^3$  organisms, and harvests were made from pools of 4 (●), 6 (▲) or 8 (■) foot pads at the intervals after inoculation shown. The doubling time during logarithmic multiplication, calculated from the best-fitting straight line, is approximately 13 days (adapted from reference no. 17).

were carried out at intervals from the pooled tissues of four to eight feet. The number of AFB per foot pad may be seen to have increased exponentially to  $> 10^6$  in the course of the 150 days following inoculation, after which a stationary or 'plateau' phase ensued. Although this process is extraordinarily slow, it has been shown to be reproducible.

#### MULTIPLICATION IN THE IMMUNE-COMPROMISED MOUSE

In a ground-breaking paper,<sup>12</sup> Rees showed that, in adult-thymectomized, lethally irradiated and bone-marrow-reconstituted CBA mice inoculated with  $10^4$  *M. leprae* per foot pad, *M. leprae* multiplied to a maximum higher by two to three orders of magnitude in thymectomized-irradiated ('T + I') than in immune-competent CBA mice inoculated with the same bacterial suspension. Also, T + I mice permitted multiplication from an inoculum of  $10^6$  per foot pad, whereas no multiplication ensued in immune-competent mice administered the same inoculum. Similar results were obtained by Gaugas,<sup>18</sup> who substituted antilymphocyte globulin for irradiation.

Colston and his co-workers subsequently demonstrated<sup>3</sup> an even higher maximum of multiplication in congenitally athymic 'nude' (*nu/nu*) mice, and reported that, between 18 and 24 months after inoculation, many of the mice demonstrated gross swelling of the inoculated feet.

#### THE MINIMAL INFECTIVE DOSE OF *M. LEPRAE*

Evidence that a single viable *M. leprae* is sufficient to give rise to multiplication in the mouse foot pad, i.e. that the mouse foot-pad technique is maximally sensitive, is presented in Table 1, in which *M. leprae* may be seen to have multiplied in at least seven of 10 feet inoculated with an average of five organisms per foot pad in eight experiments, in which passages had been made between 30 days before and 13 days after multiplication reached  $10^6$  AFB per foot pad.<sup>6</sup>

Moreover, as will be shown, a fraction of the inoculum is immediately lost from the site of inoculation. Thus, although five viable *M. leprae* were inoculated on average, the more-or-less random distribution of the AFB in the inoculum, together with the fact that some of the AFB are lost from the site of inoculation, suggest that the multiplication of *M. leprae* in many of the mice represented in Table 1 must have proceeded from no more than a single viable organism.

#### DOUBLING TIME OF *M. LEPRAE*

That *M. leprae* multiply as rapidly in immune-competent as in immune-compromised mice suggests that the rate at which the organisms multiply in the mouse foot pad may be maximal. Calculation of the doubling time from the slope of the logarithmic phase is imprecise, because the smaller numbers of *M. leprae* are calculated from the enumeration of only a very few organisms, and because the onset of the plateau phase is difficult to determine. An alternative technique consists of measuring the time-difference between the parallel growth curves that result when groups of mice are inoculated with serial 10-fold dilutions of the same bacterial suspension. In one experiment, the results of which are summarized in Figure 2, the curves were separated by 40–45 days, equivalent to about 13 days per doubling (the difference between any two adjacent inocula, i.e.  $5 \times 10^3$  and  $5 \times 10^2$ , is equivalent to 3.32 doublings).

**Table 1.** Killing of *M. leprae* during the 'plateau' phase (adapted from reference no. 6). In 21 experiments, weanling BALB/c mice were inoculated with serial 10-fold dilutions of suspensions of *M. leprae*, such that mice were inoculated with an average of  $5 \times 10^3$ ,  $5 \times 10^2$ ,  $5 \times 10^1$  or 5 AFB per hind foot pad. Harvests of *M. leprae* were performed at least 1 year after inoculation; the organisms were considered to have multiplied if the number of AFB harvested  $\geq 10^5$  per foot pad, without regard to the number inoculated

No. days after $10^6$	No. foot pads infected/no. inoculated with inoculum of:				No. viable* <i>M. leprae</i> per $5 \times 10^3$
	$5 \times 10^3$	$5 \times 10^2$	$5 \times 10^1$	$5 \times 10^0$	
-30			6/6	10/10	5000
-27				10/10	5000
-6				7/10	600
0				10/10	5000
1				9/10	2300
8			10/10	9/10	2300
11			10/10	7/10	1200
13			10/10	7/10	1200
14	2/4	2/10	0/10		1
22			9/10	5/10	350
61		6/10	2/8		12
78	4/8	2/10	1/10		1
82			10/10	6/10	900
94	6/10	2/10			1
95	1/8	7/10	0/10		1
110		9/10	0/6		23
117			7/10	3/10	155
122	10/10	2/2	1/10		18
158	7/10	2/10	0/10		2
191	10/10	7/10	0/10		12
232	10/10	6/10	0/10		9

\*These values were calculated as the MPN by means of the equation of Halvorson and Ziegler.<sup>21</sup>

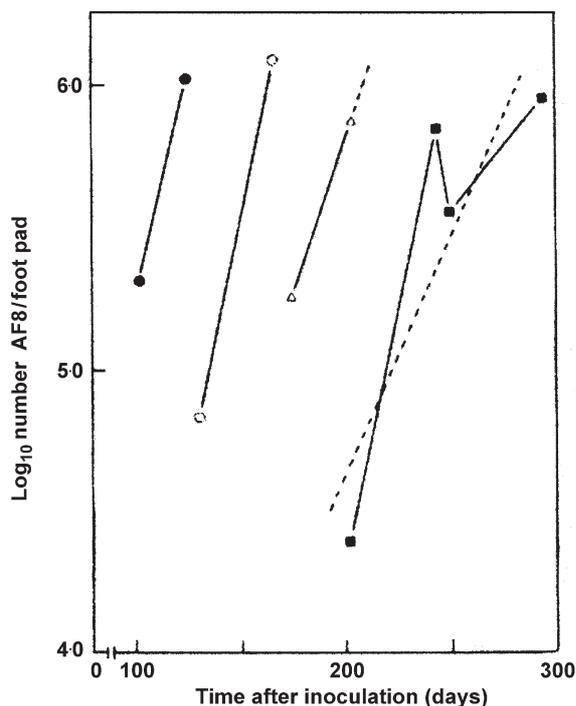
The mean value of the doubling time derived from this and six additional experiments was  $11.1 \pm 1.92$  (mean  $\pm$  95% confidence limits) days.<sup>20</sup>

