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Viability of *Mycobacterium leprae*: A Comparison of Morphological Index and Fluorescent Staining Techniques in Slit-skin Smears and *M. leprae* Suspensions[1]

Odd Odinsen, Tomas Nilson, and David P. Humber[2]

Assessment of the viability of *Mycobacterium leprae* is still far from satisfactory and until recently, it could be effectively determined by growing material obtained from patient biopsies in the mouse foot pad ([14]). However, for nearly 100 years since the time of Armauer Hansen, attempts have been made to equate morphological characteristics with viability ([3, 4, 12, 15, 18]), and it is accepted by many leprologists that *M. leprae* are dead if, after staining with carbol fuchsin, they have an irregular, "nonsolid" staining pattern. As a result, the morphological index (MI) of bacillary smears obtained from leprosy patients has been advocated as a useful parameter for indicating the viability of *M. leprae* particularly after it was shown that during chemotherapy the MI declined more rapidly than the bacterial index (BI) ([17]). *M. leprae* often vary greatly in the way in which they stain, particularly with regard to the consistency of acid-fast staining ([5]). In addition, there is no generally accepted standard of the various types of irregularly stained *M. leprae* and although a system of solid-fragmented-granular (SFG) percentages has been reported, significant differences are common between even expert observers ([10]). However, subjective interpretation and the high degree of technical skill required are not the only drawbacks to the effective use of the MI in assessing the viability of *M. leprae*. The correlation between morphology and viability has not been universally accepted. Karat, *et al.* ([7]) first reported the successful foot pad cultivation of *M. leprae* obtained from over 83% of a group of patients with zero MI; a result confirmed by a second detailed study ([1]). It has also been suggested, from data obtained by electron microscopic studies of *M. leprae,* that viability may be many times higher than that found by conventional microscopy ([16]).

It is clear, therefore, that another rapid, simple but reliable method for determining the viability of *M. leprae* would be of the greatest value, both in leprosy research and in the assessment of effective chemotherapy of multibacillary leprosy patients. Of the methods that have recently been described ([2, 8, 13]), two, thymidine incorporation and determination of ATP levels, require not only relatively large numbers of *M. leprae* bacilli from skin biopsies but also depend on sophisticated equipment and expensive, labile materials. In contrast, the use of fluorescent vital dyes offers a simple alternative, the only requirement being a fluorescent microscope (which is already in use in many tuberculosis laboratories).

The aim of this investigation was to assess the suitability of fluorescein diacetate (FDA) and Rhodamine 123 (R123) in the routine examination of slit-skin smears and to analyze the relationships between viability in *M. leprae,* their interaction with FDA and R123, and changes in the morphological index.

MATERIALS AND METHODS

Selection of patients. Biopsies and skin smears from borderline lepromatous (BL) or lepromatous (LL) leprosy patients were obtained either from the All Africa Leprosy and Rehabilitation Centre (ALERT) or from

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[2] O. Odinsen, Senior Laboratory Supervisor; T. Nilson, Senior Laboratory Technician, All Africa Leprosy & Rehabilitation Training Centre, P.O. Box 165, Addis Ababa, Ethiopia. D. P. Humber, Ph.D., Associate Professor, Department of Biology, Addis Ababa University, P.O. Box 30736, Addis Ababa, Ethiopia.

Reprint requests to Dr. Humber.

Boru Meda Hospital, Wollo Province, Ethiopia. Patients on treatment were receiving dapsone (DDS) 100 mg and clofazimine 50 mg daily, supplemented with rifampin 600 mg and clofazimine 300 mg every 4 weeks.

Slit-skin smears. Triplicate skin smears were prepared for each technique assessed (MI, FDA/ethidium bromide or EB, and R123/EB) for each of six sites routinely used at the ALERT clinical laboratory. Smears were also taken from any obvious nodular sites. Skin smears that were not examined immediately were frozen in liquid nitrogen or kept in a −70°C freezer. Examination of prepared or stained smears indicated that they may be stored at −20°C for up to 6 months without significant change in the percentage viability or loss of fluorescence (data not shown).

