# THE CONCENTRATION OF *M. LEPRAE* IN CURRENTLY AVAILABLE LEPROMINS[1]

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This report deals with the concentrations of *Mycobacterium leprae* in 16 lepromins, which were kindly made available to us by friends and associates. Since lepromin is employed to assay Fernandez and Mitsuda reactivity to antigens from *M. leprae,* judgments concerning prognosis in individual patients and resistance in populations are drawn from the readings obtained.

If the results of the Mitsuda reaction are to be reproducible, and comparisons valid in various parts of the world, it is important that all lepromins should contain adequate and reproducible concentrations of bacillary bodies.

The purpose of this study has been three-fold:

1. To simplify methods for counting *M. leprae* in lepromin in order to make them feasible in other laboratories.
2. To learn whether the bacillary content of available lepromins is sufficiently uniform to justify confidence in existing data on Mitsuda reactivity.
3. To make suggestions regarding feasible concentrations of *M. leprae*/cc. of lepromin. For this purpose, one must consider: (a) the numbers of bacilli available in lepromas, and (b) existing data on the concentrations desired for the Mitsuda reaction.

## METHODS

*Lepromins*: Requests for 5 cc. of each of two successive lots of lepromin were mailed to investigators thought to be making and using lepromin. The respondents were supplied with kits for mailing the samples and with questionnaires on which to record the methods employed for collecting lepromatous tissues and for preparation of the lepromins submitted. Each sample was transferred to a 20 cc. rubber-stoppered serum vial (containing two 3 mm. glass beads to facilitate resuspension of sediments) and stored by refrigeration. The lepromins were processed in group for uniformity of procedures, one previously counted lot being included with new lots as a control.

The method finally adopted for estimating the concentrations of *M. leprae* in lepromins will be described in an accompanying paper. It included three improvements of the original methods of Hanks ([3]). These were (a) addition of the diluent solution prior to dispersion of the declumped bacilli, (b) a simple, rapid method of transferring

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replicate samples to glass slides, by means of pin heads, and (c) elimination of the usual calibrations of the areas of films on slides and of the optical fields of microscopes.

Satisfactory dispersions of bacilli and agreements of counts were observed, except in lepromins containing relatively large particles of tissue. Since this defect prevents the drawing of uniform samples into syringes and the injection of uniform aliquots into a series of persons, it is a problem which must be solved by the elimination of tissue particles during the preparation of lepromin. This problem is now under investigation.

## RESULTS

Table 1 shows counts in the 16 samples of lepromin in which bacilli could be found. The highest count is 760 times higher than that of the lowest. In two instances two successive lots of lepromin from single sources differed by 100 and 200 times in bacterial counts.

A survey of reported methods of preparing lepromin indicates that the foremost causes of trouble are as follows: insufficient attention to established methods of selecting and pooling lepromas, excessive grinding of tissues and consequent disruption of bacilli, over-dilution, and failure to make adequate microscopic and clinical comparisons between successive lots.

TABLE 1. *Rank of concentrations of* M. leprae *in 16 lepromins; average and median counts.*

| Number of bacilli/cc. ($\times 10^6$) | Apparent sub-groups | If the median count is regarded as $25 \times 10^6$ | |
|---|---|---|---|
| | | ± 10% | ± 15% |
| 228 | | | |
| 218 | (3) | | |
| 218 | | | |
| 98 | | | |
| 62 | (2) | | |
| 37 | | | |
| 33 | | | |
| 25 | | | |
| (M) | (6) | 22-28 | 21-29 |
| 25 | | | |
| 25 | | | |
| 16 | | | |
| 2 | | | |
| 1 | | | |
| 0.9 | (5) | | |
| 0.4 | | | |
| 0.3 | | | |

62 = Average.[1]

[1] The average and the median values (M) are low in part because of known over-dilution of some samples submitted. E.g., the count of $37 \times 10^6$ = a 1:5 dilution of one lepromin. At tissue 4%, the counts of 0.4 and 0.3 would be 4 and 3 million, respectively. Incorporation of the indicated corrections, however, would not bring the average and median values into line with pre-existing data (see Table 2).