#### NATURE OF THE 'PLATEAU' PHASE

##### *Evidence of bacterial killing*

The 'plateau' phase of the growth curve of *M. leprae* in the mouse foot pad is similar in appearance to the stationary phase of the growth of cultivable bacteria in culture. However, cessation of multiplication of *M. leprae* in the mouse foot pad cannot result from exhaustion of essential nutrients or accumulation of toxic products of bacterial metabolism, which account for the stationary phase in culture. Rather, the organisms are killed by the mouse host.

Evidence for this may be found in the results of a series of experiments,<sup>6</sup> in which the proportions of viable organisms at intervals both before and after the *M. leprae* reached the level of  $10^6$  per foot pad were calculated in terms of the 'most probable number' (MPN) by the method of Halvorson and Ziegler.<sup>21</sup> The results of this series of experiments, summarized in Table 1, demonstrated that, once multiplication had ceased, viable *M. leprae* disappeared



**Figure 2.** The  $\log_{10}$  number of *M. leprae* per foot pad as a function of the number of days from inoculation to harvest and the number of AFB inoculated. The points represent the harvests of *M. leprae* from each group of mice; the best-fitting straight lines were used to measure the time from inoculation to multiplication to the level of  $10^6$  AFB per foot pad. Mice were inoculated with 5000 (●), 500 (○) 50 (△) or 5 (■) AFB per foot pad (reprinted from reference no. 20).

with a half-time of 25 days. Thus, the plateau phase is characterized by killing of the organisms.

#### *Enhanced multiplication in immune-compromised mice, and reversal by reconstitution of the immune system*

That multiplication of *M. leprae* is enhanced in the foot pads of immune-compromised mice suggests that multiplication is limited by an immune response on the part of the mouse. Moreover, Rees demonstrated<sup>12</sup> abrupt reversal of the foot-lesion in T + I mice by reconstitution of the mice with syngeneic lymphocytes. Other workers<sup>22-24</sup> have reported reversal of the pathologic process in *M. leprae*-infected nude mice by immune reconstitution. However, employing an *in vitro* assay, Adams and her co-workers<sup>25</sup> were unable to demonstrate killing of *M. leprae* in infected and reconstituted *nu/nu* mice.

#### *Resistance to superinfection*

If the mechanism by which mice limit multiplication of *M. leprae* is a cell-mediated immune response, mice challenged shortly after a prior infection, before an immune response could have been mounted, should not be protected against a secondary challenge, whereas those challenged after the onset of the immune response should be protected. A series of

experiments,<sup>26</sup> in which groups of mice were first inoculated in one hind foot pad and, at one or another interval thereafter, challenged in the contralateral foot pad with *M. leprae* of the same strain, demonstrated that this is indeed the case.

### Precision of the technique

A description of the mouse foot-pad technique, which may be found in a key paper by Shepard and D.H. McRae,<sup>27</sup> is summarized in Appendix 1. In brief, the mouse is killed, and the inoculated foot-pad tissues are dissected and homogenized. Small aliquots of the homogenate are spread on the 'circles' of a counting slide, which is then stained and examined under optimal microscopic conditions. Because it is not possible to examine the entire homogenate, only those organisms present in a small sample are actually enumerated. Moreover, like all mycobacteria, *M. leprae* tend to clump, and, in addition, not all AFB-containing tissue cells are disrupted by homogenization. As a result, the *M. leprae* are unlikely to be distributed uniformly in the homogenate, and enumeration of the AFB is subject to errors from several sources. The precision of the mouse foot-pad technique has been studied by Krushat and his coworkers.<sup>28</sup>

First, it was found that the AFB in specimens containing smaller numbers ( $\leq 2$  per microscope field) of organisms were more likely to be distributed randomly, i.e. their distribution was consistent with the Poisson distribution (see Appendix 2).

These workers also assessed the variation of the numbers of AFB among replicate circles prepared from a single homogenate and from among replicate harvests, whether from individual mice or from the pooled foot tissues of several mice. The variation among counts of AFB from replicate circles was found to be consistent with that of a normal distribution. Also, the ratio of the largest to the smallest number of AFB harvested from the individual feet of four mice of 13 groups was found to range from 1.67 to 20.0, with a mean of 6.66. As expected, the mean ratio of the yields from replicate harvests from pools of four to eight foot pads was smaller, at 4.08. These ratios suggest that the mouse foot-pad technique indeed yields reproducible results. Moreover, despite the non-random distribution of the *M. leprae* in homogenates, the mouse foot-pad technique exhibits a considerable degree of precision.

Finally, evidence has been adduced that, in the situation in which both hind foot pads are inoculated at the same time with viable *M. leprae*, the organisms multiply in each foot pad independently of that which is occurring in the opposite hind foot pad.<sup>6</sup> In a large experiment, in which harvests were performed from both hind feet of mice that had been inoculated with the same bacterial suspension, 48% of the harvests demonstrated evidence of multiplication; 23% of the mice demonstrated multiplication in both, 27% in neither, and 50% in only one hind foot pad, exactly the distribution predicted by the binomial distribution.<sup>29</sup>

Other sources of error remain to be addressed. One is the timing of harvests: performing harvests too early may prevent detection of multiplication of the *M. leprae*, whereas performing harvests too late may obscure important differences among groups of experimental animals. Another source of error is the presence in the inoculum of a very small proportion of viable organisms. If the inoculum of  $\leq 10^4$  *M. leprae* per foot pad contains only a small proportion of viable organisms, such that some foot pads are inoculated with one or more viable organisms, whereas others are inoculated only with dead *M. leprae*, as sometimes occurs,<sup>30</sup> the variation of yields of AFB from replicate harvests may be much greater than that reported by Krushat *et al.*<sup>28</sup> In such instances, the yields from replicate

harvests might well vary from less than the minimum (8875, if only one AFB is found in the examination of 40 microscope fields in the example shown in Appendix 1) to 100 or more times this number.

