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A Fluorescent Staining Procedure for Determining the Viability of Mycobacterial Cells[1]

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Fluorescein diacetate (FDA) was first used by Rotman and Papermaster to measure the viability of mammalian cells ([12]). They showed that FDA, a non-polar, non-fluorescent fatty acid ester, passes readily into living cells where it is hydrolyzed by esterases to yield polar, fluorescent fluorescein. The fluorescein rapidly accumulates inside the cells, if membrane integrity is intact, resulting in green-stained cells when viewed under fluorescent microscopy. In contrast, dead cells remain non-fluorescent. Based on cloning experiments with mammalian cells in tissue culture, it was demonstrated that a one-to-one correlation between green-stained cells and cloning ability existed ([12]). This led them to propose the use of FDA as a staining method for determining the viability of cells. Later this concept was adopted by a number of investigators to assess the viability of mycoplasma ([4]), plant cells ([13]), pollen ([5]), spores ([9]) and bacteria ([6,10]).

In contrast, Edidin developed a technique to detect damaged and dead cells by using ethidium bromide (EB) ([3]). This compound will only penetrate cells whose cell membrane integrity has been altered. Once inside the cell, EB intercalates and forms red-orange fluorescent complexes with double-stranded nucleic acid ([2,7]). Later both FDA and EB were combined into a single assay for lymphocyte cytotoxic reactions ([8]) and for measuring the viability of granulocytes ([1,11]). More recently, a semi-quantitative study was reported by Jarnagin and Luchsinger in which FDA and EB were used as a means of assessing mycobacterial viability ([6]). Our study confirmed the work of Jarnagin and Luchsinger but differed in that it is quantitative, reveals the existence of dual-stained cells, and expands the potential usefulness of the staining method to other microbes.

In our study, we adopted the FDA/EB staining procedure and modified it as a means of measuring the viability of mycobacterial cells. It has proven to be a rapid, accurate, and quantitative means of determining the viability of saprophytic mycobacterial cells, and possesses the potential for wider application to other microbial cells including *Mycobacterium leprae*.

MATERIALS AND METHODS

Bacteria. *Mycobacterium smegmatis* ATCC 607 and *M. phlei* ATCC 354 were inoculated on Löwenstein-Jensen Medium (BBL, Cockeysville, Maryland, U.S.A.) and incubated at 37°C for 72 hr. The stock slants were stored at 4°C for later use. Fresh stock slants were prepared every 16 weeks.

Media. Dubos Liquid Medium (DLM) (Difco, Detroit, Michigan, U.S.A.) containing 0.4% glycerol was used as a source of broth cultures after inoculation with the appropriate organism from a stock culture and incubation at 37°C on a rotary shaker at 180 rpm. Dubos Agar Medium (DAM) was used for plating and was the same as DLM except for the addition of 1.5% agar.

Single-cell suspensions. Due to the lipophilic nature of the cell walls of mycobacteria, the organisms form clumps in liquid medium. Therefore single-cell suspensions were prepared to ensure reliable plate and total counts. A 2.0 ml aliquot of DLM culture was centrifuged at room temperature for 15 min at 3000 × *g*, the supernatant fluid decanted, and the cell pellet resuspended in

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Hanks Balanced Salt Solution (HBSS) without phenol red (Gibco, Grand Island, New York, U.S.A.) containing 0.05% Tween-80. The suspension was aseptically transferred to a 10 ml syringe and filtered through a 5 μm membrane filter. The filtration procedure provided a single-cell suspension containing 10^6 to 10^7 cells per milliliter, and the presence of Tween-80 minimizing cell reclumping. All viability tests were performed using single-cell suspensions.

FDA/EB solutions. A stock solution of FDA (Sigma, 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 distributed to 13 × 100 mm screw cap test tubes and stored at −20°C protected from light which hydrolyzes FDA upon extended exposure. When stored in this fashion, the stock solutions remained stable for over two years.

A stock solution of EB (Sigma, St. Louis, Missouri, U.S.A.) was prepared by dissolving 20 mg of EB in 10 ml of HBSS, pH 7.4, containing 0.05% Tween-80 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 likewise 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. A 0.02 ml volume of the diluted FDA solution was added to 5.0 ml of HBSS containing 0.05% Tween-80 to give a final concentration of 2 μg per ml. A 0.01 ml volume of EB stock solution was then added to the same 5.0 ml of HBSS, containing the FDA, giving a final EB concentration of 4 μg per ml. The working solution did not deteriorate during the course of a day's work if protected from light.

