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Comparison of Radiometric Macrophage Assay and Fluorescein Diacetate/Ethidium Bromide Staining for Evaluation of *M. leprae* Viability[1]

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It is becoming increasingly evident that alternate methodologies to the mouse foot pad model are required to make possible the early evaluation of viability of *Mycobacterium leprae* for diagnosis of drug resistance, monitoring of chemotherapy, and designing of newer antileprosy drugs. Over the last decade, several *in vitro* techniques have been reported. These include the use of radiolabel incorporation in *M. leprae* maintained in macrophage cultures ([6–10, 12, 13, 15, 16, 20]) or specialized media ([1, 3]) as well as the measurement of ATP levels ([2]), the presence of esterase ([4, 14]), and intracellular cation ratios ([17]). Radiolabel uptake has been a particularly useful tool for the investigation of *M. leprae* metabolism ([21, 22]). Our laboratory has been routinely using ^{3}H-thymidine uptake by *M. leprae* resident macrophage cultures as an indicator of bacillary DNA synthesis and as a useful assay for evaluating drug resistance ([10, 16]), studying antileprosy drugs ([7, 8]), and monitoring drug therapy. This assay has been miniaturized ([6]) and compares well with the mouse model as evaluated in independent centers in double-blind trials ([10, 16]). Moreover, it has been possible to study biopsies sent to our New Delhi laboratory from field areas situated over 1000 miles away. To develop a simpler methodology which would be useful in the semi-urban field areas, we have investigated and compared our assay with the fluorescein diacetate/ethidium bromide (FDA/EB) staining reported by Kvach, *et al.* ([4]). This methodology uses the property of the enzyme, esterase, present in viable *M. leprae* to degrade FDA, a nonfluorescent fatty acid ester, to a green fluorescing compound. Dead bacilli, being unable to exclude ethidium bromide, would show red fluorescence. In the present paper, we report good correlation of the ^{3}H-thymidine uptake assay with FDA/EB as done on *M. leprae* obtained from the same biopsies. The usefulness and limitations of the FDA/EB method were also investigated.

MATERIALS AND METHODS

Patients. Of 87 lepromatous patients, 81 were from Baroda District, an endemic area in India, and 6 patients were attending the outpatient clinics of Safdarjang Hospital, New Delhi. Skin biopsies from these patients under sterile precautions were taken from active lesions using 6-mm punches (Steifel Laboratories, U.K.). The biopsies were maintained and air shipped on wet ice to New Delhi, frozen on arrival, and stored at −70°C.

Extraction of *M. leprae*. *M. leprae* from the dermal lesions were extracted by standard techniques using a Mickle's Disintegrator (The Mickle Lab., Surrey, U.K.) ([18]). The extract obtained was centrifuged at 500 rpm for 10 min to remove tissue debris. The supernate was collected and the bacterial concentration estimated by the method of Shepard and McRae ([19]). The extracted ba-

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cilli were screened for cultivable contaminants as described earlier ([13]).

Reagents for fluorescent staining. A stock solution of fluorescein diacetate (FDA; Sigma Chemical Co.) was prepared in acetone (BDH, Bombay, India) at a concentration of 5 mg per ml. One-ml aliquots were distributed in screw-cap tubes protected from light and stored at −20°C. No loss of activity was observed over a period of 6 months. Ethidium bromide (EB; Sigma) stock solution was prepared at a concentration of 4 mg per ml in phosphate-buffered saline (PBS) (0.1 M, pH 7.4); 1 ml quantities being stored at −20°C.

Fresh working solutions were prepared as required. The stock solution of FDA was diluted in acetone (1:10) to give a concentration of 500 μg per ml. The above solution was further diluted in PBS to give a concentration of 2 μg per ml. Ten μl of the EB stock solution was added to the above tube and thoroughly mixed (8 μg/ml).

The mounting fluid contained P-phenylenediamine (PDA; Sigma) at a concentration of 1 mg per ml in 90% glycerol, PBS (0.01 M, pH 7.4). The pH was adjusted to 7.8 using bicarbonate buffer (0.5 M, pH 8). Five-ml aliquots were stored at −20°C protected from light.

