Mycobacterium leprae in Mice: Minimal Infectious Dose, Relationship Between Staining Quality and Infectivity, and Effect of Cortisone

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ABSTRACT

Shepard, Charles C. (Communicable Disease Center, U.S. Public Health Service, Atlanta, Ga.), and Dorothy H. McRae. Mycobacterium leprae in mice: minimal infectious dose, relationship between staining quality and infectivity, and effect of cortisone. J. Bacteriol. 89:365-372. 1965.—The minimal infectious dose of Mycobacterium leprae in mouse foot pads was found to be on the order of 10 solidly staining bacilli. In a titration experiment, the actual number found was 3.4 to 34 solid bacilli, and the order of magnitude was confirmed by experience with inocula containing varying numbers of solidly staining leprosy bacilli from mouse passage and from clinical sources. The acid-fast staining quality of leprosy bacilli was related in a useful way to the subsequent rate at which bacillary growth appeared. When the proportion of solidly staining bacilli was high, the calculated generation time was shortest, and the lower the proportion, the longer the generation times. The results were in accord with the hypothesis that all viable bacilli are solid, and that when they die, most of them become nonsolid. Varying proportions of the dead bacilli, perhaps up to 10%, remain solid, at least temporarily. The growth curve of M. leprae in mice was followed in several experiments with total counts of acid-fast bacteria and determination of the ratio of solid bacilli. What had been called a maximal stationary phase was seen to consist of sequential phases of conversion of solid to nonsolid bacilli (death), reappearance of solid bacilli (growth), and conversion of solid to nonsolid bacilli (death). When cortisone was administered, leprosy bacilli grew somewhat more slowly during the logarithmic phase, but attained a higher level, especially of solidly staining bacilli.

The ability to grow Mycobacterium leprae experimentally in the foot pads of mice (Shepard, 1960, 1962b, 1963) makes it possible to test viability of the bacilli and to study some of their growth characteristics. Because the bacilli grow very slowly, it takes 4 to 12 months to get an answer from an experiment. We have attempted to learn whether an immediate estimate of infectivity can be obtained from the bacillary morphology, as revealed by the acid-fast stain.

It has been suggested for many years that granular leprosy bacilli were dead, and in recent years there has been much confirmatory evidence (reviewed by Rees and Valentine, 1962; Waters and Rees, 1962). Thus, in patients receiving effective chemotherapy, the number of uniformly staining bacilli dropped from 54% to about 3% in 6 months and remained at that level until at least 18 months (Waters and Rees, 1962). Studies were also done with M. lepraemurium and Escherichia coli, where viability could be determined directly by inoculating animals or by plating out.

Suspensions of these bacilli were incubated in saline, and when their viabilities had fallen to 0.1% or less, the number of uniformly filled bacilli (seen by electron microscopy) had fallen to about 5% (Rees, Valentine, and Wong, 1960). In another study, a close agreement between light and electron microscopy was observed; the acid-fast stained regions of individual M. leprae seen by light microscopy correlated very closely with the opaque regions of the same bacilli seen by electron microscopy (Rees and Valentine, 1962).

In the course of work with M. leprae in mice, we have inoculated many different suspensions of either mouse passage or human clinical material. The preparations made for counting the acid-fast bacteria (AFB) in the inoculum could be used to determine the proportion of solidly stained bacilli in the inoculum, and the results could be correlated quantitatively with the infectivity of the bacilli, as judged by their subsequent rates of growth in mice.
**Materials and Methods**

The methods are described elsewhere in greater detail (Shepard, 1960, 1962b). In brief, suspensions of tissues were prepared in balanced salt solution containing 0.1% bovine serum albumin (Hanks-BSA). The AFB in the suspension were then counted microscopically, and further dilution (in Tryptose broth) was made, if necessary, to bring the inoculum to 5 x 10^5 AFB. The mice (usually 20) were inoculated in a rear foot pad. At monthly intervals, one mouse was killed and its inoculated foot processed for histological sections. When significant numbers of AFB were detected in the sections, the foot, its hair and a piece of adjacent inguinal lymph nodes were removed, fixed, stained microscopically, and processed for histological sections.