Staining of bacteria with FDA/EB. A 0.5 ml volume of the FDA/EB working solution was added to a 1.0 ml aliquot of single-cell suspension and incubated for 10 min at room temperature. After a wet mount was prepared and sealed to prevent evaporation, the suspension was observed under incident and ultraviolet illumination at a magnification of 950× with a Leitz Dialux microscope. The microscope was equipped with a BP390-490 exciting filter, a RKP 510 beam splitting mirror, a K480 edge filter, and a BG38 red suppression filter. A total of 200 cells were counted in replicate and differentiated on the basis of their color. Green cells were considered to be live; whereas red-stained cells were considered to be dead. Percent viability was calculated by dividing the number of green cells by the total number of cells observed and multiplying by 100.

Viable plate counts. A series of 10-fold serial dilutions of the single-cell suspension were made using HBSS containing 0.05% Tween-80 as the diluent. Plating in triplicate was done by inoculating 0.1 ml volumes of the appropriately diluted single-cell suspension on DAM. The inoculum was evenly distributed on the agar surface with a flamed L-shaped glass rod. Inoculated plates were incubated at 37°C for three days before plate counts were made.

Total counts were performed with a Neubauer-Hausser counting chamber. A 1.0 ml volume of the original single-cell suspension was autoclaved at 121°C for 10 min to kill the bacteria. A 0.5 ml volume of the FDA/EB working solution was added to the killed single-cell suspension and both chambers of the hemacytometer filled with stained-cell suspension. After a 10-min incubation at room temperature, to permit staining and the cells to settle, bacterial counts were performed in duplicate under incident UV illumination at 400× magnification. Although 950× magnification can be used, we have found that the stained cells were easily discernible at 400× and that the counts could be performed more rapidly.

Since the original submission of this manuscript, it has been found that it is less time consuming but equally accurate if the cells are not first killed. The contaminated hemacytometers are sterilized by submersion in 70% ethanol followed by autoclaving. Percent viability was calculated by dividing the number of viable bacteria per ml by the total number of bacteria per ml and multiplying by 100.

Photography. Forty-second exposures were made using Kodak Ektachrome slide film ASA 400. Photographic prints were made from the slides.

TABLE 1. *The effect of fluorescein diacetate (FDA) and/or ethidium bromide (EB) on the viability of* Mycobacterium smegmatis *as determined by plate counts.*

Time (hr)	No. of bacteria (×10) per ml of HBSS containing			
	—	FDA	EB	FDA/EB
0	1.4	1.5	1.4	1.4
3	1.6	1.5	1.6	1.4
6	1.6	1.4	1.6	1.5

TABLE 2. *Comparison of mycobacterial cell viability as determined by plating and staining with FDA/EB.*

Organism	Culture age (hr)	Percent viability determined by	
		Plating	FDA/EB
M. phlei	22	95	96
	24	83	92
	27	82	92
M. smegmatis	18	87	95
		87	94
	20	92	96
		93	94
	22	81	93
		94	96
	24	90	92
		90	97
		85	90
		96	90
		91	92

RESULTS

Staining qualities of mycobacterial cells treated with FDA/EB. As the first step in the study, cell suspensions of *M. smegmatis* or *M. phlei* were treated with FDA/EB to determine whether the cells exhibited the same staining qualities as those observed with mammalian cells ([1,11]). It was observed (Fig. 1) that FDA/EB consistently stained 90% to 97% of log phase cells green and the remaining cells red-orange. After autoclaving, 100% of the cells from the same cultures stained red-orange. These observations were similar to those obtained with mammalian cells ([1,11]). These results made further study possible since they indicated that FDA entered the mycobacterial cells and that the cells had the enzymatic ability to hydrolyze FDA to fluorescein which was then accumulated intracellularly. The results also indicated that EB could penetrate the lipoidal cell wall of the mycobacteria after cell membrane disruption by autoclaving and intercalate with DNA.

Since mycobacterial cells form clumps, it was equally important to show that both FDA and EB had the ability to penetrate clumps and stain the cells within the clumps. Figure 2 shows that cell clumps are as efficiently stained as single cells and that the cells can be readily discriminated.