TABLE 2. *Patterns of bacillary concentration in lepromas and lepromins.*

| Lepromas or lepromins examined | | (Refs) | Bacilli/cc ($\times 10^6$) | Average number | Approximate median numbers assigned | Discrepancies between high and low counts |
|---|---|---|---|---|---|---|
| Individual lepromas | | (2) | 210[1] 124 | | | |
| Individual lepromins: | JM | (3) | 40 | | | |
| | W | | 38 | 76 | 39 | 11× |
| | JH | | 24 | | | |
| | | | 19 | | | |
| | | | 555[2] | | | |
| | | | 185 | 267 | 185 | 9× |
| | | | 60 | | | |
| Pooled lepromins: | | | | | | |
| Abe *et al* | | (4) | 220 | | | |
| Wade, Mw | | | 150 | 185 | 185 | 1.5× |
| Mabalay #1 | | | 213 | | | |
| Mabalay #2 | | | 208 | | | |
| Wade | | | 164 | 186 | 186 | 1.3× |
| Maeda *et al* | | (4) | 158 | | | |
| Averages of above: | | | | 179 | 151 | |
| Sixteen samples in present study: | | | | 62 | 25 | 760× |

[1] Counts adjusted to tissue 3 per cent. These counts were made in the pre-sulfone era. They are thought to be low for two reasons: the bacilli were not declumped (clumps are lost from films more readily than single bacilli); fixation of films to slides was not as carefully controlled as at present.

[2] Counts adjusted to tissue 3 per cent.

The data in Table 2 are assembled to demonstrate three points.

1. The first two sections in the table show that the bacterial concentrations in first-quality, individual lepromas tend to differ by at least 10 times. The necessity of pooling at least five or six lepromas is inescapable.
2. The next two sections in the table show that when lepromas are carefully selected and pooled the bacterial counts fall in a fairly narrow range. In the studies illustrated, comparative skin tests demonstrated only small differences in Mitsuda reactivities to 220 and to 150 $\times$ $10^6$ bacilli/cc. of lepromin.
3. At tissue 3-5 per cent, the bacterial concentrations in the first four sections of the table average 179 $\times$ $10^6$.

## DISCUSSION

Extreme variations in the concentrations of *M. leprae* in currently available lepromins raise serious questions regarding the comparative value of existing data on Mitsuda reactions. If *M. leprae* is to be re-

garded as the major factor inducing Mitsuda reactions, it is urgent that steps be taken to standardize bacterial concentrations.

As a result of careful investigations of bacterial concentrations in relation to early and late skin reactions, the Japanese workers cited in Table 2 ([1, 4]) have adopted 160 million bacilli per cc. of lepromin.

Skilled laboratory workers are not always available where lepromas may be obtained. Our laboratory, therefore, is prepared to cooperate with all laboratories that will undertake to establish counting and pooling procedures permitting re-distribution of a standard product on an institutional or regional basis.

## SUMMARY AND CONCLUSIONS

1. This survey of concentrations of *M. leprae* in lepromins available to us has been combined with a re-study of methods for estimating the numbers of bacilli and the collection of information on the preparation of lepromin.

2. Enumerations in 16 valid samples of lepromin from 10 sources revealed that: (a) the richest lepromins contained 760 times more bacilli than the poorest; (b) two lots from individual investigators may differ by as much as 100 or 200 times; (c) the average in this series was only 35 per cent of those in other base-line studies.