Ziehl-Neelsen staining and determination of MI. A modified Ziehl-Neelsen was performed on the skin smears as described previously ([10]). The number of solid-stained bacilli were determined by counting a total of 100 bacilli in each of three separate areas of each smear. Only bacilli whose length exceeded five times their width, and with both even shape and staining were considered as solid. Bacilli which were fragmented, unevenly stained, or faintly stained were considered nonsolid. The MI was determined independently by two observers (TN and OO), and their determinations were in good agreement. All slides were coded and read blind.

Preparation of armadillo *M. leprae*. Small pieces of armadillo liver (0.1 to 0.2 g) were thawed and homogenized in 2 ml of phosphate buffered saline pH 7.2 (PBS). The suspension was centrifuged at 100 × *g* × 10 min at 4°C to remove large pieces of tissue debris. The supernatant was then diluted to an appropriate concentration. Infected armadillo liver was kindly supplied by Dr. M. Lefford, Wayne State University, U.S.A. The freeze/thaw cycles were performed by alternately placing the suspension into a −70°C freezer until frozen (approximately 15 min), followed by thawing in a 37°C water bath.

Fluorescein diacetate (FDA)/ethidium bromide (EB) staining. A stock solution of FDA (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was made by dissolving 100 mg in 20 ml of acetone and kept in foil-covered tubes at −20°C until required ([8]). Immediately before use, a 1:10 dilution in acetone was made, and 20 μl of this solution added to 5 ml of PBS (final concentration of FDA 2 μg/ml). Stock EB (Sigma), prepared by dissolving 20 mg in 10 ml of PBS, was kept at −20°C until required. Before use, 10 ml of stock EB was mixed with 5 ml of the FDA working solution. Slides were stained by adding one drop of the FDA/EB solution to each air-dried smear and incubating in the dark at room temperature for 30 min. Excess liquid was poured off and the slides mounted with a coverslip using polyvinyl acetate/glycerol mounting medium, pH 8.6, containing p-phenylenediamine (PPD, 0.8 mg/ml; Sigma) or 1,4-diazobicyclo (2,2,2) octane (DABCO, 25 mg/ml; Sigma) to prevent fading. There was little difference in the effectiveness of these two compounds, but DABCO may be stored and used without special precautions to avoid skin contact and exposure to light ([6]). All slides were coded and read blind, using a Leitz microscope with epifluorescent illumination. The percentage of green-staining bacilli was determined by counting at least 100 bacilli in each of three separate areas. Doubtful or dual-stained bacteria (less than 1%) were examined using a filter which only admitted red fluorescence. Mycobacteria that could not be resolved in this way were considered to be viable.

Rhodamine 123 (R123)/EB staining. A stock solution of R123 (Sigma) was made by dissolving 1 mg of R123 in 200 ml of PBS ([4]). Immediately before use, the stock solution was diluted 1:3 in PBS (final concentration of R123 1.7 μg/ml) and 10 μl of the above stock EB solution was added to 5 ml of the R123 stain. The staining solution was applied to each air-dried smear and incubated in the dark at 37°C for 30 min. The slides were then washed gently in PBS, and the excess liquid drained off. Stained slides were mounted with a coverslip using the mountant described above. The smears were examined and counted as described for FDA/EB.

RESULTS

Correlation between FDA/EB and MI, and between FDA/EB and R123/EB. The data shown in Figure 1 a and b demonstrate

