

Standardizable Lepromins Yielding Uniform Concentration of *M. leprae* per Skin Site^{1, 2}

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Three difficulties are created by current methods of preparing lepromin. First, during manual grinding, variable proportions of the bacilli are broken and dispersed. This is undesirable as long as counting remains the primary method of standardization. Second, much of the connective tissue is reduced to suspensions that tend to flocculate. Third, it is not practical to exclude flocculating components and visible tissue particles by means of filtration. Tuma⁽¹⁾ also has emphasized these objections.

Furthermore, intact mycobacteria occur in a great variety of clump sizes, which vary from lot to lot of lepromin. These variations probably influence the rate at which Mitsuda reactions can develop.

It has been shown recently⁽⁷⁾ that the flocculating components and visible tissue particles, and also the largest clumps of bacilli, settle in syringes during the time required for 8-10 intradermal injections. This causes marked variation in the amount and kind of material in successive doses injected from the same syringe.

If lepromin is to be standardizable, it seems evident that a radically new approach is required. Such an approach requires methods that can be standardized conveniently at each step.

Advantage has been taken of the fact that human lepromas resemble geologic formations comprised of soft and hard strata, with regions rich in heavily infected cells, and streaks and bundles of connective tissue. If subjected to stress by appropriate cutting and shearing forces, such structures should cleave along the "faults," i.e., areas rich in cells and bacilli, liberating the majority of the bacilli without excessive dispersion of the tougher masses of connective tissue.

The work here reported shows that the desired mechanical forces are provided by electric blenders within 30-60 seconds. This action minimizes the disruption of bacilli and the dispersion of connective tissues, and is readily standardized. Sedimentation, with successive recoveries of supernates from the resuspended tissue residues, seems to be the most effective method for separating a uniform range of bacterial clump sizes from the floccules and particles of tissue. Since the largest clumps of bacilli remain in the rinsed tissue residues, reliable microscopic counts can be made without chloroform declumping.⁴

MATERIALS

Blenders.⁵ (a) A Waring blender (WB), modified as follows, was used: 1. The work-

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⁴Chloroform declumping is inconvenient and a potential source of error in inexperienced hands. The minimal effective concentration of chloroform is proportional to the concentrations of tissue components and of mycobacteria in each lepromin suspension. Excess chloroform increases the flocculation of tissue components and reduces the acid-fastness of the bacilli that are the basis for standardization.

⁵Sources of blenders: (a) WB—Arthur H. Thomas Co., P.O. Box 779, Philadelphia, Pennsylvania

ing volume of a 360 ml. semimicro monel metal cup was restricted to ± 75 ml. by pressing a No. 9 rubber stopper tightly against the flaring shoulders of the upper part of the shell.

2. The inadequate blades supplied with this cup were replaced with full length blades. Two of the tips were bent upright to slice large pieces of tissue, and two of the blades were bent downward so as nearly to touch the bottom of the cup.

The blades were not adequately sharpened in Experiment 101. Thereafter, all blades were kept sharpened so that the leading edges of the blades could not be seen with a 3x magnifying glass.

(b) A Sorvall Omni-mix (SvO) also was employed. This machine was used with new standard blades in a cup having a working range from 10 to 50 ml.

Lepromas. Since the bacterial concentrations in lepromatous tissues may differ by more than 1 log^(5, 6), and the quality of bacilli is variable, tissues from at least five lepromas must be included, even in small batches of lepromin.

As recommended by Wade⁽¹³⁾, the lepromatous lesions from each source⁶ were freed from epidermal, adipose, and connective tissues by dissection, and examined semiquantitatively for bacterial content.

In order to compare modifications of the basic approach, each tissue was subdivided into two or three parts, to provide strictly comparable sublots of weighed tissue. Differences in procedure were compared by processing two or three sublots simultaneously.