Influence of FDA and/or EB on mycobacterial viability. In order to determine whether FDA and/or EB were influencing the viability of the organisms during the staining procedure, a suspension of *M. smegmatis* was subdivided into 1.0 ml aliquots and exposed to FDA or EB, singly and in combination, at the concentrations used during the staining procedure. After various periods of exposure, a 0.1 ml volume of cell suspension was removed from each tube, diluted in HBSS and plated on DAM, and plate counts were performed. The data in Table 1 show that neither FDA and/or EB had any effect upon the viability of the cells during an incubation period of up to 6 hr at room temperature.

Correlation between the FDA/EB staining method and plate counts. In order to establish the accuracy of the FDA/EB method for determining mycobacterial viability, the method was compared to standard bacteriological plate counts using 18 hr to 27 hr DLM cultures of either *M. smegmatis* or *M. phlei* grown at 37°C. The results presented in Table 2 show that a high degree of correlation ($p < 0.005$) existed between the two methods with an average difference of 6%. The consistently higher percent viability yielded by the staining method is probably due to a small amount of cell reclumping after single-cell suspensions were prepared. Upon plating, two or more clumped cells would grow into a single colony just as a single cell would, and as a result give lower plate counts. Losses due to the adherence of bacteria to the L-shaped spreading rod would also account for the lower counts. These quantitative results established the FDA/EB staining method as

