Are All Nonsolid *Mycobacterium leprae* Dead? Does a Negative Finding in the Mouse Foot Pad Indicate That There is Actually no Growth of *M. leprae* in the Animals?[1]

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Ever since the discovery of the foot pad technic for growth of *Mycobacterium leprae* in mice, investigators have overemphasized laboratory results in clinical application. Response to the foot pad technic was so enthusiastic that practically all the findings obtained from the mouse were believed to be applicable to leprosy patients. Extrapolations of animal data for clinical applications were frequently made without any reservation. Unfortunately, such overenthusiasm has led to some disastrous results in the leprosy field. Two well-known examples can be cited, which were based on suppositions that: a) all the nonsolidly stained *M. leprae* are dead, and b) a negative finding in the mouse foot pad indicates no growth of *M. leprae* in the animal. The former led clinical investigators to claim a false emergence of drug resistance after one year's treatment with a potent antileprosy drug, B663, which was nearly abandoned for later clinical use. The latter led investigators to introduce a low-dose treatment, which resulted in a worldwide appearance of DDS resistance in leprosy. Comments on these questions are presented in this communication.

Are all nonsolid M. leprae dead? This question has been debated in the past, particularly during the World Health Organization Conference of 1968 in London. Unfortunately the recordings of the discussion were not made available. However, doubt surrounding this question has not diminished. Dr. Dharmendra (personal communication, 1973), after discussions with leading leprologists throughout the world, concluded that many have never accepted the idea that all nonsolid *M. leprae* are dead. The data supporting the view that all nonsolid organisms are dead have never been convincing. On the contrary, *M. leprae* showing 100% nonsolid organisms revealed good growth in the foot pads (see below). The reasoning against the idea that all nonsolids are dead is outlined here.

1. The nonsolid organisms *(Escherichia coli, Mycobacterium tuberculosis,* and *Mycobacterium lepraemurium)* used by the original investigators ([25]) were already dead before the test started. The organisms were either kept *in vitro* in phosphate buffer at room temperature for 28 days, or obtained from animals which had been treated with isoniazid for 12 months. Live nonsolid organisms were not used. These experiments could only conclude that *dead,* nonsolid organisms were not alive ([8]).

2. Comparisons were made by matching the electron microscopic image of organisms with a diagram of the same organisms under the light microscope. This obviously caused the authors difficulty, since, in three successive publications, the EM image of *M. leprae* appeared three times, each time matched with a different diagram ([23-25]).

3. By comparing the EM images with the light microscopic Morphological Index (MI), Edwards and Draper ([12]) concluded that both findings agreed qualitatively but not quantitatively. Actually they found that EM detected a slightly higher number (up to 13% more) of nonsolid *M. leprae* than in the light microscope. Contrary to these findings, Ozawa and Kobayashi ([21]) concluded that EM detected many more solid *M. leprae* (averaging 3.8-fold more) than the light microscope. Thus, EM images do not appear to be comparable to light microscopic observations.

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4. In their EM studies on the growth of irradiated *M. tuberculosis* H37Rv in macrophage cultures, Draper and D'Arcy Hart concluded that "damaged bacilli equals non-viable, but the intactness of the organisms can not be equated with viability" ([11]).

5. Nyka's method ([19,20]) has been used by at least six laboratories to improve the acid-fast staining of the mycobacteria. As shown in Table 1, the total number and the number of solidly stained *M. leprae* and/or *M. lepraemurium* were increased after periodate oxidation in all six laboratories. It is obvious that the type of stain used determines the number of solid forms, which, in turn, may be incorrect as a criterion for death.

6. Several years ago, I accidentally found smears containing very large acid-fast rods. These smears were made from the liver of a recently dead, normal mouse. There were some rods which showed acid-fast segments, but the areas between the rod segments were clearly stained with methylene blue. This indicated that the nonsolid areas are not necessarily empty as other investigators have believed ([23-25]).

