Staining Tissue-derived *Mycobacterium leprae* with Fluorescein Diacetate and Ethidium Bromide[1]

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A fluorescent staining procedure incorporating the use of fluorescein diacetate (FDA) and ethidium bromide (EB) previously has been shown to accurately measure the viability of *Mycobacterium smegmatis* and *M. phlei* under a variety of physicochemical conditions ([3]). Live mycobacterial cells were distinguished from dead and damaged cells based upon their staining characteristics which depended upon the physiological and metabolic status of the cells.

Live cells stained green due to their ability to enzymatically hydrolyze nonfluorescent FDA to fluorescent fluorescein, and the presence of an intact cell membrane which permitted the cells to intracellularly accumulate fluorescein and exclude EB. Cells which stained red-orange were shown to be dead and incapable of either enzymatically modifying FDA or excluding EB, which forms a fluorescent red-orange complex with double-stranded nucleic acid once inside a bacterial cell.

Although our previous findings have shown that armadillo liver-derived *M. leprae* cells could be stained similarly to cultivable mycobacterial species, technical problems such as a) background fluorescence, b) interfering, host tissue debris, c) extended periods for staining, and d) rapid fading of the green-stained *M. leprae* made it difficult to microscopically observe the bacteria. These problems had to be resolved to permit a clinical evaluation of the staining technique which has the potential of serving as a clinical tool for monitoring the efficacy of chemotherapy in lepromatous (LL) and borderline lepromatous (BL) leprosy patients.

The purpose of this research was twofold. First, to resolve the technical problems associated with staining armadillo-derived *M. leprae* cells, and then to apply the optimized staining method to *M. leprae* cells obtained from leprosy patients and infected mouse foot pads.

MATERIALS AND METHODS

FDA/EB staining solutions. A stock solution of FDA (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was prepared by dissolving 100 mg of FDA in 20 ml of acetone to give a final concentration of 5 mg per ml. One milliliter volumes of the stock solution were transferred to 13 × 100 mm screw-cap test tubes and stored at −20°C protected from light. When stored in this fashion, the stock solutions remained stable for over two years.

A stock solution of EB (Sigma) was prepared by dissolving 20 mg of EB in 10 ml of Hanks' balanced salt solution (HBSS), pH 7.4, to give a final concentration of 2 mg per ml. One milliliter volumes were distributed in 13 × 100 mm screw-cap test tubes and stored at −20°C. This solution was also stable for over two years.

A fresh working solution of FDA/EB was prepared daily by diluting the FDA stock solution 1:10 in acetone to give 500 μg per ml. The 1:10-diluted solution can also be stored at −20°C for at least six months without loss of activity and does away with the need to dilute the stock solution daily. A 0.02 ml volume of the diluted FDA solution was added to 5.0 ml of 0.1 M potassium phosphate buffer, pH 7.2, to give a final concentration of 2.0 μg per ml. Although HBSS was previously used to prepare the working solution ([3]), the phosphate buffer has served equally well, is cheaper, and does away with the need to work aseptically so

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as not to contaminate the HBSS. A 0.01 ml volume of EB stock solution was added to the same 5.0 ml of phosphate buffer, already containing the FDA, giving a final EB concentration of 4.0 μg per ml. The working solution did not deteriorate during the course of a day if protected from light.

Armadillo tissue suspensions. *M. leprae*-infected liver tissues (armadillo R-199 and R-277) containing approximately 2×10^{10} and 2×10^{9} bacteria per gram, respectively, were obtained from the National Hansen's Disease Center, Carville, Louisiana, U.S.A. The tissues were shipped on dry ice; thawed at room temperature upon receipt; subdivided aseptically into 1–5 gram pieces; and refrozen and maintained at −76°C until needed. After thawing the tissue at room temperature, a 20% tissue suspension was prepared by homogenizing 1 part (grams) of tissue in 4 parts (milliliters) of ice cold 0.1 M potassium phosphate buffer, pH 7.2. Homogenization was accomplished in a 100 ml stainless steel Sorvall Omni-Mix tissue homogenizer cup at 35,000 rpm for 1 min on cracked ice. The tissue suspension was transferred to a sterile 13 × 100 mm glass test tube and managed according to experimental protocol.