Calculations. If $F$ is the number of AFB inoculated into the host, and $H$ is the number harvested per foot at time $t$ after inoculation, the average generation time calculated on the assumption that solid AFB are viable is

$$G = t / (\log_2 (H/F))$$

where $H$ is the number of solidly stained bacilli and $S$ is the number of non-solid stained bacilli. The generation time of the bacillary growth from inoculation to harvest is calculated as the generation time ($G$); this provided a valuable quantitation of the uniquely slow growth of this organism.

For the acid-fast stains, excessive heating was avoided in the fixation procedure. Carbol-fuchsin was applied for 20 min at room temperature; de-staining was done with 1% HCl in 70% ethanol (Shepard, 1962a). Whenever otherwise indicated, the microscopes were equipped with phase-contrast objectives, compensating oculars, clear lenses, and careful focusing. Only bacilli with completely unobscured outlines were accepted for counts. Organisms scored solid were stained uniformly throughout their length. Organisms scored nonsolid contained unstained or irregularly stained regions. Usually 50 bacilli were scored. When possible, slides were done as "blinds." Repeat counts by the same or by other trained observers usually gave the same solid count within sampling error. Some difficulty was encountered in materials with very low solid counts. In the scoring of 50 bacilli, one or two that were stained uniformly, but were of a lesser intensity than the typical solid bacilli, were often encountered. Since they were solid by definition, they were scored so; later results led us to think that these may not have represented infectious bacilli. Rees and Valentine (1962) have pointed out a source of error in the same direction; when a bacillus loses acid-fast staining only at the end, it cannot be differentiated from a solid bacillus. Thus, in the interpretation of the results, it was deemed safer to avoid basing certain conclusions on solid ratios of 60% or less.

For the cortisone experiment, a commercial preparation of cortisone acetate for intramuscular administration, containing 25 mg/ml, was diluted to 2 mg/ml with Hanks balanced salt solution, and injected daily in 0.05-ml amounts into the thigh muscles. Alternate sides were used on alternate days. Each time a mouse was killed for foot sections or for bacillary harvests, the spleen and inguinal lymph nodes were removed, counted, and processed for histological sections.

**Results**

**Growth curves and changes in staining quality.** These are presented first in order to familiarize the reader with the experimental model. The evidence that nonsolid bacilli are dead is given later. Curves very similar to those in Fig. IA to IC have been obtained for a period of 4 years. Figures IA and B refer to a strain descending
from the hybrid black mice used by Chatterjee (1968). Results with this strain have been similar to those obtained with the CFW strain.

If attention is confined first to the total number of bacilli harvested (H) at various times, the growth curve after inoculation of $5 \times 10^6$ bacilli is seen to consist of three parts: (i) a lag phase that lasted usually 60 to 90 days; (ii) a logarithmic phase, during which multiplication occurred at a rate of 12 to 13 days per generation with more reliable data (Fig. 1A); at about 150 days, when $H$ reached a level of $10^9$ to $10^{10}$, the logarithmic phase terminated; and (iii) a period of slow increase then followed during which $H$ rose to a level of near $10^{10.8}$ at about 300 days.

In Fig. 1A to 1C are also shown the ratios of solid bacilli and the numbers of solid bacilli ($H_s$) and nonsolid bacilli ($H_n$) in the harvest. $H_s$ reached a maximum at the end of the logarithmic phase, then fell, and rose to a second maximum about 140 days after the first. The curves for $H$ also dropped slightly after the end of the logarithmic phase. In the experiment of Fig. 1C, the infectivity of the harvested bacilli was tested by subinoculation. The subinoculated bacilli grew at rates that were predictable from the ratios of solid bacilli (see below), although higher values of $G_s$ were again observed at lower ratios of solid bacilli.