7. Chang and Andersen ([8]) reported that during the early stage of the growth of *M. lepraemurium* in cultures of mouse macrophages, many organisms showed either a faintly stained, pink colored, pointed end, or an isolated acid-fast dot at the tip of the rod. Both the pointed ends and the isolated dots eventually grew together into deeply stained solid rods on continued cultivation. This suggested that the nonsolid appearance was possibly a normal form of growing acid-fast organisms.

8. Karat *et al* ([15]) inoculated *M. leprae* with a zero MI (i.e., 100% nonsolid organisms) into mouse foot pads, observed growth of the organisms in 30 of 36 experiments (83.4%), and concluded that there was no consistent relationship between the MI and viability of *M. leprae*.

9. Contrary to the fact that not all nonsolids are dead, it is probable that not all solids are alive. The well-known story of the clinical trial of the drug B663 is a good example ([6]). Browne reported that leprosy bacilli developed resistance to B663 after one year's treatment because of the recurrence of the solid *M. leprae* in the smears ([2]). Our studies in murine leprosy ([4,5]) revealed that emergence of resistance of *M. lepraemurium* did not appear even after the B663 treatment was extended up to 816 days. Fortunately, our results convinced Dr. W. Vischer, Chief of Bacteriology, Geigy, Basel, Switzerland and an additional supply of B663 was made available for further clinical trial. Later, Knight and his associates ([29]) at the National Institutes of Health, Bethesda, Maryland, U.S.A. disproved the findings of Browne. It has been more than 15 years since the first trial, and not a single B663-resistant case has been observed from the worldwide clinical use ([22]).

From the above findings, it is obvious that conclusive evidence proving all nonsolid *M. leprae* to be dead does not yet exist. I can appreciate that the EM pictures of bacteria which showed only a few patches of electron-dense material could be dead organisms. This is, however, beyond the scope of the present discussion.

To clinical investigators, the real meaning of the MI is the change of stainability of the organisms as a result of drug treatment. This finding is sufficient for the evaluation of drug activity. However, when it comes to laboratory growth experiments, why not abandon measuring probable viability by MI until some reliable criterion appears in the future?

Does a negative finding in the mouse foot pads indicate that there is actually no growth of M. leprae in the animal? Recent developments indicate the following drawbacks of the present mouse foot pad/*M. leprae* model for a quantitative evaluation of the growth of *M. leprae*.

1. *Animal-to-animal variations.* Nakamura and Hisai ([18]) reported that more than a 1,000-fold difference was observed in the growth of *M. leprae* in foot pads among individual animals in a group of 20 mice. Tsutsumi *et al* ([28]) reported marked variations in the growth of *M. leprae* between the left and right foot of the same animals. The left/right ratio varied approximately 1,000-fold in a group of 50 animals. In our laboratory the growth of *M. leprae* was studied in a group of 20 animals (CBA/J mice). Variations of growth among individual animals and between left and right foot pads were so great that the standard deviations were greater than the average numbers of organisms. These results were presented at the 9th International Leprosy Congress in 1973 and at the 8th U.S.-Japan Joint Leprosy Conference, July 30-August 1, 1973, San Francisco,

California ([7]). The findings suggest that the use of a larger number of mice is necessary for a quantitative evaluation of growth of *M. leprae* in foot pads. In the original report, the foot pad technic used only four animals in the experiments.

2. *Bacterial clumps in the Mickle-treated bacterial suspensions.* A long time ago Shepard noticed bacillary clumps in the suspension ([27]). I estimated that if there is one bacterial clump in every ten microscopic square fields and if the clump contains a minimal number of five bacilli, the total number of organisms in the clumps of two milliliters of homogenate will reach 4×10^6. If there is only one clump in the whole smear, the total number of organisms in the clump will be 1×10^3. It is clear if one misses the clump in the smear, serious errors could result in total bacterial counts ([3]). In this regard, the findings of investigators in the Leonard Wood Memorial Laboratory, Cebu, The Philippines are of particular interest. They used the standard Mickle-treated bacterial suspension and made the standard bacterial counts. Then, they re-homogenized the suspension and counted the organisms again. Surprisingly, the number of *M. leprae* was constantly 10- to 20-fold higher than the original counts. They repeated the experiments many times, and the results were similar each time ([26]).