19105, U.S.A. The specifications required are as follows: No. 4281-A—Waring blender, single speed drive and 1,000 ml. glass jar. No. 4282-F—Semimicro size (360 ml.) Monel metal jar. The stubby blades supplied should be replaced. No. 4282-I—Full length blades, to be modified and used in the above. (b) SvO—Ivan Sorvall, Inc., P.O. Box 230, Norwalk, Connecticut 06852, U.S.A. The specifications required are as follows: No. OM-1150—Sorvall Omni-mixer, Basic unit, 115 volts, AC, 60 cycles. No. OM-1961ST—Stainless steel chamber assembly, 50 ml. capacity. No. OM-1064ST—4 inch Rotor-knife assembly.

⁶Dr. C. V. Reich, Director of the Leonard Wood Memorial-Eversley Childs Sanitarium Leprosy Research Laboratory in Cebu, Philippines, kindly supplied 4.8 gm. of cutaneous lepromatous tissues (carlobes, etc.), which had been boiled for one

Concentrated stock solutions. Phenol 5% = 10x the final concentration; NaCl 9% = 20x; sodium lauryl sulfate (LS) 2% = 100x. The volumes required per liter of lepromin are: phenol 5%, 100 ml.; NaCl 9%, 50 ml.; LS 2%, 5 ml.

Working solutions. The working solutions are prepared by combining appropriate volumes of the stock solutions and by dilution.

Phenol saline (NaCl). The phenol 5 per cent and the NaCl 9 per cent are combined with 850 ml. of distilled water to yield one liter of phenol 0.5 per cent, NaCl 0.45 per cent.⁷ For simplicity this solution will often be mentioned as "NaCl" or "saline."

One half of this solution (500 ml.) is converted to phenol saline plus LS (NaCl, LS) by adding the 5 ml. of 2 per cent LS. Supers I and II wet clean glassware, while Supers III and IV do not (see definition of supers, pp. 70-72). Tween 80 cannot be used in the presence of phenol. Sodium lauryl sulfate in 0.02 per cent concentration, therefore, was included in the "saline" used to collect the last two supers.

Dewaxed glassware. Glassware that has been used for mycobacterial products absorbs waxes that cannot be removed by the usual washing. The waxy films absorb mycobacterial cells⁽⁴⁾. Methods for the removal of silicones are useful for removing the mycobacterial waxes.

Enough 5N NaOH is added to wet all internal surfaces. The vessel is warmed over a flame until it is as warm as the hands will tolerate. The solution is rinsed over the entire surface several times.

To prevent reaccumulation of miscellaneous lipids, rinsing must be limited. The vessel is inverted in a Petri dish or over the drain in a sink for 3-5 minutes. It is then

hour and then preserved in 0.5 per cent phenol saline. Dr. J. A. Cap, of the World Health Organization, supplied 12.8 gm. of subcutaneous lepromas. Dr. C. H. Binford, Medical Director of the Leonard Wood Memorial, made available smaller amounts of lepromatous tissues that had become outdated for studies on transmission to animals.

⁷The usual phenol (0.5% in NaCl 0.9%) is hypertonic and promotes flocculation of denatured tissue components. Since 0.5 per cent phenol is slightly more than one-half iso-osmolar, the actual NaCl requirement for isotonicity is 0.4 per cent.

filled to about 20 per cent of volume with tap or distilled water, shaken, and drained again. It is rinsed only once more with distilled water and allowed to drain dry.

Sedimentation chambers. Gravity separations must be conducted in columns of standard, reasonable heights. Small bacterial clumps situated near the bottom of tall columns settle to the bottom long before larger tissue fragments that are situated near the top.

In ordinary glasswares, the volumes contained at a depth of 50 mm. are as follows: (1) screw cap tubes: 13 mm., 5 ml.; 16 mm., 7.6 ml.; 20 mm., 11.5 ml.; (2) 50 ml. centrifuge tubes, 34 ml.; (3) bottles and jars: (a) Pyrex 60 ml. bottles, 50 ml.; (b) Coplin jars, 56 ml.; (c) French square bottles, 70 ml.