On the hypothesis that all viable bacilli are solid, and that nonviable bacilli are usually nonsolid, there appear to have been extraordinary sequences in which either growth or death of bacilli predominates. The period of slow increase in total bacilli, mentioned above, is seen to consist of a death phase, followed by a second growth phase, followed in turn by a second death phase. The growth rate during the initial logarithmic phase and the second growth phase was apparently about the same ($G_s$ about 8 to 10 days). In both growth phases, infectious bacilli increased to a level of $10^9$ to $10^{10}$, at which point growth abruptly ended and was succeeded by a death phase. The first death phase was profound and lowered the number of solid bacilli 63 to 95%, at a half-life rate of 8 to 20 days. The second death phase appeared to be similar. At the end of the first growth phase, the ratios of solids were higher, because nonsolid bacilli had not yet accumulated in large numbers.

Since these unusual sequences of growth and death phases were unexpected, the possibility that changes in staining technique had been responsible was examined by exhaustive re-investigations of staining details. The results were negative. Also, cyclic errors in counting were sought strenuously but unsuccessfully. For example, sets of slides covering whole growth

Fig. 1. Growth curves of Mycobacterium leprae in mouse foot pads at top. Below is number of acid-fast bacilli (AFB) and the amount of infiltrate (I) in tissue sections. At the bottom is the proportion of solidly staining bacilli in the harvest (and inoculum). (A) The events of the logarithmic phase are covered best in this figure. (B) Arrangement is the same as in A. The events of the first death phase and second growth phase show more clearly in this figure. (C) Arrangement is the same as in A. In this experiment, the harvests were diluted, if necessary, to give an inoculum of $8 \times 10^6$ acid-fast bacteria and subinoculated into new groups of mice; the average rates of growth ($G_s$ and $G_n$) in the subinoculation are given (scale at right).
curves had been kept in the unstained state, and these were stained in one batch and counted as "blinds" and found to agree with the first slides.

Histological sections were examined from mice taken chiefly during the logarithmic phase (Fig. 1A to 1C). Acid-fast bacteria appeared in the sections in significant numbers (++) or more) only after the bacterial population was above 10^4. Well-developed cellular infiltrates (Fig. 1 and 2 of Shepard, 1960) did not appear except in one instance (Fig. 1C), and this at the beginning of the first death phase. Although sections were not continued after this period in these experiments, other work has shown that the well-developed infiltrate typically appears 1 to 2 months after the appearance of significant numbers of bacteria.

**Effect of cortisone.** Forty mice were infected with a strain of leprosy bacilli in fourth mouse passage. Twenty of the mice were given daily intramuscular injections of 0.1 mg of cortisone acetate, and changes in the lymph nodes and spleens showed that a distinct pharmacological effect was obtained. In every case, the lymph nodes of the cortisone group were smaller, about half the diameter of the controls when found; and 25% were so small they could not be located. In the histological sections, follicles were indistinct or absent. The spleens were always smaller than those in the controls, especially in thickness, and the follicles were poorly developed. The results of the harvests (Fig. 2) indicate that the administration of cortisone slowed the growth of *M. leprae* during the logarithmic phase. At the first harvest, the average G was 24.1 days in the cortisone group and 20.3 in the control. The lower solid count at the end of the logarithmic phase in the cortisone-treated mice is in conformity with slower logarithmic growth, assuming that cortisone does not change the conversion rate of solid to nonsolid bacilli. However, bacterial growth in the cortisone group then proceeded to a higher level, especially in terms of solid bacilli. The first death phase and second growth phase were again evident in the untreated mice, but there was no sign of them in the treated mice.

In histological sections of the feet, a cellular infiltrate did not appear in the cortisone-treated mice even late in the growth curve.

The experiment had been undertaken to see whether the harvest of leprosy bacilli could be increased, since an increase of 100-fold or so would open up several new experimental approaches. The administration of cortisone seems to have increased the bacillary harvest, but not enough to repay the many injections.