Examples of the array of numbers that result from replicate harvests of *M. leprae* from individual feet of the untreated, control mice of six experiments are presented in Table 2. The ratios of the largest to the smallest numbers of AFB harvested range from approximately 10 to 480. Inspection of the six sets of results of harvests from individual feet suggests that the inocula differed greatly, in terms of the proportion of viable *M. leprae*; the proportion of viable organisms in the best inoculum was 10-fold that in the worst.

#### FATE OF THE INOCULUM

In addition to the problem resulting from the non-random distribution of the organisms, a further complication is loss of the inoculum from the site of inoculation. As may be deduced from the description of the technique of enumerating the AFB recovered in harvests from the inoculated foot, it is not possible to measure the proportion of the inoculum remaining in the tissues of the foot pad immediately after inoculation; recovery of the entire inoculum of  $\leq 10^4$  AFB in the harvest would result in the actual enumeration of fewer than one AFB per 40 microscope fields per circle, on average. In the course of an experiment in which mice were inoculated in the foot pad with large numbers of *M. leprae* and harvests were carried out within a few days thereafter, it was discovered that only about 30% of the inoculated

**Table 2.** Measurements of the numbers of *M. leprae* in individual foot pads of the control mice of six experiments. The results of individual harvests of *M. leprae*, representing the control groups of six different experiments, are analyzed in terms of the foot-by-foot variation of the numbers of AFB harvested

Source (no. reference)	No. feet	No. AFB per foot pad ( $\times 10^5$ )				MPN*
		Individual foot pads	Median	Ratio		
31	6	9.14, 5.50, 4.44, 2.40, 1.24, 0.889	3.42	10.2	1.25	
30	16	7.72, 4.08, 1.60, 0.71, 0.62, 0.089 (2),** <0.089 (9)	<0.089	>86.7	0.288	
32	15	33.6, 20.2, 18.9, 15.1, 11.5, 8.70, 6.39, 6.04, 5.06, 4.70, 4.62, 3.90, 3.37, 3.36, 1.69	6.04	19.9	2.77	
***	36	14.3, 5.95, 5.15, 4.86, 4.62, 3.37, 2.84, 2.31, 1.86, 1.69, 1.60, 1.51 (2), 1.33, 1.24, 1.15, 1.06 (3), 0.976 (2), 0.89, 0.621 (3), 0.266 (2), 0.178 (3), 0.089 (2), <0.089 (4)	1.06	161	0.723	
33	25	48, 37, 33, 9.6, 6.9, 4.7, 3.75, 2.65, 2.3, 1.3 (2), 0.8 (2), 0.4 (5), 0.2 (2), 0.1 (5)	0.80	480	0.280	
33	14	89.8, 45.7, 40, 30, 20.8 (2), 18.6, 12.7, 12.5, 7.2, 5.4, 5.3, 5.2, 3.4	15.6	26.4	1.36	

\* The most probable number of viable *M. leprae* per 5000 AFB.

\*\* Number of feet yielding the result indicated; in all other cases, only a single foot yielded the indicated result.

\*\*\* These results are unpublished data from M. Ngamying *et al.*

organisms were recovered,<sup>34</sup> suggesting that a large proportion of the inoculated organisms may be lost. Experiments were carried out in the attempt to quantitate the loss.<sup>35</sup>

Large inocula of *M. leprae* were prepared by centrifugation of the bacterial suspensions harvested from the inoculated feet of large numbers of mice; these concentrated suspensions were then inoculated into the hind foot pads of new mice. At intervals ranging from 1 h to 9 days after inoculation, harvests were performed and the recovered AFB enumerated. No more than 40%, and as few as 10% of the inoculated AFB were recovered within hours of inoculation, whereas an average of 75% of the AFB simply added to a homogenate were recovered. Moreover, the missing AFB were not recovered by harvesting adjacent tissues not ordinarily included by the technique. Similar results were obtained after inoculation of foot pads with viable *M. marinum* followed one and two hours later by harvest and culture of the resulting tissue suspensions.

That 70–90% of the inoculum may immediately be lost from the foot-pad that has been inoculated with as few as five organisms, leaving only one or two AFB to multiply *in situ*, supports the conclusion that a single viable *M. leprae* is sufficient to give rise to multiplication in the mouse foot pad.

### Applications of the mouse foot-pad technique

The lack of a useful method for cultivating *M. leprae* in cell-free media has been a serious handicap to the conduct of research in leprosy. This obstacle has been partially overcome by application of the mouse foot-pad technique, which has permitted investigations in a number of important areas. The applications of the mouse foot pad technique are considered here under two major headings: the mouse as a culture medium; and the mouse as a model of the human host.

Only a single example of each type of application has been selected to illustrate the manner in which the technique has been applied.