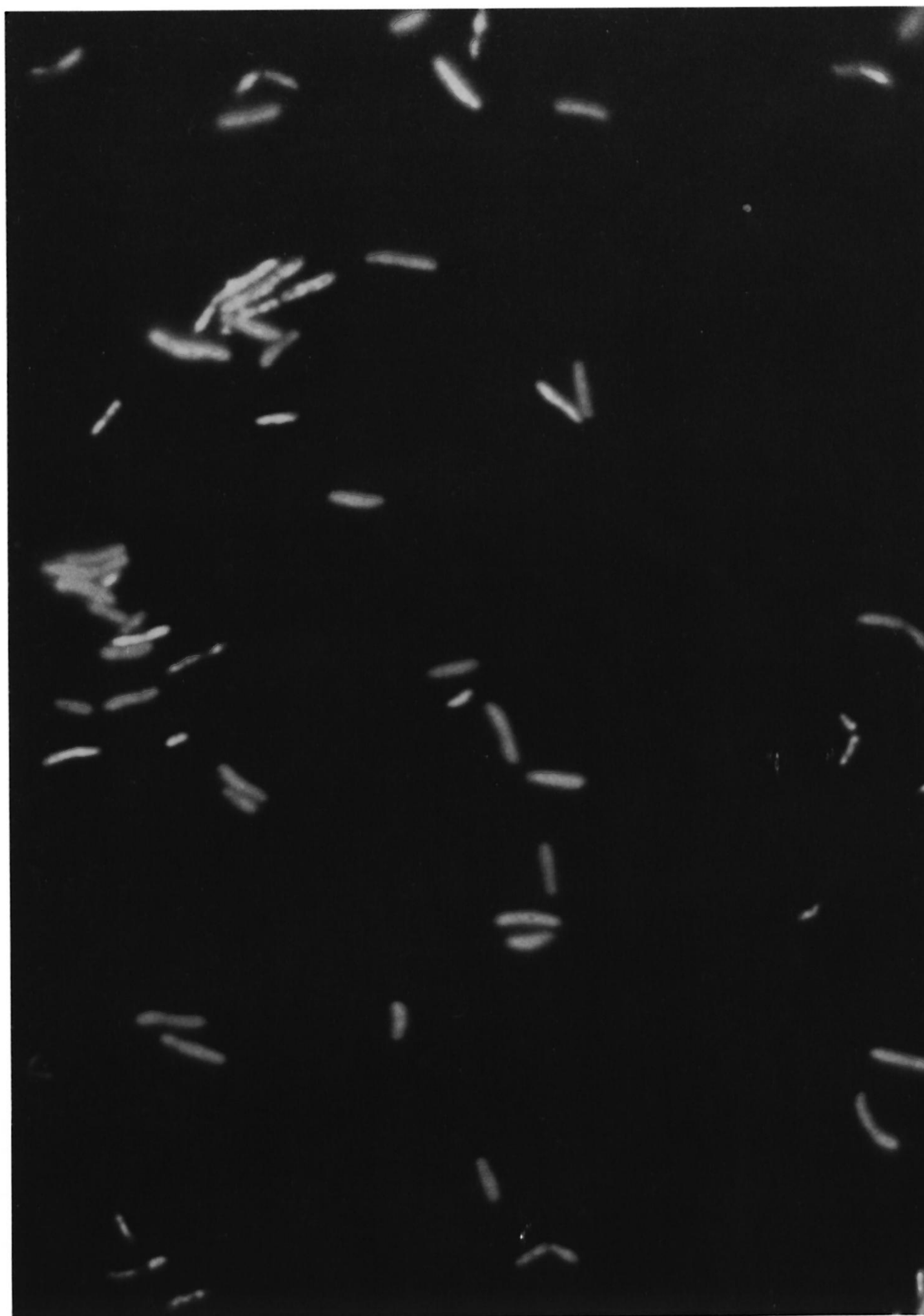

FIG. 1. A suspension of *M. smegmatis* stained with FDA/EB for 10 min at room temperature. (×950).

an accurate means of measuring mycobacterial viability.

Heating of cell suspensions. Once the accuracy of the staining procedure was established, it was further evaluated using mycobacterial cells grown and/or treated under conditions which are known to exert a detrimental effect upon the viability of bacteria. Heating is a physical method which reduces cell viability by disrupting cell