**Minimal infectious dose and effect of diluent.**

A titration of infectivity was done (Table 1). The minimal infectious dose was 14 to 140 AFB, or 5.4 to 34 solidly staining AFB. No differences among the diluents were noted. There was no evidence that bovine albumin was protective.

The average rates of growth (G) between inoculation and harvest were about the same, regardless of dilution of the inoculum.

**Staining qualities of bacilli and their infectivity.** In Fig. 3A, 3B, 4A, and 4B, the relationships are given between the proportion of solidly staining bacilli, S/(N + S) in the inoculum, and the subsequent rate of bacillary multiplication, as measured by average generation times.

The rate of growth was clearly related to staining quality. In Fig. 3A, data are presented from routine passage experience. The generation times were calculated on the basis that the inoculum was the total number of AFB, whether solid or nonsolid. In all instances, the appearance of significant numbers of AFB in the monthly sections was the signal for the harvest, which was usually done near the end of the logarithmic phase of bacterial growth. When the ratio of solid bacilli in the inoculum was greater than 50%, the average generation time was regularly between 14 and 24 days. With ratios of less than 50%, the G increased progressively as the ratio decreased.

Similar results occurred when leprosy bacilli in clinical materials were inoculated (Fig. 3B). Although the ratios of solids in the clinical materials averaged lower than those in the passage suspensions, the rate of growth after inoculation into mice was about the same with inocula of equivalent ratio. There were more "misses" and "partial misses" with clinical materials, but these arose largely from very small inocula of solid bacilli; the relationship between size of inoculum and consistency of "takes" is considered below.
### Table 1. Minimal infective dose and effect of diluent

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Dilution of inoculum</th>
<th>Diluent</th>
<th>AFB inoculated</th>
<th>Mycobacterial findings in sections</th>
<th>Harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1      2   3   4   5   6   7   8   9   10  11  12  13  14</td>
<td></td>
</tr>
<tr>
<td>1–20</td>
<td>None</td>
<td>Hanks-BSA</td>
<td>$1.4 \times 10^4$</td>
<td>0 0 0 0 0 + 4+ 2+&lt;sup&gt;*&lt;/sup&gt; 0 — — — — —</td>
<td>252</td>
</tr>
<tr>
<td>21–40</td>
<td>$10^{-1}$</td>
<td>Tryptose</td>
<td>$1.4 \times 10^4$</td>
<td>0 0 0 0 0 3+ 0 0 2+ 4+ 3+&lt;sup&gt;*&lt;/sup&gt; — 3+</td>
<td>371</td>
</tr>
<tr>
<td>41–60</td>
<td>$10^{-2}$</td>
<td>Tryptose</td>
<td>$1.4 \times 10^2$</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>371</td>
</tr>
<tr>
<td>61–80</td>
<td>$10^{-3}$</td>
<td>Tryptose</td>
<td>$1.4 \times 10^1$</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>371</td>
</tr>
<tr>
<td>81–100</td>
<td>$10^{-4}$</td>
<td>Tryptose</td>
<td>1.4</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>394</td>
</tr>
<tr>
<td>101–120</td>
<td>$10^{-2}$</td>
<td>1% BSA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>$1.4 \times 10^2$</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>371</td>
</tr>
<tr>
<td>121–140</td>
<td>$10^{-3}$</td>
<td>$D_2T$&lt;sup&gt;+&lt;/sup&gt;</td>
<td>$1.4 \times 10^2$</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>371</td>
</tr>
</tbody>
</table>

<sup>a</sup> Bacilli from a strain in fourth passage were harvested in Hanks-BSA as usual (Shepard, 1960, 1962b). Solidly staining bacilli were 24%. Dilutions were then made as indicated, and 20 mice were inoculated per dilution.

<sup>1</sup> Number of months.

<sup>2</sup> The month in which the harvest was made.