Human tissue suspensions. Punch biopsies from lepromatous leprosy patients were taken at the Leonard Wood Memorial's facility in Cebu, Philippines, and processed for mouse foot pad (MFP) inoculation. A 10 μl volume of the tissue suspension was uniformly spread over a 1 cm diameter circular area on a microscope slide and air dried prior to staining with FDA/EB.

Mouse foot pad suspensions. Tissue suspensions from infected MFPs were prepared and 10 μl of suspension distributed on a microscope slide as described above. After air drying, the smears were stained with FDA/EB. Companion slides were used for acid-fast staining and measurement of the morphological index (MI) and the number of bacteria per foot pad.

Staining of bacteria with FDA/EB. Two methods of staining were employed depending upon the source of bacteria (human, armadillo, or MFP) and the manner in which the bacterial smears were prepared. Petroleum ether partitioned cells were stained directly on the slides; whereas bacterial smears prepared from tissue suspensions from human skin biopsies or MFPs were stained in a Coplin jar containing FDA/EB working solution. Each method and the rationale for its use will be described in detail in the Results section.

Ultraviolet microscopy. FDA/EB stained mycobacteria cells were observed under incident, ultraviolet (UV) illumination at a magnification of 950× with a Leitz Dialux microscope equipped with a BP 390–490 exciting filter, RKP 510 beam splitting mirror, K480 edge filter, and BG 38 red suppression filter.

RESULTS

Staining petroleum ether partitioned *M. leprae* cells. Armadillo liver tissue suspensions of *M. leprae* contain large amounts of tissue debris and serve as a rich source of esterases. Both the tissue debris and esterases complicated microscopic observation of FDA/EB stained *M. leprae* in their own way. Dead host tissue stained red-orange and obscured red-orange stained *M. leprae* cells adhering to the tissue; whereas tissue esterases hydrolyzed FDA which created a green background fluorescence which masked green-stained bacteria. In either case, an unknown proportion of green and red-orange stained *M. leprae* were not seen, and the risk of acquiring erroneous results was possible.

In order to solve these technical problems, a petroleum ether partition technique designed to take advantage of the hydrophobicity of *M. leprae* cells and the relative insolubility of tissue components in an organic solvent was investigated. The partition technique was performed by adding 1.0 ml of petroleum ether to 0.1 ml of a 20% armadillo liver suspension and mixing for 15–20 sec at room temperature. After 1–2 min, to permit phase separation and large pieces of tissue to settle, bacterial smears were prepared from the petroleum ether layer (top layer) by placing one drop of the suspension on a microscope slide. After evaporation (10–15 sec), the process was repeated one or more times, if necessary, to concentrate bacteria in the smear. The smears were stained by placing 20–30 μl of FDA/EB working solution on the smear which was then covered with a cover glass and sealed with nail polish to prevent evaporation. The slides were incubated at room

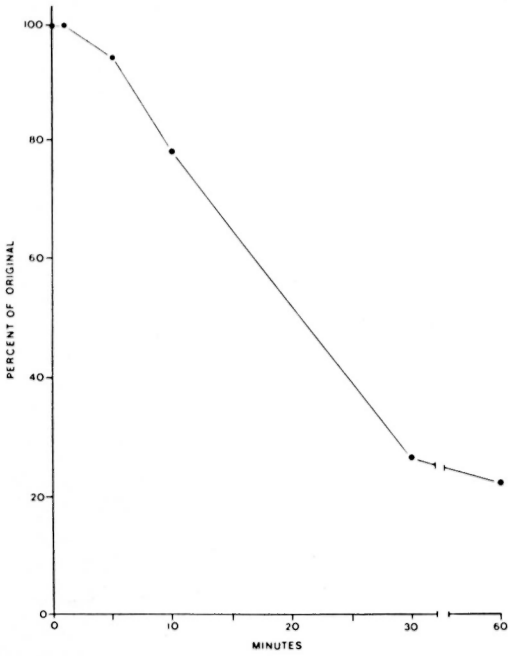

FIG. 1. The effect of petroleum ether at various periods of time on the percentage of armadillo-derived *M. leprae* which stain green.