Fifty ml. centrifuge tubes and 60 ml. Pyrex bottles (containing two or three beads of 4 mm. diameter) were convenient for volumes used in the present experiments. The necessary pieces of glassware were marked at two levels: \pm 50 mm. and at 40 per cent of that volume. The lower marking indicates when 60 per cent of a system has been recovered as supernate devoid of visible particles. After each complete settling cycle, saline can be added to the upper mark to initiate further elution and recovery of bacilli.

PROCEDURES

The principles employed in early experiments were: (1) to limit the first blending to 30 seconds; (2) to recover undamaged bacilli by removing three successive supernates during the first settling cycle (Cycle I), and pooling these as "Super I"; (3) to dilute the sediment with NaCl, shaking vigorously, and similarly recovering and pooling "Super II"; and (4) in the same fashion to recover "Super III."

In early experiments the homogenates were allowed to settle in the blenders. A further blending for 30 seconds preceded the collection of Super IV. When a single blender was used to produce successive lots of homogenate, each homogenate was transferred to a sedimentation chamber and not subjected to further blending. Glassware facilitated the complete removal

of nonparticulate supernates, while the marked levels simplified addition of the saline used for further elutions.

To ensure maximal recovery of bacilli, the three sublots of supernate collected during each sedimentation cycle were obtained as follows. All available particle-free supernate was collected after 20 or 30 minutes of settling. In order to free bacilli from tissue fragments, as well as those trapped by sediment, the system was "blended" for 2 seconds or shaken vigorously for 10 seconds, and again allowed to settle for the chosen interval. This process was repeated once more. After these three collections had been pooled as Super I, new saline was added and the system shaken to initiate Cycle II and the collection of Super II. This was repeated to obtain Super III and IV. The volume of each supernate was recorded.

The complete recovery of all obtainable, nonparticulate supernates required: (1) gentle recoveries with a capillary pipette and rubber bulb or cautious siphoning, (2) gradual tilting of the sedimentation chamber, and (3) cessation of siphoning at the moment when small particles could be seen in the stem of the pipette or siphon. Supernates containing visible particles were discharged back into the sedimentation chamber.

Microscopic counts. The concentrations of bacilli in each supernate were determined by the standard method⁽⁹⁾, without chloroform declumping. All samples from an experiment were spotted on a single slide to ensure uniformity of staining, handling, etc. The bacillary concentrations, multiplied by the volume of each supernate, revealed the total number of bacilli obtained by any stage in a procedure. The use and the analysis of such data are shown in the tables. An accompanying note provides a new Table of Conversion Factors⁽⁸⁾.

After experience was gained with the proposed method, major questions were rechecked during the preparation of lepromin batches Nos. 101 and 102. The data are designated as derived from Experiments 101 and 102E.

RESULTS

Shearing of structural elements and liberation of *M. leprae*. Both the Waring blender and the Omni-mix were satisfactory. The Waring blender is recommended on the basis of cost and general usefulness.

The premise that high speed knife blades should liberate the majority of the bacilli while shearing primarily the soft strata in lepromas was substantiated in two ways: (1) by comparing the numbers of bacilli liberated during a first and a final 30 seconds of blending, and (2) by microscopic observations on the tissue residues.

In Table 1, the averages show that about 95 per cent of the total bacilli recovered had been liberated during the first 30 seconds (Supers I + II + III), i.e., long before the majority of visible tissue fragments had been milled. A further blending for 30 seconds (Super IV) yielded only 5 per cent of the total bacilli recovered. This means that only 1/19th as many bacilli were liberated per second.

Table 1 also demonstrates the importance of sharpening the blades in blenders. The effects of the duller blades in the Waring blender in Experiment 101 were: (a) to decrease the primary liberation of bacilli, leaving more to be liberated by the second blending, and (b) to reduce the total number of bacilli recovered.

Microscopic studies demonstrated that only a small proportion of the bacilli remained encased in tissues after the first blending for 30 seconds and the removal of Supers I to III. Smaller numbers of bacilli remained in the residues after the second shearing for 30 seconds. These bacilli were distributed as follows: (1) very modest numbers within tissue particles, and (2) large clumps and globi, which had been liberated but had settled during the 20-30 minute periods of sedimentation.