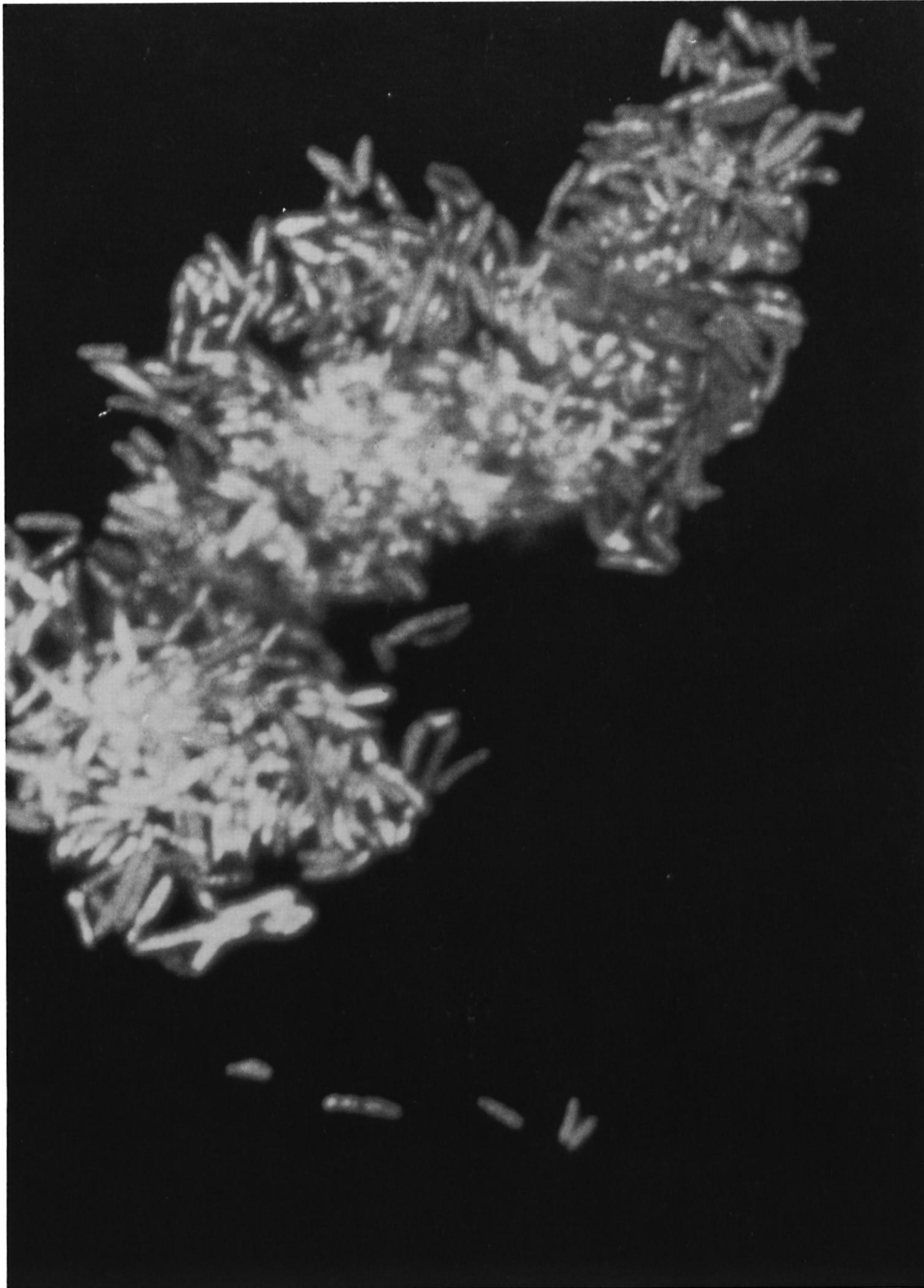

FIG. 2. A clump of *M. smegmatis* stained with FDA/EB for 10 min at room temperature (×950).

membranes and denaturing structural proteins and enzymes.

Log phase (22 hr), single-cell suspensions of *M. smegmatis* were exposed for various lengths of time to a temperature of 60°C and percent viability determined by FDA/EB and plating. The results from a representative experiment shown in Figure 3 indicate a significant overall reduction in percent viability when measured by either of

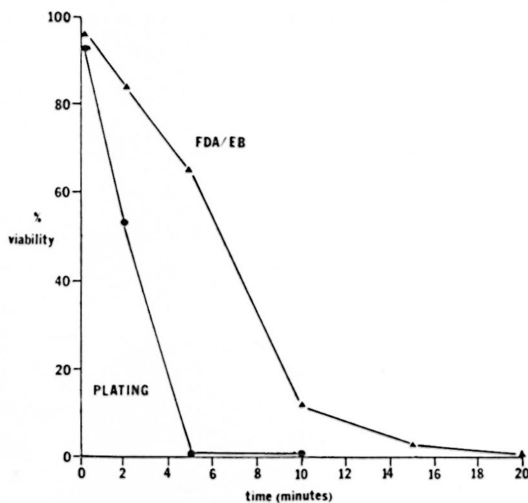

FIG. 3. The effect of heat (60°C) on the percent viability of a single-cell suspension of *M. smegmatis* as determined by plating and FDA/EB.

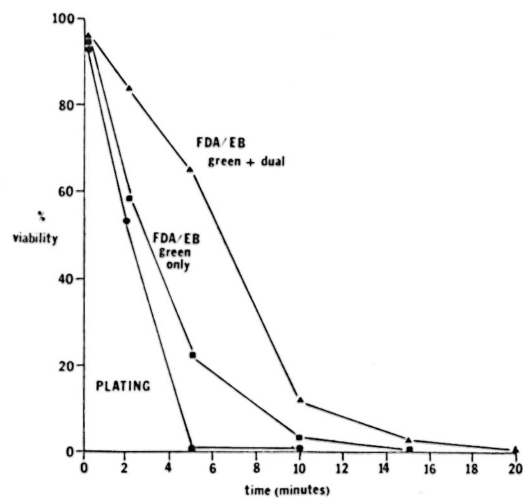

FIG. 4. The percent viability of a suspension of *M. smegmatis* heated at 60°C for 20 min.

the two methods, but not absolute agreement. During the course of the experiments, however, it was observed that in addition to the previously recognized populations of uniformly stained green (live) and red-orange (dead) cells, a population of dual-stained cells appeared. These cells are characterized by a green periphery of variable thickness which would indicate acetylesterase activity; and a diffuse to well-defined red-orange nuclear area which would indicate penetration of EB, a characteristic of cells with a damaged cell membrane ([3]).

Since the dual-stained cells were considered to be alive in the above experiment, they were considered to be the obvious source of discrepancy. The experiment was repeated, but this time the dual-stained cells were enumerated and considered dead. Figure 4 shows that when only uniformly stained green cells were considered to be alive, a much closer agreement between the two methods was observed and confirmed that dual-stained cells should be considered dead by the criterion of being unable to grow under the cultural conditions used in the experiment.

Prolonged incubation. To determine whether the FDA/EB method gave an accurate measure of viability when bacteria were exposed to other means of cell damage, and to see if the appearance of dual-stained cells was unique to cells which had been heated, the effects of prolonged incubation were studied. This condition adversely affects the viability of a bacterial population by the accumulation of toxic metabolic by-products and/or the depletion of essential nutrients.