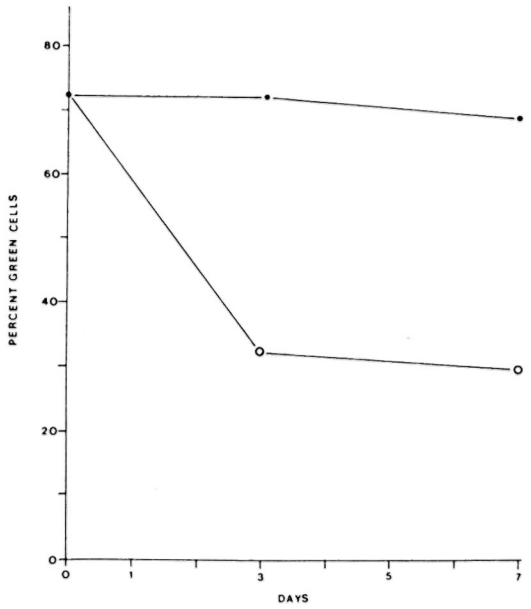

FIG. 2. The effect of time on the percentage of green-stained *M. leprae* cells stored desiccated at 4°C as unstained (●—●) or stained (○—○) smears. Bacterial smears were prepared from armadillo liver by petroleum ether separation.

temperature protected from light for 15–20 min to permit the *M. leprae* cells to stain.

It is important to prepare the smears within 5 min since an appreciable reduction in the percentage of green cells occurred after this time (Fig. 1). Once the bacterial smears were prepared by partitioning, the smears could be held unstained for at least one week at 4°C in the presence of Drierite without a change in the percentage of green-stained *M. leprae* (Fig. 2). Stained smears, however, could not be stored for the corresponding period of time (Fig. 2). Stained smears could be held for at least 3 hr at room temperature without a change in the percentage of green-stained cells (data not shown).

Upon microscopic examination, it was noted that a minimum of red-orange stained tissue debris was present in the smears; green, background fluorescence was minimal to absent; and the *M. leprae* cells stained rapidly and with an intensity of green equivalent to that previously observed with the saprophytic mycobacteria. Once the petroleum ether technique was established as an efficient means of separating *M. leprae* from armadillo liver tissue, the technique was performed with similar results on skin biopsies from LL patients.

Maintenance of green fluorescence with *p*-phenylenediamine (PDA). Johnson and Nogueira Araujo have shown that PDA reduced fading of immunofluorescence ([2]). PDA was studied to determine if it would maintain the fluorescence of green-stained mycobacterial cells. PDA was added to FDA/EB working solutions to give final concentrations of 100, 10, 1.0 and 0.1 μg PDA/ml. Each solution was used to stain *M. smegmatis* and petroleum ether partitioned *M. leprae,* and the fluorescent quality of the cells was assessed over a period of 5 min. All green-stained cells appeared brilliant when first observed, but only *M. smegmatis* cells in the presence of 100 μg PDA/ml maintained their original staining quality after 5 min. The experiment was repeated with *M. leprae,* but this time the FDA/EB staining solution contained 1000 μg PDA/ml. *M. leprae* stained with this solution maintained their original fluorescence for over 5 min and permitted color photographs to be taken. The results show

TABLE 1. *The effect of* p-*phenylenediamine (PDA) on the percentage of green-stained* M. smegmatis *and* M. leprae.

Organism	Trial	Percent green-stained cells exposed to PDA (μg/ml)		
		0	100	1000
M. smegmatis	1	84	84	—
	2	86	84	—
	3	71	75	—
	4	89	91	—
M. leprae[a]	1	59	—	59
	2	34	—	37
	3	49	—	48

[a] Petroleum ether separated, armadillo liver-derived.

that PDA can be used to maintain green fluorescing *M. leprae* if used in proper concentration.

M. smegmatis and *M. leprae* cells were then stained with FDA/EB solution with and without PDA to determine PDA's effect upon the cells. The results presented in Table 1 show that PDA had no effect upon the percentage of green-stained cells.

Staining human and mouse foot pad (MFP)-derived *M. leprae*. Human skin and MFP homogenates contain tissue debris and tissue esterases but not to the same extent as armadillo liver suspensions. An alternative and technically simpler technique was investigated for staining human and MFP-derived *M. leprae*.

