# ENUMERATION OF *MYCOBACTERIUM LEPRAE* FOR THE STANDARDIZATION OF LEPROMIN

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It is recognized that "standardization" of the regular Hayashi-Mitsuda lepromin by comparison of smears of old and new stocks, for rough approximation of the bacillus content, is but an expedient necessitated by lack of methods for making accurate bacillus counts. The preparation of declumped suspensions of tubercle bacilli and *M. leprae* for microscopic studies has been routine in this laboratory for many years. Studies on the centrifugal purification of declumped suspensions of *M. leprae* had defined conditions which permit maximal yields of single bacilli and convenient enumerations. Since modification of these methods has recently been shown to permit convenient estimations of the numbers of bacilli in lepromin, a description of the tolerances and limitations of the principle employed, and of the adopted method, may be useful. This is not intended as a new technique for preparing lepromin for clinical use.

## MATERIALS AND METHODS

Two kinds of material have been studied. The first consisted of 5 and 10 per cent tissue suspensions of lepromas, fresh, heated, Chryochem-dehydrated, or glycerinated (refrigerated). These suspensions were prepared by mincing the tissue, then grinding by hand or in a glass mill[2] while diluting in water, saline or phenolized saline (1). They were studied in some instances as crude suspensions and in others after light centrifugal clarification. After flowing known volume-ratios of chloroform beneath these homogenates, then shaking the tightly stoppered tubes end to end as vigorously as possible through an arc of 90° for different intervals, the distributions and numbers of *M. leprae* were ascertained by microscopic observation and quantitative counts.

The success in liberating and recovering single bacilli was determined after diluting these suspensions to 10 times their original volume, centrifuging 3 minutes at 130G (fluid depths = 55 mm.), and preparing standard films from the supernates. If maximal recoveries of bacilli were of interest, sediments from the satisfactory preparations were shaken for 2 minutes prior to each of two rewashings in experimental diluents. By totaling the average number of bacilli per field in each of the three successive supernates, recovery factors were established.

The second class of material consisted of prepared lepromins, from which the gross particles of tissue components already had been removed. Employing the optimal conditions defined in the foregoing work, the following procedure was developed.

[1] With the assistance of John T. Moore.

[2] Such homogenizers are now widely available, e.g., from Fisher Scientific Co., Greenwich & Morton Sts., New York 16; Macalaster Bicknell Co., Cambridge, Mass.

1. Flow 0.02 cc. of chloroform beneath a 0.2 cc. aliquot of well-shaken lepromin in a 3 cc. glass-stoppered tube (e.g. Kimball #45152). Shake vigorously for 3 minutes.[3]

2. Add 1 per cent serum in 0.5 per cent phenol water to rinse the walls of the tube and make the final tissue concentration equivalent to 1 per cent.

3. Stir each sample with a 0.7mm loop [4] which, when picked horizontally through the surface of the liquid, transfers 0.00022 cc. aliquots to a glass slide. These small droplets are allowed to spread to their natural limits while drying. Six or seven such microspots of each sample are arranged vertically (slide laid on graph paper); as many as eight vertical rows (samples) can be filmed on one slide.

4. Expose the dried films to formalin fumes for 3 minutes by placing the slide in the slot of a Coplin jar containing 0.7 cc. formalin (formaldehyde 37-40%) and sealed with a screw cap or inverted #6 rubber stopper.

5. Restrict enumerations to films which have perfect rims and uniform diameters when viewed under the low-power objective. With the oil-immersion objective record the number of bacilli seen in a standard number of fields while moving completely across the diameter of each film. (Having chosen the number of fields which are available for a given working combination, one may wish to traverse slightly above or below the maximal diameter of some films in order always to duplicate this standard distance.) The numbers of bacilli seen in three films of each sample provide an index of the consistency of sampling and of averages. The usual calculations based upon the volume of sample, the area over which it spreads, and area viewed by the oil-immersion objective, permit converting the counts to bacilli per cubic centimeter of 1 per cent lepromin.