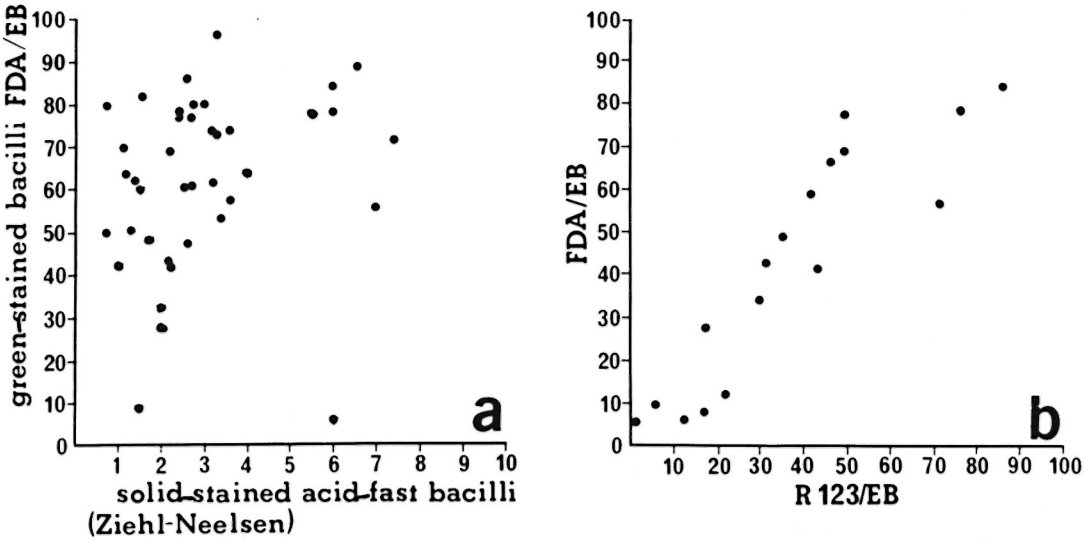

FIG. 1. Scatter diagrams showing correlations between: **a** = percent green-staining bacilli by FDA/EB and MI; **b** = percent green-staining bacilli by FDA/EB and that with R123/EB.

that while there is an excellent correlation between the findings using FDA/EB and R123/EB staining, no significant correlation is apparent between the FDA/EB method and viability as assessed by the criteria of the MI.

Viability of bacilli in skin smears from treated and untreated patients. In untreated multibacillary patients (Fig. 2) the percent viability as judged by FDA/EB staining ranged from 52%–86%, indicating that viable *M. leprae* were present in all of the skin smears examined. In contrast, following the assessment of viability of the same samples using the MI, viability ranged from 0% (in two of the sites) up to 7%. After multiple drug chemotherapy for 2–3 weeks, assessment of the MI showed that one third of the samples examined had 0% viable mycobacteria, whereas FDA/EB staining of the same samples indicated 15%–20% viable bacilli.

Freezing and thawing or incubation at 60°C of armadillo *M. leprae*. The effect of successive cycles of freezing and thawing are shown in Figure 3a, and it is apparent that even after 12 treatment cycles 20%–30% of the bacilli are still viable, as judged by FDA/EB and R123/EB staining. The two fluorescent vital staining methods show a progressive and relatively constant decline in viability, with an overall 60% decrease in viable *M. leprae*. A similar, but not significant decline in viability, as judged by assessment of the solid-staining bacilli, is also seen, although no additional decline in viability occurred after the third cycle of freezing and thawing. In contrast, incubation of *M. leprae* at 60°C (Fig. 3b) resulted in a marked discrepancy in viability as assessed by the fluorescent staining methods and the MI. The change in MI after 45 min at 60°C only indicates a similar decrease in viability to that produced by freezing and thawing. However, assessment of viability by both fluorescent staining methods shows a highly significant (80%–90%) decrease during the same period. After an additional 45 min at 60°C, staining with R123/EB showed 0% viability.

DISCUSSION AND CONCLUSION

This report compares two methods of measuring physiological viability of *M. leprae* with a morphological method (MI). The first physiological method is based on the ability of FDA, a nonpolar, nonfluorescent, fatty acid ester, to pass freely into cells where it can be hydrolyzed, by esterases in living cells, to a polar, fluorescent compound. The second method uses a fluorescent, cationic probe, R123, which only accumulates in cells by moving along a transmembrane potential (only found in living cells). The two