3. *Methods to reduce bacterial clumps.* Desikan and Venkataramaniah ([10]) compared bacterial counts made by the conventional (Mickle) method in one foot with those made by their modified technic on the other foot of the animals. Their method consisted of a gentle grinding of the whole foot (without the digits) in a glass mortar with a pestle. Bacterial counts made from their modified method were higher than those made by the conventional method.

In our laboratory a simple method was used to reduce bacterial clumps in the tissue homogenates. Tissue pieces were ground against the sides of the glass Tenbrock grinder instead of its bottom. One can apply more force to press the tissue with the pestle against the sides than against the bottom. This is Dr. Chapman H. Binford's original method. We employed this technic to make tissue homogenates for a total of 608 mouse foot pads (88 for *M. leprae* and 520 for *M. lepraemurium*). Only occasional small bacterial clumps were observed ([3]).

4. *Dependence upon staining.* Mohysen and Alemayehu ([17]) reported that sections from tuberculoid, intermediate, and lepromatous lesions all revealed an increase (averaging fourfold) of acid-fast bacilli after oxidation with periodic acid (Nyka's staining technic) (see Table 1).

Harada *et al* ([14]) reported that the source of basic fuchsin used in the Ziehl-Neelsen formula has great influence on the staining property of acid-fast organisms, i.e., *M. leprae, M. lepraemurium,* and *M. tuberculosis.* Fuchsins possessing a maximum absorption lower than 552 mμ tend to show a poor, unstable color intensity accompanied with beadings of the bacilli, while those possessing a maximum absorption of $\geqslant 552$ mμ do not. Surprisingly, many leading laboratories regularly use the inferior fuchsins in their Ziehl-Neelsen staining preparations.

5. *Why we observed marked animal-to-animal variations.* In our laboratory the bac-

TABLE 1. M. leprae *and* M. lepraemurium *stained with the original or modified Nyka's technics.*

Authors	Acid-fast organisms	Increase in the number of solid organisms	Increase in the total number of organisms
Andersen and Chang ([1])	Ml, Mlm	+	+
Ozawa and Kobayashi ([21])	Ml	+ 3.8-fold	+
Kato and Berthiaume ([16])	Ml, Mlm	+	+
Mohysen and Alemayehu ([17])	Ml	+	+ 4-fold
Fisher and Barksdale ([13])	Mlm	+	+
Delville and Richee ([9])	Ml	+	+

Ml = *M. leprae;* Mlm = *M. lepraemurium.*

terial homogenates were made by the method described in point 3, and the organisms were stained with a modified Nyka's staining method ([1]). We also employed a larger number of animals in the experiments in order to make a statistical analysis. We obtained higher counts than other investigators and, therefore, observed greater individual variations among the animals.

Since the present standard foot pad model employs: a) the Mickle-treated bacterial suspension, b) only one bacterial count from a pool of four foot pads (from which it is difficult to calculate a standard deviation), and c) the old Ziehl-Neelsen staining without periodic acid oxidation, the bacterial count obtained from such a model could be 40- to 80-fold lower (i.e., 4-fold lower by using the old Ziehl-Neelsen staining and 10- to 20-fold lower by using the Mickle disintegrator) than the organisms actually present in the foot pads. Therefore, if the present foot pad technic is employed, a negative finding in the foot pads does not necessarily indicate that there are no *M. leprae* in the tissue.