Precautions and refinements which have been described elsewhere include: inspection rather than counts to ensure reproducibility of films ([3, 4, 5]), proper differentiation after staining ([4]), reliance on the numerical distribution of bacteria in the films in order to record the counts in each field and to facilitate statistical analysis ([5]). For this purpose, we have found it useful to double the size of "field" and to pool the counts across two films of each sample before making comparison with the values in other samples.

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[3] An alternative method, which saved much labor, was to combine the lepromin and chloroform in 10 mm. lusteroid tubes, which were packed into a 10KC Raytheon sonic oscillator (water depth around tubes = 15 mm.) during vibration at 150 milliamperes for one minute. The lusteroid tubes (5LT) are obtainable from the Lourdes Instrument Corp., 53rd Street and First Avenue, Brooklyn 32, N. Y.

[4] Commercial source: A. H. Thomas #8304G, 31 gauge platinum-iridium (15%). Preferred: 28 gauge, prepared for us by Howe & French Co., 99 Broad St., Boston. Due to the larger gauge of wire, this loop deposits 0.0003 cc. and the films tend to present 20 rather than 15 blocks or fields/diameter.

EXPERIMENTAL RESULTS

*Definition of optima.*—When determining the concentration of chloroform required for dispersing the bacilli it was found that, although 1 per cent or 3 per cent by volume initiated the unravelling of clumps and globi during one minute of shaking, the bacilli could not be freed from the matrix in which they had been bound. Six per cent of chloroform was found to be a threshold concentration. It usually permitted uniform distribution of the bacilli in turbid mycobacterial suspensions and in 5 per cent tissue homogenates during three minutes of shaking. In 10 per cent tissue suspensions, 10 per cent chloroform assured effective action and caused no difficulties. Chloroform 20 and 50 per cent decreased the total numbers of bacilli and increased the acid-fast debris, clouded the suspensions with coagulated proteins, and aggregated most of the intact bacilli into the bulky sediments if the tubes were allowed to stand.

After these suspensions had been diluted to 10 times their original volumes and centrifuged lightly, enumerations of bacilli in the supernates were consistent with the foregoing observations. It was only after treatment with 10 per cent chloroform that the supernates contained maximal numbers of single bacilli.

The numbers and states of the bacilli in the sediments were as follows: 6 per cent chloroform, the majority of the bacilli as small clumps and singles, except within fragments of tissue which had not been homogenized; 10 per cent chloroform, practically all bacilli as singles, though many were not yet liberated from the tissue residues; 20 and 50 per cent chloroform, much acid-fast bacillus debris and excessive protein precipitate which trapped bacilli of poor staining quality.

The differing capacities of several diluents to liberate declumped *M. leprae* from tissue components were demonstrated in studies on maximal recovery of such bacilli into supernates. Of conveniently available reagents, the differences between water or saline and fresh or boiled 2 per cent serum in water were the more instructive. After shaking 10 per cent tissue suspensions with 10 per cent chloroform for three minutes, the dilutions to 1 per cent tissue (1% chloroform) were made in aqueous media with and without serum, and the suspensions were clarified by light centrifugation to yield supernates I and I′. The sediments were shaken with fresh aliquots of the respective diluents, brought to volume, etc., so that the respective supernates II and III were obtained. After enumerating the bacilli recovered in the successive supernates, typical recovery factors were water or saline compared with 2 per cent serum, 1:1.6.

To summarize, the most successful declumping, dispersion and recovery of *M. leprae* was accomplished by shaking 10 per cent tissue suspensions with 10 per cent chloroform and by diluting to 1 per cent tissue in the presence of fresh or heated 2 per cent serum.

*Numbers of mycobacteria in lepromin.*—In Table 1 are shown: comparisons between the alternate methods employed for shaking lepromin with chloroform; the average numbers of bacilli per square field and the probable numbers per cubic centimeter (1% tissue) in three lepromins and one "BCG lepromin" described; also the concentrations of tissue if these lepromins were adjusted to similar numbers of bacilli.