Air dried smears of tissue homogenates were immersed in a Coplin jar containing 45 ml of FDA/EB working solution for 10–20 min and gently rinsed by two successive rinses in 45 ml of 0.1 M potassium phosphate buffer, pH 7.2, in separate Coplin jars. After air drying, the stained smears were mounted under a coverslip with Eukitt Mounting Medium (Calibrated Instruments, Inc., Ardsley, New York, U.S.A.). Although tissue esterases were initially present in the tissue smears, the esterases were eluted from the smear and diluted in the staining solution and phosphate buffer rinses. As a consequence, minimal green, background fluorescence was observed. Tissue debris, although present, did not significantly hinder observation of the bacteria. Staining either human biopsies or MFP homogenates can be accomplished in this fashion and is preferable, since it is technically less burdensome. Armadillo liver homogenates, however, cannot be stained in this manner since residual tissue debris and esterase activity made observation of *M. leprae* impossible.

TABLE 2. *Percentage of green-stained* M. leprae *in tissue biopsy homogenates of untreated and drug-treated lepromatous leprosy patients.*

Patient[a]	Period of therapy	Percentage of green-stained *M. leprae*
1	0	85.2
2		66.7
3		65.3
4		57.7
5		57.6
6		56.5
7		49.3
8		49.0
9		44.3
10		37.5
11		32.6
		Avg. = 54.7 ± 14.7
12*	3 months	67.1
13		59.9
14		50.0
15		37.9
16*		35.5
17		31.8
18		16.2
19		12.0
20*		3.7
		Avg. = 34.9 ± 21.7
21*	24 months	37.8
22*		24.9
23		24.5
24		20.3
25*		10.7
26		4.9
27*		4.7
28*		1.1
29		0.9
30		0.8
31*		0.8
32		0.3
33		0.0
34*		0.0
		Avg. = 9.4 ± 12.4

[a] The ten patients annotated with an asterisk had dapsone (DDS)-sensitive infections and underwent dapsone and rifampin therapy; whereas the remaining treated patients had dapsone-resistant infections and received clofazimine and rifampin.

Percentage of green-stained *M. leprae* from tissues of untreated and drug-treated LL patients. Once a satisfactory means of staining *M. leprae* from human biopsies was

TABLE 3. *The morphological index (MI) and percentage of green-stained* M. leprae *in infected mouse foot pads.*

No. of harvests[a]	Avg. time of harvest (mo)	Avg. no. bacteria/MFP ($\times 10^4$)	Percentage of green-stained *M. leprae*	Avg. MI
13	7.2 (5.1–7.8)	344 ± 251 (61–959)	92.6 ± 5.0 (83.0–100)	2.5 ± 2.8 (0–10)
10	9.2 (8.1–9.8)	361 ± 251 (115–898)	92.6 ± 11.0 (63.1–100)	1.4 ± 1.3 (0–3)
6	11.1 (10.2–11.9)	220 ± 118 (113–372)	93.0 ± 5.7 (82.0–96.7)	0.8 ± 1.0 (0–2)
3	12.6 (12.2–13.1)	238 ± 285 (41–565)	93.3 ± 5.4 (87.0–96.6)	0.7 ± 1.2 (0–2)

[a] At each harvest, the foot pads from four mice were pooled and assayed.

developed, the percentage of green-stained *M. leprae* cells from untreated and drug-treated LL patients was determined at the Leonard Wood Memorial's research laboratory in Cebu, Philippines. Three groups of patients consisting of 11 untreated, 9 treated for three months, and 14 treated for 24 months were studied (Table 2). The drug therapy varied, as indicated in Table 2, and depended upon whether the patients were infected with dapsone (DDS)-sensitive or DDS-resistant *M. leprae* as determined by MFP inoculation.

The data (Table 2) indicate that the untreated group is significantly different from the three-month treated ($p < 0.05$) and the 24-month treated ($p < 0.001$) patients as determined by Kruskal-Wallis one-way analysis of variance. Statistical analysis further showed that, as a whole, the two groups of drug-treated patients differed significantly ($p < 0.001$) from the untreated patients and that the patients treated for three months differed significantly ($p < 0.005$) from the 24-month treated patients. The progressive and significant decrease in the percentage of green-stained *M. leprae* was directly proportional to the length of chemotherapy. This indicates that the staining method detected metabolic and physiological differences in the bacterial populations of the untreated and drug-treated patients.