6. *The minimal inhibitory concentration of DDS.* Recently, emergence of resistance of *M. leprae* to DDS has been observed worldwide in leprosy patients as a result of insufficient drug treatment ([22]). A low-dose hypothesis was introduced some years ago. This hypothesis was based on foot pad experiments in which a minimal inhibitory concentration (MIC) of DDS in the blood was established. This mouse MIC was applied directly to the clinical treatment of leprosy. Even an extraordinarily small dose of one milligram per day was recommended for treatment. It should be noted that low-dose drug treatment is renowned for inducing resistance. This is the situation I dwelt upon at the U.S.-Japan leprosy conferences. Unfortunately, the low-dose treatment has been used since the standard MIC was established. Now, regretfully, DDS-resistance has become a worldwide problem.

7. *What may result if the use of present standard foot pad/*M. leprae *technic continues?* Since the present standard foot pad technic does not detect all *M. leprae* in tissues, it could produce, apart from the low-dose incidence, more misleading information and yield more ruinous results, such as:

a. detection of fewer drug-resistance cases of *M. leprae;*

b. misinterpretation of a high degree of drug resistance as low resistance;

c. detection of fewer relapsed cases;

d. getting a low Bacterial Index (BI) or a false negative BI during treatment;

e. obtaining false negative findings in biopsies made for diagnosis of new cases;

f. getting false negative growth in foot pad experiments;

g. yielding false positive antileprosy activity to new drugs, etc.

It is still not too late to develop better technics for preparation of bacterial suspension and for staining of acid-fast organisms. I appreciate the usefulness of the foot pad/*M. leprae* model. But I do not think any exaggeration of its usefulness is necessary. It has been more than 17 years since the first discovery of this model. It is time to have some modification to keep the "mighty mouse" as mighty as possible.

SUMMARY

Ever since the discovery of the foot pad technic for growth of *M. leprae* in mice, investigators have overemphasized the laboratory results in clinical applications. Overenthusiasm has led to some dire results in the leprosy field. Two well-known examples can be cited, which are based on the presumption that: a) all the nonsolidly stained *M. leprae* are dead, and b) that a negative finding in the mouse foot pad indicates no growth of *M. leprae* in the animals. The former led clinical investigators to claim a false emergence of drug resistance after one year's treatment with a potent antileprosy drug, B663, which was almost abandoned for later clinical use. The latter led investigators to introduce a low-dose drug treatment, which resulted in a worldwide appearance of DDS resistance in leprosy. This paper outlines the reasoning that not all nonsolid *M. leprae* are dead, and that not all the organisms in the foot pads are detectable by the present standard foot pad/*M. leprae* technic.

RESUMEN

Desde la introducción de la técnica de los cojinetes plantares para crecer al *M. leprae* en ratones, los investigadores han sobreenfatizado los resultados del laboratorio en las aplicaciones clínicas. Este sobreentusiasmo ha conducido a algunos resultados desastrosos en el campo de la lepra. Se pueden citar dos ejemplos bien conocidos, los cuales estan basados en las suposiciones:

a) que todos los *M. leprae* teñidos en forma no homogénea estan muertos, y b) que los hallazgos negativos en los cojinetes plantares del ratón indican que no hubo crecimiento del *M. leprae* en los animales. La primera suposición condujo a los investigadores clínicos a proclamar una falsa emergencia de resistencia a drogas después de un año de tratamiento con una potente droga antileprosa, B663, la cual fue casi abandonada en su uso clínico posterior. La segunda suposición condujo a los investigadores ha introducir un tratamiento con dosis bajas de una droga, lo cual dio como resultado la aparición mundial de resistencia de la lepra al DDS. Este trabajo señala las razones que llevan al autor a considerar que no todos los *M. leprae* que se tiñen en forma no homogénea están muertos y que no todos los microorganismos en los cojinetes plantares son detectables por la técnica "estándard" de los cojinetes plantares del ratón.