<sup>3</sup> Bovine serum albumin, fraction V, 1% in distilled water.

<sup>4</sup> NaCl (0.9 m), 8% sucrose, and 0.1 m tris(hydroxymethyl)aminomethane, adjusted to pH 8 with HCl (Sprott et al., 1960).
It is noteworthy that there was no evidence to suggest any "adaptation" to mice; i.e., leprosy bacilli in primary clinical materials grew at the same rate as those in mouse passage, when allowance was made for number of bacilli inoculated and their staining quality.

In Fig. 4A are given generation times, $G_s$, calculated on the basis that all the harvested bacilli arose from solid bacilli in the inoculum. $G_s$ is more nearly the same at all solid ratios, than was G (Fig. 3A). If nonsolid bacilli had contributed significantly to growth, some unusually low $G_s$ would have appeared at very low ratios of solids, where relatively large numbers of nonsolid AFB were inoculated. Instead, the $G_s$ were somewhat high with ratios below 20%; the most likely explanation for this was that in this range the ratio of solids had overestimated the number of infectious bacilli.

One explanation for the overestimate might be that a constant proportion of solid bacilli were not infectious; however, when the $G_s$ are re-
calculated on this basis, the higher values at low ratios persist. If, conversely, one assumes that when the bacilli die, a constant proportion does not convert to nonsolid, a more logical pattern emerges. When the unconverted proportion is assumed to be 10%, the G₈, with exceptions, fall in the same range, regardless of ratios. In practice, this proportion was estimated by taking 10% of the nonsolid fraction, so that infectious bacilli in any inoculum consisted of solid bacilli less 10% of nonsolid bacilli (Fig. 4B). When the ratio of solids was 11% or less, a ratio of 2% was assumed in order to obtain an estimate of the upper limit of the generation time. All of the exceptions to the orderly pattern in Fig. 4B are high G₈. These might have arisen because deleterious conditions during collection and preparation of the specimens killed some of the bacilli without allowing them time to convert to nonsolid. Also, many of the exceptions were with small inocula in terms of solid bacilli; since there is a tendency for some of the mice to escape infection with small inocula (unpublished observations), the effect would be to delay the harvest and lower the average harvest per mouse.

In other calculations, the results of which are not shown, percentages higher and lower than 10% of nonsolid bacilli were assumed and subtracted from the solid bacilli, and it was found that 10% seemed to give the most constant generation times. Obviously, the percentage may sometimes be less than 10%, since certain inocula with less than 10% solid bacilli had normal G₈ values (Fig. 4A).

The data shown in Fig. 3A and 3B could also be used to estimate the minimal infectious dose. To do this, the proportion of “takes” was written down according to number of bacilli inoculated. With log₁₀F of greater than 4.50, 4.50 to 4.00, 3.90 to 3.50, 3.40 to 3.00, 2.90 to 2.50, and 2.40 to 2.00, the proportion of “takes” was 100% (3/3), 75% (6/8), 95% (76/80), 67% (10/15), 73% (8/11), and 33% (1/3), respectively. For this purpose, “takes” were defined as the appearance of AFB in either sections, or counts of AFB at harvest. Similar results were calculated for solid AFB in the inoculum, excluding from consideration ratios of 6% or less. With log₁₀F of greater than 3.00, 3.00 to 2.50, 2.40 to 2.00, 1.90 to 1.50, and 1.40 to 1.00, the proportion of “takes” was 100% (53/53), 100% (26/26), 78% (7/9), 100% (3/3), and 100% (1/1), respectively. Thus, inocula containing as few as 10 to 100 solid bacilli produced “takes” consistently. This is in agreement with the results of the titration experiment (Table 1) where the minimal infectious dose of solid bacilli was 3.4 to 34.