Concentrations of bacilli in Supers I-IV. Table 2 illustrates calculations for analyzing the number and percentages of liberated bacilli recovered in Supers I-IV. The

TABLE 1. Proportions of bacilli liberated from autoclaved lepromas during first and second blendings for 30 seconds.

Lepromin number	Blendings (in sec.)	Supers	Total bacilli (x10 ⁶) recovered	Per cent of bacilli liberated during	
				1st 30 sec.	2nd 30 sec.
101A ^a	1st = 30 2nd = 30	I-III IV	7,190 490	93.7	6.3
Total			7,680	100.0	
101B ^a	1st = 30 2nd = 30	I-III IV	8,980 280	96.9	3.1
Total			9,260	100.0	
Average %		1st 30 sec. 2nd 30 sec.		95.4	4.6

^a Comparison of two blenders:

A = Waring blender, old blades.

B = Sorvall Omni-mix, new blades.

calculations demonstrate that to resuspend the tissue three times in fresh diluent, each time taking off three supernates, brings recoveries to the point where further work may not be justified. It will be noted that all recoveries in Super IV were low and that this will determine policy in pooling supernates to attain standard counts (see DISCUSSION).

Sedimentation rates. In order to secure maximal recovery of liberated bacilli from the objectionable masses of tissue and globi, one question was whether the visible tissue particles should be permitted to settle for 30 minutes or for 20 minutes before siphoning off the supernates.

Supers II-IV, recovered after 30 minutes of sedimentation (Experiment 101), were devoid of all clumps that would require the use of chloroform prior to counting. Modest numbers of small and medium sized clumps remained in the residues. Supernates recovered after 20 minutes of sedimentation (Experiment 102) contained higher numbers of bacilli and larger bacterial clumps. Because of the higher concentrations of bacilli in the microscopic films, the net result tended to smooth out irregularities in counting data. Sedimentations for 20 minutes, therefore, are recommended in order to recover the maximal concentrations of bacilli compatible with stable suspensions of lepromin.

The results shown in Table 2 demonstrate that policy in respect to Cycle I should differ from that in Cycles II-IV. In Cycle I, both the proportions of homogenate and the percentage of bacilli recovered varied markedly. Low percentage recoveries in Cycle I tend to be followed by higher percentage recoveries in Cycles II and III (see Table 2, Experiment 102E). These effects are attributed to a combination of factors: (1) the softness of the original tissues, (2) the extent of autoclaving (formation of gelatin), and (3) the period of blending. Each of these factors tends to increase the weight of colloids and the viscosity, and to decrease sedimentation rates.

Further experience with lepromas submitted from several sources and blended for 60 seconds has demonstrated even greater variations in sedimentation rates

during Cycle I. This suggests the role of differing concentrations of gelatin and the desirability of standardizing the autoclaving of lepromas. While further experience is being gained, we will request that autoclaving periods at 121° be as follows: 12 minutes with 1-3 gm. of leproma/vial and 15 minutes with 4-10 gm. of leproma/vial.

PROCEDURES RECOMMENDED

Equipment, stock solutions, and precautions for the selection and pooling of lepromas have been described under MATERIALS. The following procedures are recommended:

Trim each lesion. Incise it and touch the tip of the knife to a mapped site on a clean slide for microscopic evaluation. Include only those lesions that seem to rate 2+ or more on your familiar bacteriologic index.

Place a known weight or volume of lepromas in the blender. To provide a concrete example, 4 gm. of leproma will be assumed and the corresponding volume of saline shown in parentheses. For each gram of leproma, add 9 volumes (36 ml.) of saline. Blend the tissues for 60 seconds, not more. Although supernates can be recovered from the blender, it is assumed that sedimentation chambers will be used and that the blender will be used to process more tissue.

Transfer the homogenate to an appropriate sedimentation chamber. Use 2.5 volumes of saline (10 ml.) to rinse the blender. You now have 4 gm. of tissue in a 50 ml. system, i.e., tissue diluted 1:12.5, or tissue 8 per cent (T_8). Set a timer for 20 minutes.