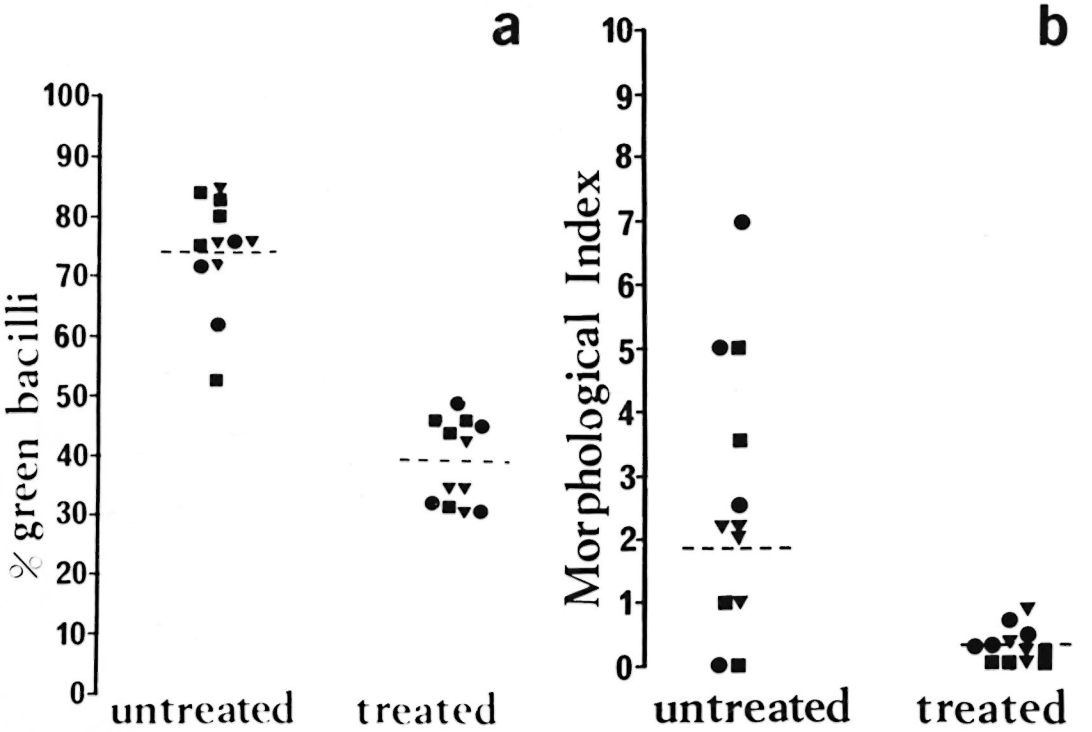

FIG. 2. Effect of multiple drug therapy on the viability of *M. leprae*. **a** = assessment by FDA/EB; **b** = assessment by MI. ▼ = ears; ■ = elbows; ● = knees.

methods were further enhanced by incorporating a second fluorescent compound, ethidium bromide, which cannot enter cells with intact plasma membranes (i.e., it will only stain dead cells). The results obtained with these techniques do not correlate with those obtained using the conventional estimator of viability, the MI. The discrepancy between the fluorescent techniques and the MI is evident, both from the slit-skin smears and in the data obtained from suspensions of armadillo *M. leprae* which were killed by freezing and thawing or by heating at 60°C. In the latter experiments, the MI showed a small and not statistically significant decrease, only during the initial part of the time courses. In both experiments, the R123/EB method gave consistently lower results than the FDA/EB method. It is likely that the difference between the two methods is a reflection of their theoretical basis. FDA depends on the presence of esterase enzymes which may still retain slight activity even at 60°C; whereas R123 is dependent on the maintenance of a transmembrane potential (an all or nothing phenomena) which would be more easily destroyed by both high temperatures and freezing and thawing.

The data from the freezing and thawing experiment, together with the results of viability from treated patients, show that samples of *M. leprae* which contain no, or very few, solid-staining bacilli may, in fact, contain an appreciable percentage of "physiologically viable" bacilli. This is a disturbing finding, since it is important to know whether the *M. leprae* in a smear are dead or alive. Mycobacteria which no longer possess esterase enzymes and/or an intact transmembrane potential cannot survive for more than a short time. Thus, methods assessing these criteria would accurately estimate the number of dead *M. leprae*. Although FDA/EB staining patterns accurately measure viability in other cultivable mycobacteria (9), the relationship between "physiological viability" and replicative potential is difficult to assess in *M. leprae*, and some of these physiologically viable cells