M. smegmatis were cultured over a period of ten days at 37°C on a rotary shaker in 100 ml of DLM. After inoculation the culture was periodically sampled, and after single-cell suspensions were prepared, viability was determined by plating and the FDA/EB method. Figure 5 shows the results of a representative experiment. The data are plotted to show percent viability, as determined by FDA/EB, when uniformly stained green and dual-stained cells were considered as a group to be alive, and when only uniformly stained green cells were considered to be alive. Once again, a much closer agreement between the two methods of determining viability was observed when only uniformly stained green cells were considered to be alive. The data also indicate that the appearance of dual-stained cells is not restricted to cells that have been heated and, therefore, represent a more generalized phenomenon which would be expected if dual-stained cells represent damaged cells.

Staining of *M. leprae*. As a result of the

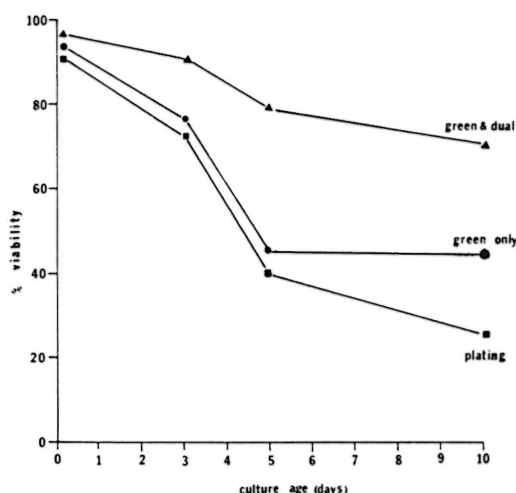

FIG. 5. The effect of extended incubation at 37°C upon the percent viability of *M. smegmatis* as determined by plate counts and FDA/EB.

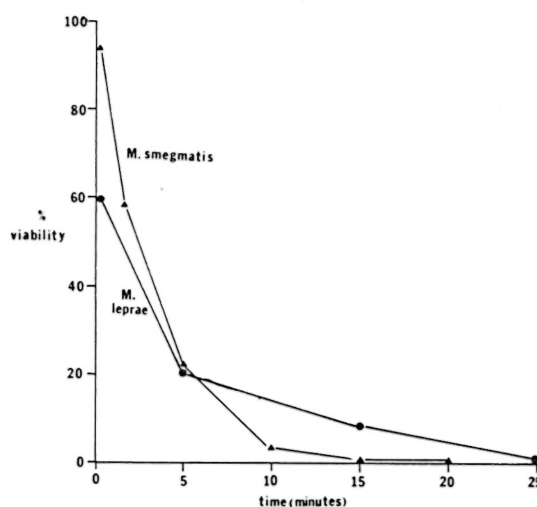

FIG. 6. The effect of heat on the percent viability of *M. leprae* and *M. smegmatis* as measured by FDA/EB. The *M. leprae* cells were heated at 50°C; whereas the *M. smegmatis* cells were heated at 60°C.

successful staining of the saprophytic mycobacteria, application of the FDA/EB method to *M. leprae* became feasible. A suspension of armadillo-derived *M. leprae* containing a 4×10^6 cells per ml of 0.1 M phosphate buffer, pH 7.4, was stained for 45 min at room temperature. This period of incubation was necessary to achieve the same intensity of fluorescence as was previously observed with the saprophytes, and may reflect upon either the rate at which FDA can enter the cells or the rate of enzymatic generation of fluorescein. Three distinctly stained populations of cells (green, dual, and red) were observed, as previously seen with the saprophytes. This indicates that *M. leprae* cells possess acetylesterase with adequate cell membrane function for retention of fluorescein and the exclusion of EB.

In order to determine whether *M. leprae* would be affected by heat in a fashion similar to *M. smegmatis*, a suspension of *M. leprae* was heated at 50°C which is approximately 15°C higher than its temperature for growth in animals, and is the same temperature differential used for *M. smegmatis*. The data presented in Figure 6 show the decrease in percent viability of *M. leprae* closely paralleled that of *M. smegmatis*. This would be expected if the staining method was giving an accurate measure of the viability of *M. leprae*.