If no further tissues are to be homogenized, this is a convenient time to clean the blender and bench top, to label needed glassware, etc.

To collect supernates. The essential points in technic have been described under PROCEDURES. The following are procedures for the preparation of Supers I-IV:

Super I. It will be recalled that standard sedimentation rates did not occur in Cycle I. After the first 20 minutes of settling, pipette off all obtainable nonparticulate supernate and estimate the volume of this

TABLE 2. Analysis of *M. leprae* recovered in Supers I-IV.

Method of blending (in sec.)	Lepromin No.	Super	Tissue or residue	NaCl added (ml.)	T% ^a	Recoveries				Final Bac./ml. x10 ⁶	
						Supers		Bac./ml. x10 ⁶	Total bacilli/cycle		% Ratios among cycles
						(ml.)	% of system				
WB 30	101A	I	2.4 gm.	21.6	10.0	16	67.0	343	5,500	72.0	
		II	(8 ml.)	12.0	4.0	14	70.0	99	1,380	18.0	
		III	(6 ml.)	9.0	1.6	12	80.0	26	310	4.0	
		IV	(3 ml.)	9.0	0.4	6	50.0	81	490	6.0	
Total						48			7,680	100.0	160
SvO 30	101B	I	2.4 gm.	21.6	10.0	14	58.0	520	7,300	79.0	
		II	(10 ml.)	14.0	4.2	17	71.0	87	1,480	16.0	
		III	(7 ml.)	8.0	2.0	10	67.0	20	200	2.0	
		IV	(5 ml.)	15.0	0.5	12	60.0	23	280	3.0	
Total						53			9,260	100.0	174
WB 60	102E	I	4.0 gm.	36.0	10.0	20	50.0	400	8,000	54.0	
		II	(20 ml.)	30.0	4.0	30	60.0	166	4,980	33.0	
		III	(20 ml.)	30.0	1.6	30	60.0	44	1,320	9.0	
		IV	(20 ml.)	20.0	0.8	20	50.0	30	600	4.0	
Total						100			14,900	100.0	149

Calculation: $X \quad Y = XY$

^a T% = The *theoretic* concentration of tissue that should remain after each cycle, if the tissues were soluble. Such data indicate that four elution cycles should recover more than 99 per cent of any soluble or readily suspendable materials that were present.

fraction (Ia). If the volume is less than 20 ml. (one-third of the volume to be recovered in Cycle I), shake the contents of the sedimentation chamber vigorously and reset the timer to extend the next settling period accordingly. For example, if only 10 ml. was recovered, double the settling time.

After the chosen interval, recover the available supernate (Ib), and readjust the settling period if it seems necessary. Repeat this step a third time (Ic). These three recoveries of supernate complete Cycle I and yield Super I.

In experimental work the volumes and the microscopic counts are recorded separately for each of Supers I-III or I-IV. In batch production, simply pool the successive supernates I, II and III in a stock bottle that should accommodate the entire batch when (after microscopic counts) the final dilution of the lepromin is to be made. If suitable lepromas were selected, the final volumes usually will be 25 to 30 times the original weight of tissue. For the sake of having a rich suspension of bacilli that can be used to fortify any low-count lepromins, it pays to set aside a portion of the earliest Super I's produced.

Super II. To the tissue residue, add saline to the original volume. Shake the sample 10 seconds. Set the timer for 20 minutes and proceed as above until Super II has been collected.

Super III. Repeat the above.

Super IV. At this point a further elution could be made, as in Cycles II and III. If so, remember that the bacterial yields will be low. If further blending or exceptional shaking is desired, step IV is the time to do it.

Super IV should not be pooled with Supers I-III during routine production. Count Supers I-III and Super IV separately, and then calculate whether Super IV can be added in whole or in part to adjust the final concentration of bacilli to 160×10^6 /ml. For further comment on the production of standard batches, see DISCUSSION.