RÉSUMÉ

Depuis que la découverte de la technique du coussinet plantaire a permis la croissance de *M. leprae* chez la souris, les chercheurs ne se sont pas privés d'extrapoler les résultats de laboratoire aux applications cliniques. Un enthousiasme exagéré a mené à des conclusions parfois effarantes dans le domaine de la lèpre. Deux exemples bien connus peuvent être cités, où les conclusions ont été basées sur les présupposés suivants: a) tous les *M. leprae* colorés de façon non uniforme sont morts; b) une constatation négative dans le coussinet plantaire de la souris constitue la preuve d'une absence de croissance de *M. leprae* chez les animaux. La première de ces conclusions a mené des chercheurs cliniques à affirmer à tort l'apparition d'une résistance médicamenteuse après une année de traitement par un médicament antilépreux efficace, le B663, qui en conséquence a été alors presque abandonné en utilisation clinique. Le deuxième postulat a conduit certains investigateurs à introduire un traitement basé sur l'administration de médicaments à doses faibles, ce qui a entraîné l'apparition de résistance à la DDS dans la lèpre à l'échelle mondiale. Cette communication s'attache à démontrer que tous les *M. leprae* non colorés de façon uniforme ne sont pas nécessairement morts, et les organismes présents dans coussinet plantaire de la souris ne sont pas tous détectables par les méthodes coutumières actuelles de coloration de *M. leprae* dans le coussinet plantaire.

REFERENCES

1. Andersen, R. N. and Chang, Y. T. Morphological changes produced by various Ziehl-Neelsen staining technics in *M. leprae* and *M. lepraemurium*. Int. J. Lepr. (Abs.) **38** (1970) 344.
2. Browne, S. G. and Hogerzeil, L. M. Apparent resistance of *M. leprae* to B663. Lepr. Rev. **33** (1962) 185-189.
3. Chang, Y. T. Bacterial clumps in tissue homogenates prepared by the Mickle disintegrator. Widely circulated communication, Dec. 1974-Feb. 1975.
4. Chang, Y. T. Effects of B.663, a rimino-compound of phenazine series, in murine leprosy. *In:* Antimicrobial Agents and Chemotherapy. Washington, D.C.: Am. Soc. Microbiol., 1962, pp 294-307.
5. Chang, Y. T. Further studies on B663 in murine leprosy. Absence of resistance of *M. lepraemurium* to B663 and delay in development of resistance to isoniazid. Int. J. Lepr. **34** (1966) 1-6.
6. Chang, Y. T. Story behind the clinical trial of B663 in leprosy. Int. J. Lepr. **35** (1967) 78-80.
7. Chang, Y. T. and Andersen, R. N. Animal-to-animal variations in the growth of *Mycobacterium lepraemurium*. Int. J. Lepr. (Abs.) **41** (1973) 514; Animal-to-animal variations in the growth of *Mycobacterium lepraemurium* and *Mycobacterium leprae*. 8th U.S.-Japan Joint Leprosy Conference, July 30-August 1, 1973, San Francisco, California, pp 19-20.
8. Chang, Y. T. and Andersen, R. N. Morphological changes of *Mycobacterium lepraemurium* grown in cultures of mouse peritoneal macrophages. J. Bacteriol. **99** (1967) 867-875.
9. Delville, J., and Pichel, A. M. Does Hansen's bacillus exist in a nonacid-fast stage? Comparison of different staining technics in detecting bacilli in leprous lesions. J. Int. Res. Commun. (IRCS) **2** (1974) 1192.
10. Desikan, K. V., and Venkataramaniah, H. N. A modified method of harvesting *M. leprae* from foot pads of mice. Lepr. India **48** (1976) 157-162.
11. Draper, P., and D'Arcy Hart, P. Phagosomes, lysosomes and mycobacteria: cellular and microbial aspects. *In:* Mononuclear Phagocytes in Immunity, Infection and Pathology. R. van Furth, ed., London: Blackwell Scientific Publishing Co., 1975, pp 575-594.
12. Edwards, R. P., and Draper, G. J. The effect of antibacterial drugs on the ultrastructure of *Mycobacterium leprae* in human skin. Lepr. Rev. **43** (1972) 173-180.
13. Fisher, C. A., and Barksdale, L. Cytochemical reactions of human leprosy bacilli and mycobacteria: ultrastructural implications. J. Bacteriol. **113** (1973) 1389-1399.
14. Harada, K., Gidoh, S., and Tsutsumi, S. Staining mycobacteria with carbolfuchsin: properties of solution prepared with different samples of basic fuchsin. Microsc. Acta **78** (1976) 21-27.