TABLE 1.—*Comparison of methods; average number of* M. leprae *per cc. of lepromin (1% tissue).*

| Lepromin No. | Average bacilli per square | | | | Average bacilli per sample[a] | | | | Per cent tissue for constant numbers of bacilli |
|---|---|---|---|---|---|---|---|---|---|
| | A. Hand shaken 3 min. | | | B. Sonic shaken 1 min. | | Bac/ sq. | Bac/cc x10[7] | Ratios | |
| 1. Wade | 2.6 | 3.0 | 2.7 | 2.0 2.5 | | 2.6 | 6.5 | 1.0 | 3.0 |
| 2. JH[b] | 0.9 | 0.6 | 0.7 | 0.8 0.9 | | 0.8 | 2.0 | 0.3 1.0 | 9.9 |
| 3. JM[b] | 7.1 | 6.5 | 7.1 | 7.3 9.0 | | 7.4 | 18.5 | 2.8 9.3 | 1.1 |
| 4. BCG[c] | 2.7 | 2.5 | 2.7 | 2.5 2.6 | | 2.6 | 6.5 | 1.0 | 3.0 |

[a] Based on the five films counted for each sample. It was not necessary to calculate the significance of differences between samples #1 and 2 or #1 and 3 by the method described (5). In each of the sets of 20 field-by-field comparisons, no overlapping of values occurred.

[b] Prepared from individual lepromas kindly supplied in 1947 by Dr. George L. Fite and in 1953 by Dr. Rolla R. Wollcott of the U. S. Public Health Service Hospital, Carville, La.

[c] A BCG suspension processed by Wade's method in the presence of 3 per cent human spleen, to prepare a "lepromin" matching that of Wade.

The total numbers of bacilli per film diameter (20 fields of 16 squares each) ranged from 259 in Sample 2 to 2,417 in Sample 3. The distributions of bacilli within films were appreciably less uniform after shaking by hand than after sonic vibration. There were, however, no significant differences between results produced by the two methods. Comparisons between diluting the chloroform-treated suspensions in water and in 1 per cent serum showed that the latter delineated the margins of the stained films more clearly and afforded an appreciable improvement in the reliability of the counts.

Since Wade's lepromin was prepared from pooled lepromas ([8]), the counts in it may afford a clue to the concentrations of *M. leprae* now employed to elicit the Mitsuda reaction. The numbers of bacilli in the Nos. 2 and 3 lepromins substantiate previous observations that natural concentrations of bacilli may differ in the order of 10 times ([2]) and emphasize the necessity of pooling lepromas as the first step in preparing for standardization of ordinary lepromins.

Successive counts during the preparation of the "BCG lepromin" substantiated the earlier evidence that chloroform declumping can be conducted without destruction of mycobacteria. Measurable clearing of the carefully-prepared BCG suspension (turbidity = nephelometer 8) occurred during autoclaving at 10 pounds steam pressure for 10 minutes. Nevertheless, the microscopic counts did not decrease after this treatment, or during subsequent declumping of the autoclaved suspension alone and of the same suspension diluted in the autoclaved suspension of human spleen.

## DISCUSSION

Although the procedures outlined in the first part of the Results section of this report permit efficient recoveries of declumped and purified *M. leprae* for microscopic study, they are not proposed as a basis for the preparation of purified leprosy antigens from heated lepromas. Mycobacteria heated in such circumstances are readily permeated by human tissue proteins. Mechanical washing and/or enzyme treatment seem unlikely to free such bacilli of human components.

The fractionation data are included primarily because successful use of chloroform as a declumping agent depends upon adherence to relatively narrow tolerances, which are determined by the properties of the chloroform, the mycobacteria, and the tissue lipids and proteins. Of the many solvents used, namely ethyl ether, petrol ether, xylene, benzene, toluene, carbon tetrachloride and chloroform, the last is the most effective declumping and "concentrating" agent (6). Its major liabilities are: coagulating action on proteins, and superior capacity to extract from mycobacteria those lipids (chiefly waxes) which are particularly important as adjuvants and as components which bind the Ziehl-Neelsen complex during staining. Its action as a concentrator disappears whenever declumped suspensions are diluted to a point where no free solvent remains. Since the solubility of chloroform in water at 22° is 0.6 per cent, a 10-times dilution of systems treated with 6 per cent chloroform obviates the concentrator effect.