Storage of tissue residues. Screw cap bottles should be used to pool all tissue residues remaining after step III or step

IV. They will occupy a volume that is $\pm 5x$ the initial volumes of the tissues. After further study, recommendations will be made for recovery of *M. leprae* from stored residues.

Microscopic counts. For routine purposes (pools of Supers I-III), dilute the samples 1:5 as recommended earlier (⁹). During experimental work on individual Supers I-IV, take into account the differing concentrations of bacilli anticipated. In this laboratory we have found it useful to dilute Supers I 10x, Supers II 5x, and Supers III 2x, and to use Supers IV undiluted. For Supers IV, 0.01 ml. of serum 90 per cent (phenolized at 0.5%) was added to 0.5 ml. of the sample, and then the usual 0.03 ml. was added to the spot plate.

In view of the presence of clumps of modest size, at least 600 bacilli should be encountered per strip counted, in order to obtain duplicate counts that agree within ± 10 per cent.

Dilution and standardization of the final product. Dilution of the pooled Supers I-III to contain 160×10^6 bacilli/ml. (^{1, 10}) can be accomplished with saline or with Super IV, if that has been prepared. The need for enrichment with the recommended reserve stock of Super I would indicate either poor selection of lepromas or inadequate liberation of the bacilli.

DISCUSSION

Two of the factors that interfere with the purification of *M. leprae* from tissues and, indeed, with the preparation of lepromins that can be standardized in bacterial content and in dosage per patient, are: (1) the marked fibrosis in human lepromas and the great preponderance of suspendable (but rapidly settling) tissue components, and (2) the wide range of bacterial clump sizes, which prevents differential behavior of tissue and bacteria fractions during sedimentation or centrifugation.

It was found useful, as proposed, to minimize the rupture of bacilli and the suspension of unwanted, fibrous, insoluble components of lepromas, and then to undertake the recovery of only that proportion of the bacilli exhibiting nearly uniform rates of sedimentation.

The methods described seem to have contributed five fundamental steps toward the goal of injecting known and uniform numbers of intact *M. leprae* into each skin test site. These contributions are:

1. **Ensurance of minimal and consistent damage to the bacterial cells.** This is a fundamental consideration as long as bacterial counts remain the basis for standardizing lepromin. We have a lepromin in our collection that was made by hand-grinding for four hours, in which it is almost impossible to find intact acid-fast cells. Nevertheless, it is reputed to give "good Mitsuda reactions." Tuma *et al.* (¹²) have shown that Mitsuda reactions are produced by lepromins in which nearly all the bacilli had been ruptured. Abe *et al.* (²) more recently demonstrated that Mitsuda reactions are produced by lepromin that had been sonicated until devoid of stainable bacilli. They attributed this reactivity to fractured cell walls. It is suspected also that the dispersion of cell walls into a colloidal state may exaggerate the Fernández and the early phases of Mitsuda reactions. Comparisons made by Dr. J. A. Cap, WHO, Geneva (³) of a "blended" and a hand-ground lepromin that produced similar Mitsuda reactions, revealed that the hand-ground preparation caused significantly stronger Fernández reactions.

2. **Maximal recovery of *M. leprae* in a reproducible range of clump sizes.** Such suspensions should cause Mitsuda reactions that evolve at standard rates.

3. **Suspension of only a modest proportion of the fibers and ground substance in lepromas.** Failure to observe standard sedimentation rates in the crude homogenates (Cycle I) has been emphasized and discussed in the RESULTS. It is evident that the periods of autoclaving and of blending must be standardized.

4. **Production of suspensions that are sufficiently stable to permit injection of 10 replicate dosages from a syringe during a reasonable period of time.** Sedimentation is a tedious method for separating the bacilli from the tissue flocs and particles. To obtain the desired result by filtration would be even more troublesome. Efforts to eliminate flocs and tissue particles from

homogenates of rat leprosy bacilli, which contain much less collagen, have been frustrating. The difficulties are: (a) the need for a series of 6-8 screens of decreasing mesh size; (b) the fact that different homogenates overload and plug a different screen on nearly every occasion, and (c) the difficulties of convenient or efficient rinsing of the tissue residues. For the reasons indicated, no attempts were made to remove tissue particles by filtration.

