New Method for Concentration and Quantitation of *Mycobacterium leprae*

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A new method of enumerating *Mycobacterium leprae* has been developed. Suspensions containing the organisms were filtered through a polycarbonate membrane filter (25-mm diameter, 0.4- μ m pore size, 10- μ m thick; Nuclepore) to concentrate the organisms. The membrane was then mounted on a glass slide and stained with a standard acid-fast stain. Finally, the membrane was treated with a small amount of chloroform to fix it to the slide and make it transparent. This method enabled us to detect *M. leprae* in quantities as small as 4.98×10^2 regardless of the total volume of the original material. Comparison with a standard method for enumerating *M. leprae* showed that both methods gave similar results when the organisms counted by the standard method were present in sufficient quantity for reproducibility. Because the least number of organisms that can be detected with the standard method is 10^4 ml and because the organisms detected with the new method could be concentrated on the polycarbonate filter from a large amount of infected fluid, a substantial number of suspensions were shown by the new method, but not by the standard method, to contain *M. leprae*.

Because *Mycobacterium leprae* cannot be cultivated, the enumeration of these organisms when present in small numbers is accomplished with great imprecision (W. M. Krushat, K. E. Schilling, J. A. Edlavitch, and L. Levy, *Lepr. Rev.*, in press). Two methods of enumerating acid-fast bacilli (AFB) are generally used at this time: the microspot technique described by Hanks et al. (2) and the method of Shepard and McRae (SM; 4). Because both methods employ only a small volume of bacillary suspension (30 μ l or less), the precision of the data is poor when the concentration of the organisms is smaller than 10^3 /ml, and it is virtually impossible to detect fewer than 10^4 organisms/ml.

This paper describes a new method for enumeration of AFB. It includes the use of an extra-thin (10 μ m) polycarbonate membrane for trapping and concentrating the organisms by filtration followed by an acid-fast staining of the filter and counting the organisms on the filter itself. Our method has an advantage over established methods, because it permits a more accurate count of the organisms, especially when the number is low, and because the organisms can be concentrated and readily detected when present in small numbers in a large volume of infected material.

MATERIALS AND METHODS

Polycarbonate membranes. These were obtained from the Nuclepore Corp., Pleasanton, Calif., and

were 25 mm in diameter with a pore size of 0.4 μ m. For filtration, the membrane was placed in a stainless-steel microsyringe holder with Teflon gaskets (Millipore Corp., Bedford, Mass.).

Source of *M. leprae*. The *M. leprae* used in these experiments was a strain that had been originally isolated by Shepard from a patient with lepromatous leprosy and subsequently maintained by passage in the footpads of mice (3). The organisms used in these experiments were obtained from the hind footpads of BALB/c mice or neonatally thymectomized Lewis rats (1) or from tissue culture fluids from tissue cultures that had been inoculated with *M. leprae* from one of those sources.

Normally, the footpads of three mice from an experimental group that had been inoculated at least 8 months earlier were pooled, minced finely with iris scissors, suspended in 2 ml of Hanks balanced salt solution, and homogenized in a Mickle tissue disintegrator (4). The suspension was then centrifuged at $270 \times g$ for 1 min, and 1 ml of the supernatant fluid was removed. For comparative purposes, the AFB in this supernatant were enumerated either by the SM method (4) or after filtration through the polycarbonate membrane.

Filtration and staining. Unless otherwise stated, 0.5 ml of each *M. leprae* supernatant was diluted to 4 to 5 ml with Eagle minimum essential medium supplemented with 10% fetal bovine serum. The diluted sample was drawn into a 10-ml syringe and filtered through the polycarbonate membrane, after which the filter assembly was flushed with air from the syringe, forcing any remaining fluid through the filter. The filter was removed from the holder, placed in the center of a glass slide (taking care not

to trap air bubbles), and allowed to dry. After drying, the filter was fixed in formalin fumes and stained by a standard acid-fast stain (4). During the staining process, a small amount of dye occasionally remained between the filter and the slide. This was readily removed by flooding the slide with acid-alcohol, lifting the filter with a pair of sharp forceps, and rapidly shaking it through the acid-alcohol for a few seconds until all excess stain was removed. It was then rinsed in tap water and air-dried. Finally, the slide was placed on a leveled platform, quickly covered with several drops of chloroform to increase transparency, and permitted to dry.