#### THE MOUSE AS A CULTURE MEDIUM

##### *Drug screening*

Several methods have been employed in drug-screening. The ‘continuous’ method, which was the first employed to demonstrate the antimicrobial activity of a drug against *M. leprae* in mice,<sup>36</sup> requires that the drugs be administered to the *M. leprae*-infected mice from the day of inoculation until the animals are killed; active drugs are those that inhibit multiplication of the organisms, as indicated by harvests performed at the time of death. This is the most sensitive method for detection of antimicrobial activity against *M. leprae*. However, it cannot discriminate between bactericidal and merely bacteriostatic activity; although many compounds exhibit bacteriostatic activity, only the few that are bactericidal against *M. leprae* are potential components of multi-drug regimens to be employed in the treatment of leprosy.<sup>37,38</sup>

The ‘kinetic’ method is identical to the continuous method, except that the compound to be screened is administered for only a limited period, usually beginning 60 days after inoculation, at which time the organisms are early in the logarithmic phase of multiplication, and continuing for as long as 90 days,<sup>39,40</sup> although drug administration may be limited to a

single dose.<sup>41</sup> The activity of the drug is assessed in terms of the 'growth delay', determined by comparing the number of days required for multiplication to  $10^6$  AFB per foot among the treated mice to that among the untreated controls. A purely bacteriostatic drug inhibits multiplication of *M. leprae* only as long as the drug is administered; a growth delay significantly longer than the period of drug administration, i.e. failure of bacterial multiplication to resume immediately following cessation of drug-administration, suggests that *M. leprae* were killed during treatment, or that prolonged bacteriostasis occurred. Prolonged bacteriostasis may result from persistence of the drug in the tissues or within the organisms, or it may reflect the time required for the recovery of organisms that have been reversibly damaged. Thus, although the kinetic method can distinguish between purely bacteriostatic and so-called 'bactericidal-type' activity, but not between bactericidal activity and prolonged bacteriostasis, the absence of bactericidal activity is reliably demonstrated by this approach.

The 'proportional bactericidal' technique, described by Colston,<sup>42</sup> provides a means of measuring the bactericidal activity of a compound. Serial 10-fold dilutions of the suspension of *M. leprae* are made, and mice are inoculated with from  $5 \times 10^3$  to  $5 \times 10^{-1}$  (or from  $10^4$  to 1) organisms per foot pad. Control mice are left untreated, whereas the remaining groups of mice are treated for a period of time that varies, depending upon the drug, from a single dose to 60 days. After treatment, the mice are held for 12 months, a period of time more than sufficient to permit a single surviving organism to multiply to a readily detectable level. Harvests of *M. leprae* are then performed from individual feet, usually 10 per dilution of inoculum for each treatment-group; the organisms are considered to have multiplied in those feet found to contain  $\geq 10^5$  AFB. The proportion of viable *M. leprae* surviving treatment may then be calculated from the 'median infectious dose' ( $ID_{50}$ ), the number of organisms required to infect 50 percent of the mice.<sup>43</sup> If the largest inoculum is  $5 \times 10^3$  *M. leprae* per foot pad, a proportion of viable *M. leprae* as small as 6 per 100,000 organisms may be measured. One calculates the proportion of viable *M. leprae* killed by the treatment by comparing the proportion of viable organisms in the treated mice to that in the control mice.

Shown in Table 3 are the results of an experiment in which several drugs and drug-combinations were screened for bactericidal activity against *M. leprae*.<sup>44</sup> The drugs screened were HMR 3647, a new macrolide; clarithromycin; moxifloxacin, a newer fluoroquinolone; ofloxacin; rifampicin; and rifapentine, a long-acting analogue of rifampicin. The drug-combinations screened were: ofloxacin + minocycline (OM); moxifloxacin + minocycline (MM); rifapentine + moxifloxacin + minocycline (PMM); and rifampicin + ofloxacin + minocycline (ROM). Each drug or drug-combination was administered by gavage to four groups of mice that had been inoculated 3 days earlier with  $5 \times 10^3$ ,  $5 \times 10^2$ ,  $5 \times 10$  or 5 *M. leprae* per foot pad. The macrolides were administered daily for 5 consecutive days, whereas only single doses of the remaining drugs were administered. One year later, harvests were performed. The results demonstrated, among other findings, that PMM killed 99.9% of the viable *M. leprae*, a significantly greater proportion than that killed by ROM or by rifampicin alone. This was most interesting, because no combination of drugs including rifampicin had been found more active than rifampicin alone.

Although the proportional bactericidal method requires more mice and more time than do either of the other methods, and is incapable of detecting bacteriostatic or bacteriopausal activity, it is the most reliable method of demonstrating bactericidal activity, and permits quantitation of the degree of this activity.

**Table 3.** Comparing the bactericidal effects against *M. leprae* of various drugs or drug combinations by the proportional bactericidal method (adapted from reference no. 44). HMR = HMR 3647; CLARI = clarithromycin; MXFX = moxifloxacin; OFLO = ofloxacin; MINO = minocycline; RPT = rifapentine; RMP = rifampicin. Mice were inoculated in a hind foot pad with the numbers of *M. leprae* shown, and treatments were administered 3 days later. Unless otherwise noted, all the treatments were administered as a single dose. Harvests of *M. leprae* were performed from individual feet after approximately 1 year

Treatment (mg/kg)	No. feet showing multiplication*/no. feet harvested, by inoculum					% viable <i>M. leprae</i> **	% <i>M. leprae</i> killed
	$5 \times 10^3$	$5 \times 10^2$	$5 \times 10^1$	$5 \times 10^0$	$5 \times 10^{-1}$		
Untreated control	10/10	10/10	10/10	7/10	0/10	21.82	–
HMR (100 × 5 doses)	10/10	10/10	7/10	0/10	–	2.18	90.0
CLARI (100 × 5 doses)	10/10	10/10	8/10	3/10	–	5.48	74.9
MXFX (150)	10/10	10/10	6/10	0/10	–	1.73	92.1
OFLO (150)	10/10	10/10	10/10	3/10	–	8.69	60.2
MXFX (150) + MINO (25)	10/10	9/10	6/10	0/10	–	1.38	93.7
OFLO (150) + MINO (25)	10/10	8/10	10/10	3/10	–	5.48	74.9
RPT (10)	9/10	3/10	1/10	0/10	–	0.09	99.6
RMP (10)	9/10	10/10	7/10	0/10	–	1.73	92.1
RPT (10) + MXFX (150) + MINO (25)	5/10	2/10	0/10	0/10	–	0.02	99.9
RMP (10) + OFLO (150) + MINO (25)	10/10	9/10	5/10	0/10	–	1.09	95.0

\* *M. leprae* were considered to have multiplied if the harvest yielded  $\geq 10^5$  AFB per foot.