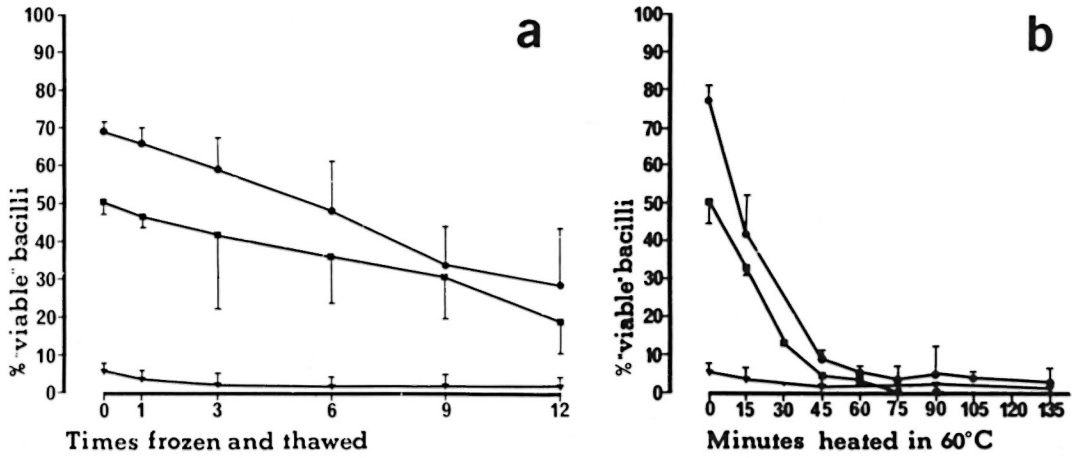

FIG. 3. Effect of **a** freeze/thawing and **b** incubation at 60°C on the viability of *M. leprae* as assessed by FDA/EB (●), R123/EB (■), and MI (▼).

might be dying and incapable of replicating. However, a mycobacterial cell which is incapable of the necessary cellular functions for division is unlikely to continue synthesizing enzymes, or to maintain an intact plasma membrane for very long. Thus, if it exists, a dead or dying cell which was esterase positive and had an intact transmembrane potential would, at best, be a transient phenomena.

The accurate assessment of viable *M. leprae* is important for successful monitoring of chemotherapy and for the detection of relapse due to drug-resistant organisms or inadequate therapy. The ideal method of assessing viability should fulfill a number of requirements. It should: a) accurately reflect the number of viable organisms; b) be sensitive and capable of detecting a low percentage of viable bacilli; and c) be simple to perform, inexpensive and, if possible, objective.

We believe that the data presented in this paper, together with previous investigations ([1, 7, 8, 16]), strongly indicate that the morphological index method is not reliable in the assessment of viable *M. leprae.* In contrast, both of the fluorescent staining methods are simple to perform, inexpensive and capable of detecting a low percentage of viable bacilli. In addition, since dual-staining bacilli are rare, the distinction between live and dead (green and red) bacilli is less subjective than the estimation of solid-staining characteristics.

SUMMARY

In a comparison of the estimation of *Mycobacterium leprae* viability by morphology and the fluorescent vital dyes FDA/EB and R123/EB, the latter techniques were more satisfactory using suspensions and slit-skin smears of *M. leprae* bacilli. Both FDA/EB and R123/EB seem to more accurately reflect viability after freeze/thaw cycles and heating, and are able to detect lower percentages of viable bacilli. In addition, the fluorescent vital dye techniques are both simple and less open to subjective interpretation than the conventional estimation of the morphological index.

RESUMEN

Comparando los métodos que pretenden evaluar la viabilidad del *Mycobacterium leprae* por morfología y por tinción fluorescente con los colorantes vitales FDA/EB y R123/EB, se encontró que las tinciónes de extendidos de linfa cutánea con los colorantes vitales fueron más satisfactorias que los métodos morfológicos. Tanto la FDA/EB como el R123/EB, parecen reflejar más exactamente la viabilidad después de varios ciclos de congelación y descongelación y después del calentamiento de las preparaciones bacilares, y son capaces de identificar porcentajes más bajos de bacilos viables. Además, las técnicas fluorescentes son más simples y menos subjectivas que los métodos morfológicos convencionales.

RÉSUMÉ

Une comparaison de deux méthodes utilisées pour évaluer la fiabilité de *Mycobacterium leprae*, à savoir l'examen morphologique, et la coloration par des colorants vitaux fluorescents FDA/EB et R123/EB, ont permis de démontrer que cette dernière technique était beaucoup plus satisfaisante lorsqu'on utilise des suspensions et des frottis cutanés de bacilles de la lèpre. Tant la méthode par les colorants FDA/EB que celle par R123/EB semblent refléter de manière plus exacte la viabilité après des cycles successifs de congélation et de décongélation, et après chauffage. Ces méthodes permettent de détecter des pourcentages plus faibles de bacilles viables. De plus, les techniques à base de colorants vitaux fluorescents sont à la fois simples et moins susceptibles d'être interprêtées de manière subjective, quand on les compare aux méthodes conventionnelles basées sur l'index morphologique.

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