Since chloroform is lost to the atmosphere in shaken systems and is bound by mycobacterial and tissue lipids, 10 per cent by volume assures declumping without appreciable modification of tissue proteins and without danger of a concentrator effect following dilution. The use of 20-50 per cent chloroform in lepromin suspensions is inadvisable for three reasons: destruction of mycobacteria, coagulation of proteins (bulky sediments), and concentration rather than dispersion of the mycobacteria.

By working within the tolerances defined above, it has been demonstrated that chloroform declumping did not lower the microscopic counts in a BCG suspension which had been autoclaved at 10 pounds steam pressure for 10 minutes; also that the numbers of *M. leprae* in

ordinary lepromins can be estimated and compared by simple procedures. Since standardization of the numbers of bacilli in lepromin seems feasible, the reasons for continuing to pool as many lepromas as possible must be emphasized. (1) The possibly different antigenic qualities of the bacilli in different lepromas has not been assayed. (2) There is increasing evidence that autoclaved human tissue may be a reactive component in the Mitsuda test ([7]). Even with pooling, the concentration of tissue components may not readily be standardized. It previously has been shown ([2]), and is here substantiated, that the numbers of bacilli per gram of normal-appearing nodules may vary over a 10-fold range. Among the previous determinations the average numbers were twice the median number. Simple adjustment of bacterial numbers in the nonpooled lepromins 2 and 3 (Table 1) would cause a 9-times difference in the concentrations of autoclaved human tissue.

## SUMMARY AND CONCLUSIONS

1. Optimal conditions for declumping and dispersing mycobacteria in turbid suspensions and tissue homogenates for microscopic purposes have been defined.

2. Shaking 10 per cent leproma suspensions, or ordinary lepromins with tissue in 3-5 per cent concentrations, for 3 minutes with 10 per cent chloroform by volume releases *M. leprae* from clumps and globi. Further dilution to 1 per cent tissue, with shaking in the presence of 1 per cent serum, permits the preparation of stained films containing suitable numbers of uniformly distributed bacilli. Methods for microscopic enumeration and for comparing the concentrations of bacilli have been given.

3. It has again been shown that the numbers of *M. leprae* in lepromas may vary over a 10-fold range.

4. Even with simple methods for counting and adjusting the bacterial content of lepromin, the pooling of lepromas must be continued in order to obtain reasonable uniformity in the quality of the bacilli and the concentration of autoclaved human tissue components.

## RESUMEN Y CONCLUSIONES

1. Se han definido las condiciones óptimas para desaglutinar y dispersar micobacterias en suspensiones turbias y homogeneatos de tejidos destinados a fines microscópicos.

2. El sacudimiento de suspensiones de lepromas al 10 por ciento, o de lepromina corriente con tejido a concentraciones de 3-5 por ciento, durante 3 minutos con 10 por ciento de cloroformo por volumen, desprende los *M. leprae* de los grumos y globos. La dilución ulterior a 1 por ciento de tejido, con sacudimiento en presencia de 1 por ciento de suero, permite la preparación de películas teñidas que contienen cantidades adecuadas de bacilos distribuídos uniformemente. Se desriben métodas para la enumeración microscópica y para compara las concentraciones de bacilos.

3. Se ha demostrado de nuevo que las cantidades de *M. leprae* en los lepromas pueden ser hasta 10 veces mayores en unos que en otros.

4. Aun disponiendo de métodos sencillos para contar y ajustar el contenido bacteriano de la lepromina, hay que continuar la mezcla de lepromas para obtener una uniformidad razonable en la calidad de los bacilos y en la concentración de los componentes de tejido humano tratados al autoclave.

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