\*\* % viable *M. leprae* =  $0.69 \times 100/\text{ID}_{50}$ .

### Drug-susceptibility testing

Treating all known leprosy patients with effective chemotherapy is the basis of the current strategy for leprosy control. Since Rees' first demonstration,<sup>45</sup> by inoculation of mice, of dapsone-resistant strains of *M. leprae*, it has been recognized that the emergence of drug-resistant strains of the organism may seriously jeopardize the treatment of patients, and make the control of leprosy far more difficult to achieve. To prevent the emergence and dissemination of drug-resistant strains, patients must be treated with an adequate multi-drug regimen; in addition, it is most important to identify those patients in whom drug-resistance has already developed, and to treat them with an alternative regimen consisting of antimicrobials to which the organisms are still susceptible.

Measurement of the susceptibility to drugs of *M. leprae* recovered from leprosy patients has been one of the most important applications of the mouse foot-pad technique. At present, susceptibility of the organisms to the three components of the multi-drug therapy (MDT) regimen, rifampicin, dapsone and clofazimine, is of interest, particularly that to rifampicin, which is the backbone of the regimens. In addition, because of the increasingly important role of fluoroquinolones in the treatment of leprosy, and the fact that ofloxacin-resistant *M. leprae*<sup>46,47</sup> have been reported, susceptibility to the fluoroquinolones should also be tested among patients who relapse after treatment with a fluoroquinolone-containing regimen.

The susceptibility of *M. leprae* to a drug can be tested only among patients with multibacillary leprosy whose skin-smears are positive. An active-appearing skin lesion is biopsied, the organisms are recovered from the biopsy-specimen, and immune-competent

mice are inoculated with  $\leq 10^4$  AFB per foot pad. The inoculated mice are divided among groups of 10–20 mice each; one group is held without treatment, and the mice of the remaining groups are treated as in the continuous method of drug-screening. The drugs are administered in one or several concentrations. If the drugs are well-absorbed from the gastrointestinal tract, they may be administered *per os*, either incorporated into the mouse diet or by gavage.

Harvests of *M. leprae* are performed approximately 6 months after inoculation from the inoculated feet of two to four untreated mice, and repeated at intervals of 2 months until the organisms are found to have multiplied to a mean  $\geq 5 \times 10^5$  AFB per foot pad, at which time the organisms are harvested from the feet of all of the treated mice. Employing this criterion of multiplication, if the organisms have multiplied only in untreated mice but in none of the treated mice, the strain is considered susceptible, whereas the strain is considered resistant if the organisms are found to have multiplied in as few as one treated mouse. Finally, if the organisms have not multiplied in treated mice, but have multiplied in so small a proportion of untreated mice that the proportion of inoculated feet demonstrating multiplication is not significantly different from zero by Fisher's exact probability calculation,<sup>48</sup> the susceptibility test of the strain must be considered inconclusive.

Recently, associations between certain missense mutations of the genome of *M. leprae* and resistance to dapsone,<sup>49,50</sup> rifampicin,<sup>47,51–53</sup> and ofloxacin<sup>46,47</sup> have been described, and DNA-based assays for the detection of drug-resistant *M. leprae* have been developed. Thus, in the future, it may be possible to detect drug-resistant *M. leprae* by rapid and reliable DNA-based assays, so that it will no longer be necessary to employ the mouse foot-pad technique for this purpose.

#### *Measuring the efficacy of antileprosy treatment in clinical trials*

The major newer anti-leprosy drugs, rifampicin, ofloxacin, clarithromycin, and minocycline, were all demonstrated to be actively bactericidal against *M. leprae* by the mouse foot-pad technique before they were employed in the treatment of patients with leprosy. Because the pharmacokinetics of the drugs and pathogenesis of *M. leprae* infection in mice are quite different from those in man, it is necessary to evaluate a promising new drug or a new combined regimen in controlled clinical trial before the new drug or regimen may be applied in the field.

The most efficient means of assessing the therapeutic effect of a new antimicrobial drug or regimen in multibacillary leprosy is to measure the rate at which patients' *M. leprae* are killed by treatment with the drug or regimen. This has been accomplished by means of mouse foot-pad inoculation. Before and at intervals during treatment, skin-biopsy specimens are obtained, preferably from the same lesion, and *M. leprae* are recovered from the specimens for mouse foot-pad inoculation.<sup>37</sup> The sensitivity of the measurement has been improved by a modification of the proportional bactericidal method,<sup>54,55</sup> in which groups of mice are inoculated with four 10-fold serial dilutions of the bacterial suspension, i.e.  $5 \times 10^3$ ,  $5 \times 10^2$ ,  $5 \times 10^1$  and 5 AFB per foot pad.

Some of the results of a clinical trial,<sup>54</sup> in which the bactericidal effects of treatment were measured in terms of the proportion of viable *M. leprae* before and after treatment, are presented in Table 4. Although the treatment exhibited bactericidal activity in all of the patients, the activity was demonstrated in two patients (patients 5 and 38) only by measurement of the proportion of viable organisms. Had only a single inoculum of 5000 AFB

per foot pad been employed, the bactericidal activity of treatment would not have been recognized in these two patients, because the proportion of foot pads showing multiplication of *M. leprae* among those inoculated with  $5 \times 10^3$  AFB per foot pad after treatment did not differ significantly from that before treatment.

Another important application of the mouse foot-pad technique is proof of relapse of multibacillary leprosy, and the demonstration that the *M. leprae* are susceptible or resistant to the drugs that had been employed in the treatment of the patient.<sup>56-58</sup>

THE MOUSE AS HOST

Little work has been carried out in which the response of the mice to infection with *M. leprae*, of possible relevance to the response of humans, has been studied.