Staining of other microbes. In order to determine whether the staining method might have wider application, a number of other microorganisms were treated with the staining solution by the method previously

TABLE 3. *FDA/EB staining of various microorganisms.*

Type of microbe	Genus and species	Successful staining
Bacteria	*M. bovis*	+
	M. vaccae	+
	M. tuberculosis H37Ra	+
	M. scrofulaceum	+
	M. lepraemurium	+
	Escherichia coli[a]	+
	Salmonella typhimurium[a]	+
	Neisseria gonorrhoeae	+
	Bacillus megaterium	+
	Staphylococcus aureus	+
	Treponema pallidum[b]	–
Fungi	*Trichophyton rubrum*	+
	Trichophyton mentagrophtes (spores)	+
	Microsporum gypseum (spores)	+
Protozoa	*Leishmania donovani*	+

[a] Suspensions were prepared from Trypticase Soy Agar slants.

[b] Suspensions were prepared in 0.1 M phosphate buffer from infected rabbit testicles.

described. Although no attempt was made to correlate percent viability as determined by staining and plate counts, the staining method was shown to stain a variety of other microorganisms to yield both green- and red-stained cells (Table 3). Of the 15 organisms used, only *Treponema pallidum* did not stain to yield both green and red cells. Only red cells were observed with this organism even though it was known that the suspension contained live cells as evidenced by their motility. These results would indicate that the staining method may eventually be of use in the measurement of the viability of a wide range of microbial species.

DISCUSSION

The feasibility of using the FDA/EB staining method for determining the viability of mycobacterial cells was investigated and based on previous studies by Rotman and Papermaster ([12]), Edidin ([3]) and Dankberg and Persidsky ([1]). The staining method was shown to be an accurate, quantitative, and rapid means of measuring mycobacterial viability. Our results show that all the cells are either stained green, dual, or red since there is a high degree of correlation between FDA/EB staining and plate counts/total counts. The concentrations of FDA (2 μg/ml) and EB (4 μg/ml) used by us were clearly satisfactory (Table 2) and were without effect upon the viability of the saprophytic cells (Table 1).

The successful staining of a variety of other microorganisms (Table 3) suggests that the staining method has the potential for broader use by both clinicians and basic researchers, especially when non-cultivable organisms (i.e., *M. leprae*) are involved. Our ultimate goal is to further develop the staining method and determine whether it provides an accurate measure of the viability of *M. leprae*. This could serve as an aid in attempts to cultivate the organism *in vitro* and also provide an economical (1000 assays/10 cents), technically simple, and timely way of determining the prognosis of leprosy patients undergoing chemotherapy. It could also be of equal value to the clinical management of other diseases if the staining method is shown to accurately measure the viability of the etiological agent.

Several technical difficulties remain unresolved regarding the staining of *M. leprae*. Host-derived esterases generate fluorescein in the suspending liquid, which results in considerable background fluorescence. This interferes with the visualization of the bacteria when fresh homogenates were used. This problem dissipates after several weeks of incubation during which time the host-derived esterases become inactivated. Prior treatment of fresh homogenates with a protease may help reduce the amount of host-derived esterases and thereby the amount of background fluorescence. The period of time required to achieve the same intensity of staining as observed with the other microbes (Table 3) is considerably longer for *M. leprae*. This phenomenon is being studied to determine whether adjustments in temperature, pH and/or the concentration of FDA will accelerate the staining. It has also been observed that highly fluorescent green-stained *M. leprae* cells lose their fluorescence in less than 1 min when exposed to ultraviolet light from the microscope. This not only makes it difficult to take photographs but requires that *M. leprae* suspensions be adjusted to a concentration which enables a worker quickly to discriminate and enumerate the number of green-stained cells within a microscopic field. We have found that 400× magnification is satisfactory for observing *M. leprae* cells and that the quenching of the fluorescence is not as rapid at this magnification as it is at 950× magnification.

Although the data to date are encouraging and would indicate that an alternative method of measuring the viability of *M. leprae* may be possible, work must yet be accomplished to show that a green-stained *M. leprae* cell is in fact a live cell.

SUMMARY

A fluorescent staining procedure has been developed which rapidly, accurately, and economically measures the viability of mycobacterial cells. *M. smegmatis* and *M. phlei* have served as prototype organisms to establish conditions which ensure optimal staining.