15. KARAT, A. B. A., SAMUEL, I., ALBERT, R., and KUMAR, A. S. J. Experiments in cultivation of *M. leprae* in monkeys and in foot pads of mice: an interim report of six years' study. Lepr. India **45** (1973) 138-147.
16. KATO, L. and BERTHIAUME, L. Viable, non-stainable *Mycobacterium leprae* and *lepraemurium*. Widely circulated communication, May 1973.
17. MOHYSEN, A. M. and ALEMAYEHU, W. Application of Nyka's method for the staining of mycobacteria in leprous skin sections. Acta Pathol. Microbiol. Scand. Sect. A Pathol. **81** (1973) 71-74.
18. NAKAMURA, K. and HISAI, S. Multiplication of *M. leprae* in the hind foot pad of golden hamsters. A comparison with the results of *M. leprae* in the mouse foot pad. Lepro **38** (1969) 147-152.
19. NYKA, W. Influence of oxidation and reduction on the acid-fastness of mycobacteria. Infect. Immun. **4** (1971) 513-515.
20. NYKA, W. Studies on *Mycobacterium tuberculosis* in lesions of the human lungs. A new method of staining tubercle bacilli in tissue sections. Am. Rev. Respir. Dis. **88** (1963) 670-679.
21. OZAWA, T. and KOBAYASHI, K. On the solid ratio of *M. leprae*. Widely circulated communication, September-November 1973.
22. PROCEEDINGS OF THE WORKSHOP ON LEPROSY CHEMOTHERAPY, National Institutes of Health, October 26, 1975. Widely circulated communication, December 1975-February 1976.
23. REES, R. J. W. and VALENTINE, R. C. Application of quantitative electron microscopy to the study of *M. lepraemurium* and *M. leprae*. *In:* Leprosy in Theory and Practice. R. G. Cochrane and T. F. Davey, eds., Baltimore: Williams and Wilkins Co., 1964, pp 26-35.
24. REES, R. J. W. and VALENTINE, R. C. The appearance of dead leprosy bacilli by light and electron microscopy. Int. J. Lepr. **30** (1962) 1-9.
25. REES, R. J. W. and WATERS, M. F. R. Applicability of experimental murine leprosy to the study of human leprosy. *In:* The Pathogenesis of Leprosy. G. E. W. Wolstenholme and M. O'Connor, eds., Boston: Little, Brown & Co., 1963, pp 39-60.
26. REICH, C. Personal communication.
27. SHEPARD, C. C. The experimental disease that follows the injection of human leprosy bacilli into foot pads of mice. J. Exp. Med. **112** (1960) 445-454.
28. TSUTSUMI, S., SAKAMOTO, Y., NAKAMURA, K., NAKAMURA, K., HISAI, S. and KASHIMA, E. Studies on the improvement of antileprous drugs. III. The inhibitory effect of some drugs on the growth of leprosy bacilli in the foot pads of mice (Part I). Lepro **39** (1970) 33-47.
29. WILLIAMS, T. W., JR., MOTT, P. D., WERTLAKE, P. T., RUBIO, J. B., ADLER, R. C., HILL, G. J., SUAREZ, G. P. and KNIGHT, V. Leprosy research at the National Institutes of Health. Experience with B663 in the treatment of leprosy. Int. J. Lepr. **33** (1965) 767-777.