Studies of candidate vaccines

The *M. leprae*-infected mouse has been used to study the protective effects of potential components of an anti-*M. leprae* vaccine. In a typical experiment,<sup>32</sup> mice were vaccinated intracutaneously in the flank with one of a number of materials, some suspended in saline and some emulsified in Freund's incomplete adjuvant (FIA), on several occasions separated by several weeks, after which they were challenged with  $5 \times 10^3$  *M. leprae* in the hind foot pad. Harvests were performed at intervals from the unvaccinated control mice until the organisms were noted to have multiplied, at which time harvests were carried out from all of the surviving mice.

**Table 4.** Determination of the proportion of viable *M. leprae* in five patients before and after treatment with various regimens (adapted from reference no. 54). In a short-term clinical trial, patients with multibacillary (MB) leprosy, whose *M. leprae* multiplied in the mouse foot pad, were administered an experimental drug-regimen, after which the same skin-lesions were biopsied; *M. leprae* were recovered from the biopsy specimens, and groups of mice were inoculated in a hind foot pad with organisms in the numbers shown. The patients were treated as follows: no. 5, a single dose of 2000 mg clarithromycin plus 200 mg minocycline; no. 6, a single 600-mg dose of rifampicin; no. 7, 1 month of the standard MDT regimen for MB leprosy; no. 21, a single dose of 2000 mg clarithromycin plus 200 mg minocycline plus 800 mg ofloxacin; and no. 38, 30 days of the dapsone and clofazimine components of the standard MDT regimen for MB leprosy

Patient no.	Day of biopsy	No. feet showing multiplication*/ no. feet harvested, by inoculum				% viable <i>M. leprae</i> **	% <i>M. leprae</i> killed
		$5 \times 10^3$	$5 \times 10^2$	$5 \times 10^1$	$5 \times 10^0$		
5	0	9/10	9/10	4/10	3/10	1.38	
	31	7/10	2/10	0/10	0/10	0.04	97.5
6	0	10/10	10/10	6/10	4/10	4.35	
	31	0/10	0/10	0/10	0/10	<0.006	>99.9
7	0	10/10	5/10	2/10	1/10	0.28	
	31	0/10	0/10	0/10	0/10	<0.006	>97.9
21	0	10/10	7/10	4/10	0/10	0.55	
	31	0/10	0/10	0/10	0/10	<0.006	>98.9
38	0	10/10	10/10	10/10	8/10	27.4	
	31	10/10	7/10	3/10	1/10	0.45	98.0

\* *M. leprae* were considered to have multiplied if the harvest yielded  $\geq 10^5$  AFB per foot pad.  
 \*\*  $0.69 \times 100/\text{ID}_{50}$ .

The results of this experiment, presented in Table 5, show that the cell-wall skeleton did not protect mice against the challenge, whereas both *M. leprae* cytosol (soluble *M. leprae* antigen from which soluble carbohydrates and lipids had been extracted) and the pooled membrane fractions conferred protection, as did a small dose of heat-killed *M. leprae*, the positive control.

A potential source of experimental error in such experiments is variation of the response of individual mice to an intervention, e.g. drug or vaccine, that affects multiplication of the *M. leprae*. It appears certain that not all mice in an experimental group will respond in identical fashion. Although one cannot measure the variation resulting from this source, it may be minimized by employing only inbred mice of the same age and sex. Moreover, it is necessary to use enough mice, and to apply the appropriate statistical techniques.

Only two papers, those of Krushat *et al.*<sup>28</sup> and Shepard<sup>43</sup>, have presented statistical analyses of the results of the mouse foot-pad technique. However, neither paper exploited the enhanced sensitivity conferred by the increasing the numbers of harvests, or by employing nonparametric statistical techniques, which offer the advantage of freedom from the requirements of parametric techniques that the data be both normally distributed and continuous.<sup>48</sup> In addition, non-parametric techniques are particularly suitable for the analysis of small samples, and permit comparison of data-sets of differing size. Finally, the computations involved are simple, particularly if appropriate computer software, such as MEDSTAT<sup>®</sup> (Astra-gruppen A/S, Copenhagen, Denmark) or STATA<sup>®</sup> (StataCorp, College Station, Texas), is employed.

#### *Use of genetically altered mice for immunological studies*

The development of transgenic and gene ‘knock-out’ technologies has led to the development of many strains of mice that exhibit defects in specific immune functions (the term ‘transgenic’ refers to the incorporation into the mouse genome of foreign, i.e. non-murine genes; the term ‘knock-out’ refers to the disrupting of specific mouse genes). Applying the mouse foot-pad technique, several strains of knock-out mice have been employed by James

**Table 5.** Protection of mice against challenge with *M. leprae* by vaccination with refined components of the organism (adapted from reference no. 32). PBS = phosphate-buffered saline; CW = *M. leprae* cell wall; ML = *M. leprae*; HKML = heat-killed *M. leprae*. The various materials, suspended in PBS, were administered intradermally into each flank of groups of 12 to 15 mice in a dosage of 20 µg per mouse on three occasions 3 weeks apart. Twenty-eight days after the third injection, the mice were inoculated in a hind foot pad with  $5 \times 10^3$  *M. leprae*. Between 120 and 143 days after inoculation, harvests of *M. leprae* were performed from the foot pads of all of the surviving mice

Material	No. feet	No. <i>M. leprae</i> / foot ( $\times 10^5$ )		<i>P</i> *
		Range	Median	
PBS	15	1.69–33.6	6.04	–
CW skeleton	10	1.42–73.3	7.59	0.782
ML cytosol	12	0.18–3.37	1.24	0.00004
ML membrane	10	0.80–4.44	2.04	0.00034
HKML, $2 \times 10^7$	10	0.089–1.60	0.32	0.00003

\*The probability that these results were drawn from the same population as those of the corresponding control, determined by the Mann–Whitney *U*-test.

Krahenbuhl, Linda Adams, and their colleagues to examine immune mechanisms involved in the resistance of mice to infection with *M. leprae*.