Percentage of green-stained *M. leprae* from MFPs. The percentage of green-stained *M. leprae* present in MFPs infected with homogenates from skin biopsies of LL patients was determined in Cebu by staining the smears from MFP homogenates with FDA/EB in a Coplin jar. The morphological indices were also determined for comparison. The results presented in Table 3 show an average of 92.9% green-stained bacilli in the MFPs with little variation over a course of five months. The total number of bacteria per MFP and the MI decreased with time. There was no correlation between the percentage of green-stained *M. leprae* and the MIs.

DISCUSSION

The ultimate goal of this research is to determine whether the FDA/EB staining procedure can be used to monitor accurately the effectiveness of chemotherapy in LL and BL patients. The technical problems associated with staining tissue-derived *M. leprae* have been solved and permitted the organisms to be stained qualitatively equal to the cultivable mycobacteria. The feasibility of routinely using the FDA/EB staining procedure in a clinical laboratory has been established during the course of this study.

The data (Table 2) suggest that the staining method is providing a measure of the viability of *M. leprae* as evidence by the significant decrease in the percentage of green-stained bacteria upon increased periods of chemotherapy. Although the study does not conclusively prove that a green-stained *M. leprae* cell is viable, the data are consistent with what theoretically would be expected. A comprehensive study has been initiated which will compare the FDA/EB data from serial biopsies to the MI, the bacterial index (BI), MFP infectivity data and, most importantly, to the clinical status of the patients. Patients who respond favorably to therapy should show a progressive

decrease in green-stained *M. leprae*. Conversely, patients who relapse due to secondary drug resistance or noncompliance or who experience primary drug resistant infections should show no decrease or, possibly, an increase in the percentage of viable bacterial cells.

Additional data will also help to confirm and interpret the results already acquired. The data in Table 2, for example, would indicate that, as groups, both treated and untreated patients display a spectrum of viable bacteria in their tissues. This might be a reflection of subtle differences in immunological competence among the patients and/or the stage of the disease process. Variation within the treated groups could be a function of the above as well as of the drug type, dosage, and concentration achieved *in vivo*.

The presence of variable percentages of green-stained bacteria in biopsies of 24-month treated patients is of potential importance since these organisms might represent persistors which have been associated with patient relapse. Follow-up study will determine whether the presence of any detectable green-stained bacteria results in relapse or if some undefined but critical infectious dose is necessary for relapse.

The MFP data (Table 3) are considerably more homogeneous than the patient data with a high percentage of green-stained bacteria. The homogeneity can be explained in part by standardization of inocula size, route of infection and the uniform susceptibility of the inbred mice. The high percentage of green-stained bacteria at 7.2 months (Table 3) would correspond to early stationary phase based upon the growth kinetics of *M. leprae* in MFPs reported by Shepard ([5]). Maintenance of a high but constant proportion of viable cells (Table 3) at later sampling times, i.e., 9.2 months, is characteristic of a bacterial population in the stationary growth phase when a steady-state is achieved. The subsequent reduction (30–36%) in the total number of viable cells per MFP (total count × percent viability) at 11.1 and 12.6 months (Table 3) is indicative of a bacterial population in a death phase and is likely due to the immunological response of the mice. It is, therefore, concluded that the FDA/EB staining data, in combination with total bacterial counts, are providing a reasonable measure of the growth kinetics of *M. leprae* in MFPs.

Comparison of the FDA/EB data and the MI shows that considerable disparity existed between the two methods for measuring the viability of *M. leprae*. Recent reports by Dhople ([1]) and Sathish, *et al.* ([4]) have shown similar disparity. Dhople has shown that there is no correlation between the MI and the ATP content of *M. leprae*; whereas Sathish, *et al.* have shown no correlation between the MI and the ability of *M. leprae* cells to take up tritiated thymidine while in macrophage culture.