The staining method incorporates the use of the fatty acid ester fluorescein diacetate (FDA) and ethidium bromide (EB). Non-

polar, non-fluorescent FDA enters live cells where it is enzymatically hydrolyzed by acetylesterase to polar, fluorescent fluorescein which rapidly accumulates in the cytoplasm. These cells appear green when viewed under incident ultraviolet illumination. Ethidium bromide enters dead cells and intercalates between the bases of DNA molecules. These cells appear red-orange under UV illumination. Live cells are, therefore, identified on the basis of possessing acetylesterase and their ability to exclude EB; whereas dead cells are identified on the basis of lacking acetylesterase and their inability to exclude EB. The feasibility of applying the staining procedure of *M. leprae* has been investigated and the results are encouraging. Our findings reveal that armadillo-derived *M. leprae* possess acetylesterase and, therefore, stain green. *M. leprae* cell suspensions exposed to adverse physico-chemical conditions give rise to high proportions of red-stained cells as would be expected if the cells are being killed. An alternative means of determining the viability of *M. leprae* appears to be feasible.

RESUMEN

Se ha desarrollado un procedimiento de tinción fluorescente para medir rápida-, precisa- y económicamente, la viabilidad de células micobacterianas. El *Mycobacterium smegmatis* y el *M. phlei* sirvieron como prototipos para estudiar y establecer las condiciones óptimas de tinción.

El método de tinción incorpora el uso de un éster de la fluoresceína (diacetato de la fluoresceína, FDA) y del bromuro de etidio (EB). El FDA, no polar, no fluorescente, entra a las células vivas donde es hidrolizado enzimáticamente por la acetilesterasa hasta fluoresceína que es polar y fluorescente y que se acumula rápidamente en el citoplasma. Estas células aparecen verdes cuando se observan bajo iluminación ultravioleta. El bromuro de etidio entra a las células muertas y se intercala entre las bases de las moléculas de DNA. Estas células se ven de color rojo bajo luz ultravioleta. Las células vivas son, por lo tanto, identificadas porque poseen acetilesterasa funcional y por su capacidad para excluir al EB, mientras que las células muertas se identifican por carecer de acetilesterasa y por su incapacidad para excluír al EB. Se ha investigado la posibilidad de aplicar esta técnica de tinción para el *M. leprae* y se han obtenido resultados alentadores. Nuestros hallazgos revelan que el *M. leprae* crecido en el armadillo posee acetilesterasa y por tanto se tiñe de verde. Las suspensiones de *M. leprae* expuestas a condiciones fisicoquímicas adversas contuvieron altas proporciones de microorganismos teñidos de rojo, como se esperaría en el caso de suspensiones de células muertas; por lo tanto, es posible aplicar esta técnica como una forma alternativa de evaluar la viabilidad del *M. leprae*.

RÉSUMÉ

On a mis au point un procédé de coloration fluorescente qui permet de mesurer rapidement, de manière précise, et à faible coût, la viabilité des cellules mycobactériennes. On a utilisé *Mycobacterium smegmatis* et *Mycobacterium phlei* comme organismes prototypes, en vue d'établir les conditions qui permettent d'obtenir la meilleure coloration.

La méthode de coloration était basée sur l'utilisation du diacétate de fluoresceine (FDA), un ester d'acide gras, et du bromure d'ethidium (EB). Le diacétate de fluoresceine non fluorescent, étant dépourvu de polarité, pénètre dans les cellules vivantes où il est hydrolysé par une enzyme, l'acetylesterase, en fluoresceine, fluorescente et polaire, qui s'accumule alors rapidement dans le cytoplasme. Ces cellules prennent une coloration verte lorsque l'on les illumine par des rayons ultraviolets incidents. Le bromure d'ethidium pénètre dans les cellules mortes, où il s'intercale entre les bases des molécules d'ADN. Ces cellules prennent une coloration rouge orangée, sous illumination par les ultraviolets. Les cellules vivantes peuvent dès lors être identifiées, en prenant comme critère la présence d'acetylesterase, et leur capacité à exclure le bromure d'ethidium; par contre, les cellules mortes sont dépourvues d'acetylesterase, et ne peuvent exclure le bromure d'ethidium. On a exploré dans quelle mesure il était pratique d'appliquer ce procédé de coloration à *M. leprae*. Les résultats ont été encourageants. Les observations menées en recourant à ce procédé de coloration ont permis de montrer que les cellules de *M. leprae* recueillies chez le tatou possèdent de l'acethylesterase, et dès lors elles se colorent en vert. Les suspensions de cellules de *M. leprae* soumises à des conditions physico-chimiques défavorables livrent une proportion élevée de cellules colorées en rouge, ainsi qu'on peut s'y attendre lorsque ces cellules ont été tuées. Il apparaît donc possible d'utiliser une nouvelle méthode pour déterminer la viabilité de *M. leprae*.

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