Adams *et al.* examined<sup>59</sup> the importance of the production of interferon- $\gamma$  (IFN- $\gamma$ ) in the immune response of the mouse to infection with *M. leprae* by studying the evolution of the process in the foot pads of BALB/c IFN- $\gamma^{-/-}$  (GKO) mice, in which the gene encoding the production of IFN- $\gamma$  had been disrupted. GKO and normal BALB/c mice were inoculated in a hind foot pad with  $6 \times 10^3$  *M. leprae* per foot pad; mice of each group were killed at intervals in the course of 1 year thereafter, and the *M. leprae* were harvested. Initially, as shown in Figure 3, the organisms multiplied similarly in both groups of mice. After 3 months, and throughout the remainder of the experiment, the mean number of AFB per foot pad remained unchanged in the control mice, whereas, in the GKO mice, the number of AFB per foot pad increased by 1 order of magnitude during the 4th month after inoculation, and remained unchanged thereafter, confirming an important role of IFN- $\gamma$  in the immune response of the mouse to the organism.

This experiment and others carried out by these workers suggest the continuing importance of studies in genetically altered mice to define the components of the murine, and probably, also, the human response to infection with *M. leprae*, and indicate an important area of research in which further progress depends upon application of the mouse foot-pad technique.

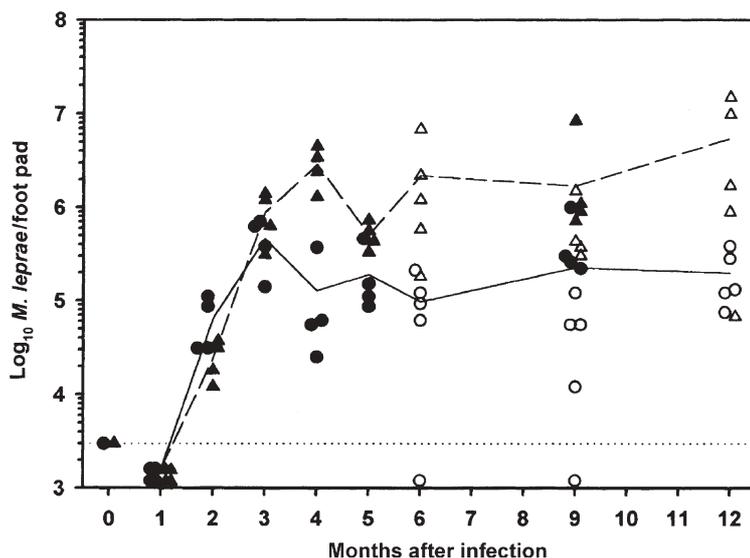
### **Evaluation of mouse foot-pad data**

The evaluation of data derived from application of the mouse foot-pad technique may be difficult for researchers unfamiliar with the technique. Given the inherent imprecision of the technique, papers reporting the results of experiments in which the mouse foot-pad technique has been employed must present all of the data, so that they may be carefully evaluated in terms of the sources of error already described. Important questions to be asked are whether enough mice were employed, whether the timing of harvests was correct, and whether the appropriate statistical analyses were performed.

### **The future of the mouse foot-pad technique – the search for an alternative technique**

The attempt has been made in this paper to review both the strengths and the weaknesses of the mouse foot-pad technique. Its greatest strength is that it is capable of discriminating between viable, i.e. capable of multiplying, and non-viable, i.e. incapable of multiplying, *M. leprae*. Its weaknesses are that it is labour-intensive, expensive, and time-consuming, and that it lacks both the sensitivity and precision that characterize the culture of cultivable bacteria in cell-free medium. These weaknesses notwithstanding, the mouse foot-pad technique is currently the standard by which all proposed alternative techniques must be judged. On the other hand, because of these weaknesses, research directed at the development of an accepted alternative to this technique must be an objective of the highest priority.

The demonstration that a given technique yields results consistent with expectations regarding the viability or non-viability of *M. leprae* is insufficient to permit acceptance of the technique as a substitute for the mouse foot-pad technique. The problem inherent in such a situation is exemplified by a study of the reconstitution of immune-compromised mice.



**Figure 3.** Growth curves of *M. leprae* in the hind foot pads of BALB/c (O, ●) and GKO (Δ, ▲) mice. The mice were inoculated in the hind foot pads with  $6 \times 10^3$  AFB per foot pad. At the times indicated, mice were killed and the *M. leprae* harvested. Each symbol represents one mouse; the open and closed symbols represent two independent experiments. The solid (BALB/c) and dashed (GKO) lines connect the mean values at each time-point. The dotted line represents the lower limit of detection of the assay (reprinted from reference no. 59).

Basing their conclusion on the results of an *in vitro* assay that had, in earlier work, yielded results consistent with those expected, the investigators reported<sup>25</sup> that reconstitution of infected nude mice *did not* cause death of the *M. leprae*. One must wonder what would have been their conclusion, had the investigators employed the mouse foot-pad technique in place of, or in addition to, the *in vitro* assay.

To be addressed is the question of how to demonstrate that any alternative technique is capable of providing information of the same quality as that provided by the mouse foot-pad technique, at the same time that it provides that information much more rapidly, and at a much lower cost. Although the demonstration of drug-resistance by the presence of genetic markers represents one such alternative, demonstrating the viability of *M. leprae*, the basic objective in most of the applications of the mouse foot-pad technique described, represents an as yet unsolved problem. Briefly, the investigator must demonstrate by experiments in which the proposed alternative is compared *directly* with the mouse foot-pad technique, that the alternative produces essentially identical results. This can be accomplished only by employing the mouse foot-pad technique in its most sensitive form, with respect to measurement of the proportion of viable *M. leprae*, i.e. the proportional bactericide technique.<sup>42</sup>