**Discussion**

The suggestion by Rees and co-workers that nonsolid leprosy bacilli are dead has been confirmed and expanded by direct measurements of infectivity in mice. Our evidence indicates that a proportion of the solid bacilli is also noninfectious, and the proportion seems larger at low solid ratios. An anatomical interpretation that conforms with the observations is as follows. Acid-fast staining depends upon the presence of protoplasm inside the cell envelope; cell walls of *M. leprae* and other mycobacteria do not stain (unpublished data) when prepared as described by Shepard and Kirsh (1961). Living leprosy bacilli are filled with protoplasm and always stain solidly. When the bacilli die, most of them degenerate to nonsolid (at a half-life rate of 8 to 20 days) by partial and regional loss of protoplasm through lesions of the cell envelopes. In a few bacilli (up to 10%), the remaining protoplasm is not lost, or is distributed in a confusing manner so that they are still scored solid. The next step in the degradation process is complete loss of protoplasm (at a half-life rate of at least several months). The final step, disappearance of cell walls, cannot be observed through acid-fast staining.

It is unfortunate, from a clinical standpoint, that all dead bacilli do not convert quickly to nonsolid forms, since this makes it difficult to know whether living bacilli remain when the proportion of solids is 5% or less. The usually recommended therapeutic practice is to continue treatment until AFB of any sort have disappeared; in lepromatous leprosy, this requires many years.

The sequences of death and growth during what had been called the phase of slow increase were unexpected. No such events have been observed in the *M. lepraemurium* infections of mice; in that infection, rapid bacillary growth continues until death of the animal (unpublished data). In chronic pulmonary infections of mice with *M. tuberculosis*, the viable population rises to a characteristic level and then remains constant without much fluctuation (Batten and McCune, 1957; Rees and Hart, 1961). Perhaps it is the timing of the cellular response in the leprosy infection that is responsible for the difference, since an infiltrate does not appear until the bacterium numbers reach 10⁶ to 10⁷ (Shepard, 1960). The results of the cortisone experiment also suggest that the cellular response plays a role, since in the mice receiving cortisone the cellular response was depressed, and the bacterial population, especially of solid bacilli, increased to a higher level.
It is not clear whether a specific immune response to the antigens of M. leprae is involved in the death phases. The first death phase is very short-lived, since it is followed by bacillary multiplication in the same foot pad at an apparently normal rate. The first death phase seems to be a local phenomenon, since the mouse is not systemically immune at this time, and, when inoculated in the other hind foot, will develop the second infection as rapidly as a first infection. However, the mouse is capable of developing a generalized immunity to M. leprae, and does so when vaccinated by other routes with large numbers of mycobacteria, especially BCG (Shepard, Amer. J. Epidemiol., in press).

The effect of cortisone on infections with M. leprae in mice seemed to be a mixture of the two effects produced by cortisone on murine infections with M. lepraemurium and M. tuberculosis, respectively. As mentioned, with M. lepraemurium there is no stationary phase. Rees (personal communication) found that cortisone decreased the number of bacilli on the order of five- to eightfold; the infection was by intravenous route and the organs studied were liver and spleen. Naguib and Robson (1956), and Buttle, D’Arcy, and Howard (1958) have reported that the tissue response in M. lepraemurium infections is decreased by cortisone. In the chronic pulmonary experimental infections of mice with M. tuberculosis, the logarithmic phase is usually short, and the stationary phase is prominent. Most observers have found that cortisone increases the level of tubercle bacilli during the stationary phase. Batten and McCune (1957) found the increase in viable bacilli to be about 10-fold, approximately the same increase as that found for solidly staining M. leprae in the present work.

In human leprosy, as usually seen in the clinic, the bacilli probably are usually in a slow increase phase or a stationary phase. Cortisone treatment in the absence of chemotherapeutic control might result in an increase in bacteria. The problem is perhaps more frequent in leprosy than in tuberculosis, because, in leprosy, cortisone has frequently been used in treatment-reactions, that is, in situations when chemotherapy is withdrawn.

Acknowledgment
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Literature Cited


