Distribution of *Mycobacterium leprae* in Circular Counting Films

TO THE EDITOR:

It would seem from the letter of Shepard and Levy that my paper ([3]) did not clearly explain the principle behind the use of stratified sampling in determining the number of bacilli in circular counting fields. All workers, including Levy and Shepard (see first paragraph of their letter), appreciate that bacteria spread over a circular area are not randomly distributed. One of the principal advantages of stratifying a sample is that the observer may calculate the optimal sampling rate in each stratum ([1]), i.e., the percentage of the total number of observations that should be devoted to each stratum in order to obtain the best estimate of the population size. Thus, even if bacilli were distibuted at random most of them would be in the outer 4 strata of the circle since this comprises over 70% of the film area. It is obvious, therefore, that a better estimate of the population size would be obtained by using stratified sampling whenever the distribution is not random, and even when it is random no additional error would be introduced.

The central question regarding the analysis of circular bacterial fields is whether or not there is concentric variation in the distribution of the bacilli. Although I have been unable to find an adequate discussion of physical droplet evaporation, the everyday observation of dried droplets of coffee or other liquids clearly demonstrates that deposition of soluble or suspended substances occurs in a concentric manner across a drying drop. The concentric distribution of bacilli which I describe in my paper ([3]) was first reported some 45 years ago by Hanks and James ([2]). Shepard (see paragraph 4 of their letter) has also observed a rim effect with purified suspensions of *Mycobacterium leprae* which, as the title of my paper indicates, is what I was reporting. However, Shepard and Levy claim that while purified mycobacteria do show a rim effect they were "satisfied that, although the organisms (from tissue containing suspensions?) were not randomly distributed, they were not concentrated in any portion of the circle of the counting slide" that they used. In support of this claim, they present data from their own laboratories using their routine bacterial counting system. Unfortunately, however, they have not sampled an entire radius or diameter. I have attempted to construct a profile of their bacterial distribution by the summation of the data from both laboratories, allocating the counts to their appropriate places in the strata (assuming that the numbers given corresponded to their positions in the diameter of their fields). Contrary to the claim of the authors, there is an apparent 60% greater number of bacilli in the middle strata of their films (Fig. 1a). However, because of the infrequent sampling and the generally low numbers of bacilli in their films, together with the fact that their data is from non-replicate samples, any interpretation of the distribution from their data can only be tentative. I have therefore analyzed the distribution of *M. leprae* across a number of replicate 10 mm films and, in order to ensure that the bacterial distribution was not an artefact of purified suspensions, the smears were made from tissue-containing *M. leprae* suspensions.

A suspension of *M. leprae* was prepared from armadillo liver by homogenizing a small piece of infected liver in a Potter mill together with 1 ml of phosphate buffered saline. The resultant suspension was allowed to stand for 2 min and either 20 μl or 100 μl of the suspension was pipetted onto a 10 mm diameter circle, and then allowed to air dry. The bacterial film was then fixed and stained with Ziehl-Neelsen as described previously ([3]). In order to determine the distribution of bacteria across the film and to make a comparison with the previous publication easier, these larger bacterial films were also stratified into 13 concentric rings (one ring being equal to about 2.5 high power microscope fields). As can be seen from Figure 1b, the distribution of bacilli across these larger fields is similar to that described previously ([3]): with low numbers of bacilli at the extreme periphery, higher numbers in the next few strata, followed by a slight decrease, with increasing numbers toward the center. The principal difference between the distribution found in large circles and small circles is that in the

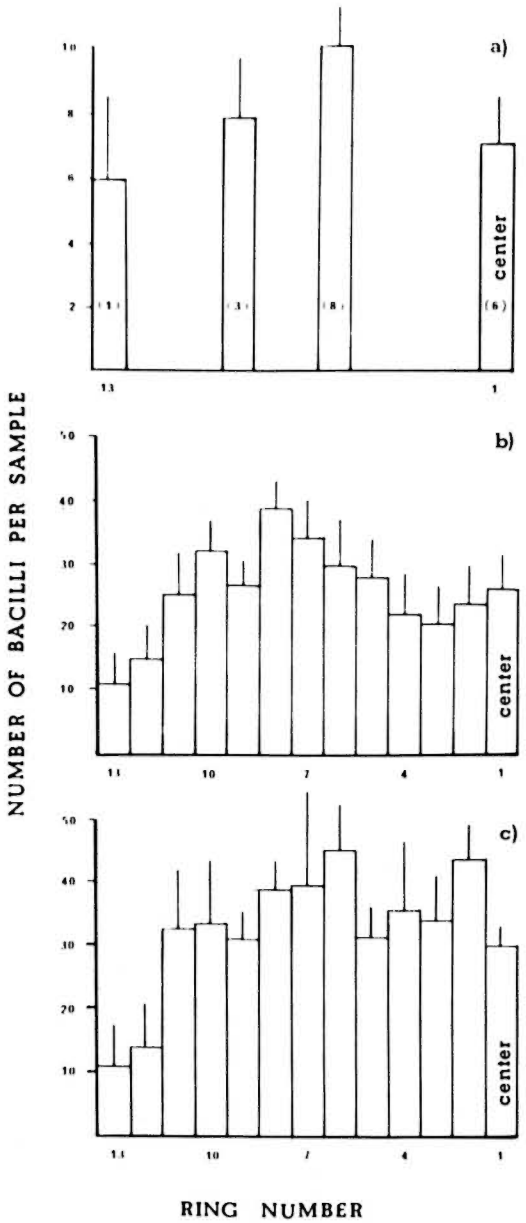

FIG. 1. Bar charts showing the stratified distribution of *M. leprae* bacilli spread across the radii of 10 mm circular fields. All fields were stratified into 13 rings. Values are means ± standard errors of the mean. **a)** Bacterial profile using data of Shepard and Levy. The numbers in rings correspond to their field numbers (N = 30). **b)** Bacterial profile using *M. leprae*-infected armadillo liver suspension (N = 10). **c)** Bacterial profile using a fivefold higher sample volume (N = 10).

former the mycobacteria tend to concentrate more in the middle strata of the circle and the distribution is more spread out. From these results, it is clear that a concentric distribution is also present when the mycobacteria are spread over a larger area, even in tissue-containing suspensions.

Shepard and Levy suggest that one explanation for my findings might be the geometry of the drop, and I have therefore analyzed the distribution of bacilli across a 10 mm circle when the drop volume was increased 5 times. As can be seen from Figure 1c, the distribution is broadly similar to that found with the smaller drop volume (20 μl) although the distribution is less pronounced, contrary to the prediction of Shepard and Levy.

In summary therefore, I feel that the criticisms of Shepard and Levy are unfounded. Firstly, my original paper discussed the distribution of purified suspensions of *M. leprae* which, as they report, do show a concentric rim effect. Secondly, their own data and that in Figures 1 b and 1c demonstrate that bacteria spread over even a large circular film do concentrate in certain strata. The exact distribution of the bacilli is not so important as the fact that there are local concentrically arranged concentrations of *M. leprae* in circular bacterial films. Thus, the application of stratified sampling will enable the investigator to make a more accurate estimate of the bacterial population, while the slightly more complex calculation involved is easily accomplished with any programmable calculator.

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