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## Appendix 1. Technique

The mouse is killed by cervical dislocation and pinned on a dissecting board. The inoculated foot is then cleansed with soap and water, rinsed with sterile water, and dried with sterile gauze. The foot-pad tissues are removed in three layers, skin and subcutaneous tissue, tendon, and muscle, by means of a sterile scalpel and haemostat. The tissues are pooled, thoroughly minced in a few drops of a saline solution, and homogenized in a measured volume (usually 2 ml) of Hanks' buffered salt solution or phosphate-buffered saline, employing a 15-ml tenBroek tissue grinder. A 10  $\mu$ l aliquot of the resulting homogenate is delivered by a micropipette or calibrated capillary tube to the centre of a fused ceramic circle on the surface of a counting slide, carefully spread over the surface of the circle, and allowed to dry in air. Because heat-fixation by exposure to flame is difficult to control, and because excessive heating may adversely affect the acid-fastness of *M. leprae*, the preparation is fixed by exposure first to formalin fumes for 3 min and then to heating for 2 min on the lid of a boiling water bath or a hot plate adjusted to 60°C. The slide is then stained at room temperature by covering the preparation for 20 min with carbol fuchsin (1% basic fuchsin in 5% phenol), which has been prepared from a batch of basic fuchsin with an absorption maximum no lower than 552 nm.<sup>60</sup> The stained slide is carefully decolourized with 1% HCl in 70% ethanol, counterstained for 1 min with 0.3% methylene blue in 30% ethanol, and air-dried. The AFB are then enumerated by microscopic examination of the stained preparation under optimal conditions, employing immersion oil both below and above the slide, an apochromatic objective and Köhler illumination at  $\times 1000$ –1250 magnification. Finally, the bacterial suspension is diluted in saline to yield a concentration of  $5 \times 10^3$  or  $10^4$  AFB per 0.03 ml, and this volume is inoculated subcutaneously into the plantar surface of the mouse foot.

It is simply impossible to count the *M. leprae* in the entire 2-ml sample, and even counting the organisms in all of the 10-ml aliquot is far too time-consuming to be performed as a routine. Therefore, the following procedure, described in the paper by Shepard and McRae,<sup>27</sup> has generally been adopted.

First, the diameter of the circle on the counting slide is measured by means of the Vernier scale on the microscope stage, and the diameter of the microscope field by means of a slide micrometer. Typical measurements are, respectively, 9.10 and 0.216 mm; thus, each microscope field represents  $1/1775$  ( $0.216^2/9.10^2$ ) of the area of the circle. Usually, 20 fields equally spaced across a diameter of each of two or three circles are examined.

If, on average, one AFB is counted per field, a 10- $\mu$ l aliquot may be calculated to contain 1775 AFB, and the entire volume of the homogenate, typically 2 ml, to contain  $3.55 \times 10^5$  AFB. Thus, if 40 microscope fields are examined, the AFB present in only 1/8875 (approximately 0.01%) of the entire homogenate have actually been counted. These

measurements also determine the lower limit of sensitivity of the technique: if only one AFB is detected in all 40 microscope fields, the harvest has yielded 8875 *M. leprae* per foot.

## Appendix 2. Poisson distribution

The distribution in space or time of independent objects or events, which is not *uniform* but *random*, is described by the Poisson distribution,<sup>29</sup> by which one may predict the numbers of red blood cells (RBC) per cell of a haemocytometer or of radioactive disintegrations per unit of time. Thus, if the mean number of RBC is 5 per cell of a haemocytometer, some cells will contain fewer than 5 RBC, whereas others will contain more than 5. By analogy, an inoculum containing a mean of 5 AFB per inoculated volume should deliver fewer than 5 AFB to some mice, and more than 5 AFB to other mice.

As shown in Table 6, the Poisson distribution predicts that, given 10 mice inoculated in a hind foot pad with an average of 5 AFB per foot pad, one or two mice will actually have been inoculated with no more than 2 AFB, seven will have been inoculated with from 3 to 7 AFB, and one or two will have been inoculated with more than 7 AFB. The calculations presented in this table describe the ideal situation, in which the organisms do not clump; clumping might be expected to result in the inoculation of a larger than predicted number of mice with portions of the inoculum in which no AFB are present, whereas a larger than predicted number of the mice will have been inoculated with a number of AFB greater than the mean.

The method of Halvorson and Ziegler,<sup>21</sup> referred to in the text, is based upon the Poisson distribution.

**Table 6.** The distribution of *M. leprae* in the inoculum, assuming that 10 mice are inoculated with a mean of 5 AFB in a hind foot pad (FP) (adapted from reference no. 28). The number of mice actually inoculated with the number of organisms indicated has been calculated according to the Poisson distribution.<sup>29</sup> The number of mice in the right-hand-most column actually inoculated with the number of organisms indicated in the left-hand most column =  $n \times (\lambda^a \times e^{-\lambda})/a!$ , where  $n$  is the total number of mice,  $\lambda$  is the mean number of AFB inoculated per mouse, and  $a$  is the actual number of AFB inoculated. 'e', the base of the system of natural (Napierian) logarithms,  $\approx 2.71828$ ; '!' represents the mathematical symbol for 'factorial', e.g.  $2! = 2 \times 1$ ,  $3! = 3 \times 2 \times 1$ ,  $4! = 4 \times 3 \times 2 \times 1$ , etc

Actual no. AFB inoculated per FP	Calculation	No. FP so inoculated
$\leq 1$	$10 \times e^{-5} + 10 \times 5 \times e^{-5}$	0.40
2	$10 \times (5^2 \times e^{-5})/2!$	0.84
3	$10 \times (5^3 \times e^{-5})/3!$	1.40
4	$10 \times (5^4 \times e^{-5})/4!$	1.76
5	$10 \times (5^5 \times e^{-5})/5!$	1.76
6	$10 \times (5^6 \times e^{-5})/6!$	1.46
7	$10 \times (5^7 \times e^{-5})/7!$	1.04
8	$10 \times (5^8 \times e^{-5})/8!$	0.65
$\geq 9$		0.69
Total		10.0