5. **The possibility of establishing regional laboratories for the preparation and standardization of lepromin.** This step seems highly desirable if uncontrolled disruption of bacilli is to be avoided and if bacterial numbers are to be standardized. With a modest outlay for equipment and sedimentation chambers, large scale production is feasible. The optimal period for autoclaving lepromas and for blending full batches in the 360 ml. cup of the Waring blender has not yet been determined.

Several factors facilitate standardization of bacterial concentrations in successive lots of lepromins. Points to be noted are as follows:

1. On the average, it seems safe to use volumes of diluent that will prepare 100 ml. of lepromin from each 4 gm. of selected lepromas.

2. When possible, it pays to stockpile some of the high-count Super I's, in order to enrich substandard batches.

3. Our routine method consists of making separate counts in pooled Supers I-III and in Super IV. Calculation will then show whether a portion of Super IV should be withheld for subsequent inclusion in a richer lot. With 4 gm. of leproma per 100 ml., one usually can include all of Super IV. Occasionally, even further dilution is possible.

SUMMARY

This paper describes a method for the preparation of lepromins that are standardizable by means of bacterial counts without declumping, and permit the injection of uniform concentrations of *M. leprae* per skin site. An electric "blendor" is used to minimize the disruption of bacilli and the dispersion of unwanted connective tis-

sues. Rapidly settling tissue particles and large and variable clumps of bacilli are eliminated by gravity sedimentation and by rinsing the tissue residues during the collection of successive supernates. The result is the recovery of *M. leprae* in a reproducible range of clump sizes and free from particles that interfere with the injection of uniform dosages. The method is designed for large scale production of lepromin in regional laboratories. Four gm. of properly selected lepromas produce approximately 100 ml. of lepromin containing 160×10^6 bacilli per ml.

RESUMEN

Este trabajo describe un método para la preparación de leprominas que son uniformes por medio del recuento bacteriano sin desagrupación y permite la inyección de concentraciones uniformes de *M. leprae* por localizaciones de piel. Una "mezcladora eléctrica" se usa para reducir al mínimo la ruptura de los bacilos y la dispersión de los tejidos conjuntivos que no se desean. La rápida localización de partículas de tejidos y los grandes y variables conjuntos de bacilos son eliminados por la sedimentación causada por la gravedad y por el enjuague de los residuos de tejidos durante la recolección de los sucesivos sobrenadantes. El resultado es la recuperación del *M. leprae* en un margen reproducible de grupos y libre de partículas que interfieren con la inyección de dosis uniformes. El método está hecho para preparar lepromina en gran escala en laboratorios regionales. Cuatro gr. de lepromas propiamente seleccionados producen aproximadamente 100 ml. de lepromina que contiene 160×10^6 bacilos por ml.

RÉSUMÉ

Cette communication décrit une méthode pour la préparation de lépromines susceptibles d'être standardisées au moyen de numérations bactériennes et ceci sans séparation des bacilles (declumping). Cette méthode permet l'injection de concentrations uniformes de *M. leprae* dans chaque endroit de la peau. Un agitateur (blender) électrique est utilisé pour minimiser la rupture des bacilles et la dispersion du tissu conjonctif indésirable. Les particules de tissu sédimentant rapidement, de même que les amas variables et de grandes dimensions de bacilles, sont éliminés par sédimentation (gravity sedimentation) et par rinçage des résidus tissulaires au cours de la collection des frac-

tions supernageantes successives. Le résultat en est que *M. leprae* peut être recueilli dans une gamme reproductible quant à la dimension des amas, et sans particules qui peuvent interférer avec l'injection de doses uniformes. Cette méthode est proposée pour la production de lépromine sur une grande échelle dans des laboratoires régionaux. Quatre grammes de lépromes choisis de façon adéquate produit environ 100 ml. de lépromine contenant 160×10^6 bacille par ml.

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