Calculation of the number of organisms. Although the diameter of the filter is 25 mm, the effective filtering area of the filter depends on the size of the gasket used in the filter assembly. The inner diameter of the gaskets from seven filter assemblies measured 2.256 cm. The field area of a 100× oil immersion objective in our microscope was determined with 10× wide-angle oculars to be 1.606×10^{-4} cm². Therefore, a microscope factor (MF; ratio of areas) for a 25-mm polycarbonate filter can be shown to be:

$$MF = (2.256/2)^2\pi / (1.606 \times 10^{-4}) = 2.489 \times 10^4$$

Fifty fields across the equator of the filter were routinely examined, and the AFB was counted. The total number of organisms on the filter can then be calculated as follows.

$$\text{Total number of AFB} = (\text{number of AFB counted} / \text{number of fields examined}) \times MF$$

The concentration of AFB per milliliter in the original suspension is calculated as:

$$\text{Number of AFB/ml} = \text{total number of AFB} \times (1 / \text{volume of original suspension filtered [ml]})$$

RESULTS

Harvest of *M. leprae* from mouse footpads. *M. leprae* from the footpad tissues of 15 groups of mice that had been inoculated 6 months earlier were harvested, and the numbers of AFB in the harvests were compared by the two methods. The results are shown in Table 1. In these experiments, exactly 0.5 ml of each bacillary suspension, which had been diluted with 4 to 5 ml of minimal essential medium, was employed for filtration and counting by the new method. Thus, counting 1 AFB in 50 microscopic fields represented a yield of 9.96×10^2 /ml. However, since there was only 0.5 ml in the original AFB suspension, one organism represented a count of 4.98×10^2 *M. leprae*. This number is actually the minimum number of AFB that can be counted by this method regardless of the volume filtered.

In seven instances when sufficient numbers of AFB could be counted by the SM method, there was very close correlation between the two methods. However, between 10.5 and 24.8 times more AFB were counted by the new

TABLE 1. Comparative counts of suspensions of *M. leprae* from the footpads of infected mice as determined by the Nuclepore and SM methods

Group	Nuclepore method		SM method	
	No. of AFB/fields counted	AFB/ml	No. of AFB/fields counted	AFB/ml
438	0/50	<10 ³	0/60	<10 ⁴
443	1,813/50	1.81×10^6	144/60	1.45×10^6
444	776/50	7.73×10^5	74/60	7.50×10^5
445	364/50	3.62×10^5	28/60	2.84×10^5
448	1,367/50	1.36×10^6	111/60	1.16×10^6
449	1,661/50	1.65×10^6	67/60	6.79×10^5
450	54/50	5.38×10^4	1/60	1.01×10^4
451	0/50	<10 ³	0/60	<10 ⁴
457	1/50	9.96×10^2	0/60	<10 ⁴
458	7/50	6.96×10^3	0/60	<10 ⁴
460	3,507/50	3.50×10^6	260/60	2.64×10^6
461	1/50	9.96×10^2	0/60	<10 ⁴
464	3/50	2.98×10^3	0/60	<10 ⁴
476	11/50	1.10×10^4	2/60	2.03×10^4
578	3,425/50	3.41×10^6	255/60	2.58×10^6

method than by the SM method.

In the two instances when no AFB were found by the Nuclepore method, none were found by the SM method. In four other instances when no AFB were found by the SM method, one to seven AFB were found by the Nuclepore method. In the two instances when the SM method showed a minimal count (groups 450 and 476), at which level the total counts should not have been very precise, it is interesting that they did not greatly differ. In group 450, we counted 54 AFB in 50 fields by the new method and only 1 AFB in 60 fields by the SM method, the total counts being 5.38×10^4 and 1.01×10^4 , respectively. In group 476 the total counts were even closer. Where 11 AFB in 50 fields were counted by the new method and 2 AFB in 60 fields were counted by the SM method, the total counts were 1.10×10^4 and 2.03×10^4 , respectively.