SUMMARY

A fluorescent staining procedure incorporating the use of fluorescein diacetate (FDA) and ethidium bromide (EB) has previously been shown to accurately measure the viability of saprophytic mycobacterial cells. Green-stained cells were shown to be viable and red-stained cells, dead. Staining *Mycobacterium leprae* cells with FDA/EB, however, was complicated by interfering tissue components which masked the presence of stained bacteria.

A petroleum ether separation technique enables *M. leprae* to be segregated from armadillo liver tissue components and permitted *M. leprae* to be stained qualitatively equal to the saprophytic mycobacteria. An alternative and technically simpler method of staining *M. leprae* from human skin biopsies and mouse foot pads was developed which permitted the initiation of a clinical assessment of the staining method. Preliminary data indicate that patients who have undergone three or 24 months of chemotherapy possess a significantly lower percentage of green-stained *M. leprae* in their tissues than untreated patients. This would be expected if the FDA/EB staining method was providing an accurate measure of viability.

M. leprae cells obtained from mouse foot pads which were harvested 5–13 months post-infection displayed more than 90% green-stained cells. There was no correlation between the FDA/EB staining method and the morphological index.

RESUMEN

Previamente se ha demostrado que el procedimiento de tinción fluorescente con diacetato de fluoresceína

(DAF) y bromuro de etidio (BE) permite medir con gran precisión la viabilidad de micobacterias saprofíticas. Las células viables se tiñen de verde en tanto que las no viables se tiñen de rojo. Sin embargo, la tinción del *Mycobacterium leprae* con FDA/EB se ha visto complicada por la interferencia de los componentes tisulares que enmascaran la presencia de las bacterias.

Aquí se describe una técnica de separación con éter que permite segregar al *M. leprae* de los componentes tisulares hepáticos, facilitando entonces su tinción. El método desarrollado es técnicamente simple y permite la tinción del *M. leprae* en biopsias de piel humana y en tejido de cojinete plantar del ratón a la vez que permite la valoración clínica del especímen según sus características de tinción. Los datos preliminares indican que los pacientes que han sido tratados por 3 ó 24 meses poseen en sus tejidos porcentajes significativamente menores de *M. leprae* teñidos de verde que los pacientes no tratados. Esto es lo que se esperaría si el método de tinción con DAF/BE realmente proporcionara una medida exacta de viabilidad.

Los bacilos (*M. leprae*) obtenidos de los cojinetes plantares del ratón 5–13 meses después de la infección, tuvieron una alta proporción (más del 90%) de células teñidas de verde. No hubo correlación entre el método de tinción con DAF/BE y el índice morfológico.

RÉSUMÉ

On a montré antérieurement qu'un procédé de coloration fluorescente utilisant le diacétate de fluorescéine (FDA) et le bromide d'éthidium (EB) permettent de mesurer de façon précise la viabilité de cellules mycobactériennes saprophytes. On a montré que les cellules colorées en vert étaient viables, alors que les cellules colorées en rouge étaient mortes. La coloration des cellules de *Mycobacterium leprae* par FDA/EB est cependant rendue compliquée suite à l'interférence de constituants tissulaires qui masquent la présence des bactéries colorées.

Une technique de séparation à l'éther de pétrole rend possible la séparation de *M. leprae* des constituants tissulaires du foie de tatou, et permet ainsi une coloration qualitative de *M. leprae* similaire à celle des mycobactéries saprophytes. On a développé une méthode de remplacement, d'ailleurs plus simple techniquement, pour colorer des bacilles de la lèpre obtenus dans des biopsies de lèpre humaine et dans le coussinet plantaire de la souris; cette méthode permet d'entreprendre une évaluation clinique de la méthode de coloration. Les données préliminaires indiquent que les malades qui ont été soumis à la chimiothérapie pendant une durée de 3 à 24 mois, hébergent un pourcentage significativement plus faible de *M. leprae* colorés en vert dans leur tissu, que ne le font des malades non traités. C'est ce à quoi on pourrait s'attendre si la méthode de coloration par le FDA/EB fournissait une mesure précise de la viabilité.

Des cellules de *M. leprae* recueillies à partir de coussinets plantaires de la souris, 5 à 13 mois après l'infection, ont montré plus de 90% de cellules colorées en vert. Il n'y avait aucune corrélation entre la méthode de coloration par le FDA/EB et l'index morphologique.

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