3. A survey of reported methods indicates that foremost problems in the preparation of lepromins are: (a) insufficient attention to established methods of selecting and pooling lepromas, (b) excessive grinding of tissues and consequent disruption of bacilli, (c) over-dilution, and (d) failures to make adequate microscopic and clinical comparisons between successive lots. This is taken to mean that the preparation of lepromin has been left too long for persons who are occupied with other duties, and that more assistance is needed from national and regional laboratories.

4. Assembled data indicate that an international standard containing 3-5 per cent tissue and $160 \times 10^6$ bacilli per cc. should be attainable by proper care in pooling of lepromas. An offer has been made to cooperate with laboratories interested in achieving this standard.

## RESUMEN

1. Esta investigación de concentraciones de *M. leprae* en leprominas disponibles ha sido combinada con un re-estudio de los métodos para la estimación del número de bacilos y la recolección de información en la preparación de lepromina.

2. Enumeraciones en 16 ejemplares válidos de lepromina de 10 fuentes, revelaron que: (a) las leprominas mas ricas contenian 760 veces mas bacilos que las mas pobres; (b) dos lotes de investigadores individuales pueden diferir tanto como 100 a 200 veces; (c) el término medio en estas series fué solamente el 35% de aquellos en otra línea básica de estudio.

3. Una investigación de métodos comunicados indica que los principales problemas en la preparación de leprominas son: (a) insuficiente atención a métodos establecidos de selección y pool de lepromas; (b) excesiva pulverización de tejidos y consiguiente disrupción de bacilos; (c) demasiada dilución, y (d) fallas en las comparaciones

adecuadas microscópicas y clínicas entre los sucesivos lotes. Esto es dicho para significar que la preparación de lepromina ha sido dejada demasiado tiempo a personas que estan ocupadas con otros tareas, y que mayor asistencia es necesaria desde los laboratorios nacionales y regionales.

4. Los datos juntados indican que un standard internacional conteniendo 3-5% de tejido, y $160 \times 10^6$ bacilos por cc. debe ser obtenible con cuidado apropiado al pool de los lepromas. Una oferta ha sido hecha para cooperar con los laboratorios interesados en lograr estos standards.


## RESUMÉ

1. Les auteurs ont profité de cette étude systématique de la concentration de *M. leprae* dans des lépromines mises à leur disposition pour procéder à un nouvel examen des méthodes utilisées pour estimer le nombre des bacilles. Ils ont également analysé la manière dont les informations se rapportant à la préparation de la lépromine ont été réunies.

2. L'énumération des bacilles dans 16 échantillons valables de lépromine provenant de 10 origines différentes a révélé les faits suivants: (a) les lépromines les plus riches contiennent 760 plus de bacilles que les plus pauvres; (b) deux lots de même origine peuvent différer dans une proportion de 100 à 200; (c) la moyenne du nombre de bacilles dans cette série de lépromines n'a été que de 35% inférieure à celles trouvées dans d'autres études de reference.

3. Une revue des méthodes de préparation telles qu'elles ont été rapportées permet de mettre en évidence le fait que les problèmes rencontrés lors de la préparation de la lépromine sont les suivants: (a) trop peu d'attention est accordée aux méthodes établies pour la sélection et le rassemblement des lépromes; (b) le broyage des tissus est excessif, entraînant une destruction des bacilles; (c) la dilution est trop grande; (d) on néglige de procéder à des comparaisons microscopiques et cliniques d'un lot au suivant.

Ces observations donnent à penser que la préparation de la lépromine a été trop longtemps laissées entre les mains de personnes qui sont absorbées par d'autres responsabilités, et suggèrent que plus d'assistance devrait être fournie sur ce plan par des laboratoires nationaux ou régionaux.

4. Les données réunies montrent qu'un standard international, à savoir une lépromine contenant 3 à 5% de tissu et $160 \times 10^6$ bacilles par cc., devrait pouvoir être obtenue, si l'on prend soin de mêler des lépromes de diverses origines. Les auteurs proposent de collaborer avec des laboratoires interessés à préparer des lépromines répondant à ce standard.


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