Isolation of *M. leprae* from infected tissue culture fluids. The original need to develop a method for concentrating and counting small numbers of *M. leprae* arose from the fact that we were attempting to cultivate *M. leprae* in tissue culture. After infection of the cultures, we frequently observed a gradual and steady disappearance of the AFB over a period of time. Examination of the tissue culture fluids did not reveal the presence of *M. leprae*. Since we could detect no less than 10^4 AFB/ml by the SM method, it was possible that in cultures containing 10 ml of tissue culture fluid, up to 10^5 AFB could be discarded without being accounted for or counted. Therefore, tissue culture fluids from *M. leprae*-infected cultures that had been previously found to be negative

by the SM method were submitted to Nuclepore filtration. The results (Table 2) indicate that all fluids contained AFB, with counts ranging from 6.47×10^3 to 2.69×10^4 . More importantly, up to 54 AFB were detected, albeit in 12 ml, where none had been detected by the SM method.

DISCUSSION

Although it has been repeatedly shown that the SM method of counting *M. leprae* is highly accurate, its limitation lies in the fact that only a very small sample (0.03 ml) is examined. For practical purposes, fewer than 10^4 *M. leprae*/ml cannot be detected in the sample examined.

For our purposes, it was essential to have not only a counting method that would detect fewer AFB, but also one in which small numbers of *M. leprae* could be detected in relatively large volumes of infected material. The only practical way this can be accomplished is by concentration by filtration followed by counting the organisms on the filter. Several types of cellulose

filters were tested and had to be discarded because they were unsuitable for examination by direct light microscopy by virtue of their excessive thickness (up to 150 μ m) and lack of transparency. The polycarbonate membranes were ideally suited for our purposes. They are only 10- μ m thick and have a smooth flat surface compared with the rough surface of the cellulose filters. The only minor drawback in the polycarbonate filters is that they are only semi-transparent. However, the addition of a few drops of chloroform to the fixed and stained membranes made them sufficiently transparent so that AFB could be readily counted, using the same operating procedures employed for counting AFB by the SM method. Further, the chloroform treatment acted as a fixative to permanently affix the membrane to the slide.

The obvious advantage in the new method is that it is possible to detect as few as 4.98×10^2 AFB regardless of the amount of material in which they are suspended. Furthermore, the quantity of material to be examined is limited only by the amount that can be passed through the filter. It should now be possible to detect *M. leprae* in many types of specimens in which hitherto it has not been possible.

TABLE 2. Results of counting *M. leprae* by the Nuclepore method in tissue culture fluids previously found to be negative by the SM method

Tissue culture fluid	Volume filtered (ml)	No. of AFB/fields counted	Total AFB
1	10	19/50	9.46×10^3
2	10	21/50	1.05×10^4
3	8	34/50	1.61×10^4
4	12	15/50	7.47×10^3
5	12	54/50	2.69×10^4
6	8	13/50	6.47×10^3
7	8	17/50	8.46×10^3

LITERATURE CITED

1. Fieldsteel, A. H., and A. H. McIntosh. 1971. Effect of neonatal thymectomy and antithymocyte serum on susceptibility of rats to *Mycobacterium leprae* infection. Proc. Soc. Exp. Biol. Med. 138:408-413.
2. Hanks, J. H., B. R. Chatterjee, and H. F. Lechat. 1964. A guide to the counting of mycobacteria in clinical and experimental materials. Int. J. Lepr. 32:156-167.
3. Shepard, C. C. 1960. The experimental disease that follows the injection of human leprosy bacilli into the foot pads of mice. J. Exp. Med. 112:445-454.
4. Shepard, C. C., and D. H. McRae. 1968. A method for counting acid-fast bacteria. Int. J. Lepr. 36:78-82.