Staining of bacteria with FDA/EB. *M. leprae* smears were made in 8-mm ring slides. Five μl of the suspension was carefully layered in the ring and air dried. The smears were fixed by flaming once, overlaid with the staining mixture, covered with Whatman filter paper strips, and further saturated with the staining solution. The slides were incubated in a humidified chamber at 37°C for 30 min protected from light. They were subsequently washed with PBS and air dried. The smears were covered with the PDA mountant and viewed under epi-illumination using an HB 50 mercury lamp as a source of ultraviolet light and an oil immersion 100× objective. A total of 10 fields were examined for green and red fluorescing bacteria. Bacilli which exhibited dual fluorescence, i.e., red and green, were counted as red. Beaded or bipolar green fluorescing bacilli were considered positive and included in the counting. Clumps of bacteria were excluded from enumeration. The above smears were subsequently stained by the standard acid-fast staining technique, and the total number of acid-fast bacilli (AFB) was determined as above.

Uptake of ^{3}H-thymidine by *M. leprae* resident murine macrophages. The details of the methodology are described elsewhere ([4]). In brief, differentiated, residential, murine peritoneal macrophages maintained in 96-well flat-bottomed plates maintained in RPMI 1640 with 20% fetal calf serum (FCS) were inoculated with "live" and heat-killed *M. leprae* (5×10^5/well). Fluorescent staining was carried out in parallel with an aliquot of the "live" bacterial suspension. Nonphagocytosed bacilli were removed after 16 hr of incubation at 37°C. The cultures were incubated with ^{3}H-thymidine (0.2 μCi/well, 49 Ci/mMole; Amersham, U.K.) for a 10- to 12-day period. The cultures were harvested after stripping the cells with 2% xylocaine (Astra; IDL, India). The cell suspension was transferred onto glass fiber (Whatman paper GF/C, U.K.) using a cell harvester (Ilacon, U.K.). The discs were dried and processed for liquid scintillation counting in a LKB Rackbeta 1712 (Finland). The incorporation index was expressed as the mean counts per minute (cpm) of cultures infected with "live" *M. leprae*/mean cpm of cultures infected with heat-killed *M. leprae*.

Slit-skin smear examination. Slit-skin smears were made from the earlobes and eyebrows of treated and untreated patients. The smears were fixed by flaming once and stored at 4°C. They were stained with FDA/EB solutions within 1–2 weeks, and examined as described earlier.

The statistical significance of the data was established by the Student's *t* test. Comparative analysis of the incorporation index versus the number of green bacilli in an inoculum was done by Pearson product moment correlation analysis.

RESULTS

Comparisons of both *in vitro* methodologies for the evaluation of *M. leprae* viability were concurrently undertaken on bacillary extracts obtained from lepromatous patients from endemic areas. The bacilli extracted from skin biopsies of 73 bacilliferous patients were screened for viability using: a) the radiometric macrophage assay

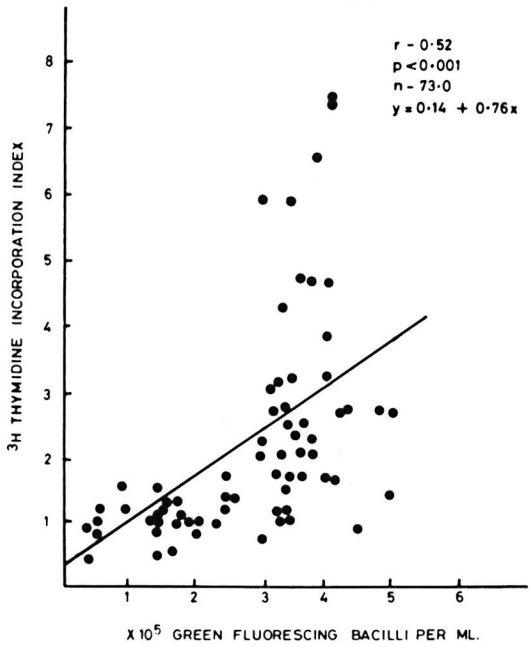

FIG. 1. Correlation of ^{3}H-thymidine incorporation index in the radiometric macrophage (Mø) assay and the number of green fluorescing bacilli by FDA/EB staining in the same bacillary extracts. Individual values of 73 samples (●) are plotted. Pearson correlation coefficient is 0.52 ($p < 0.001$ by the t test). Incorporation index = mean cpm of Mø cultures with "live" bacilli/mean cpm of Mø cultures with heat-killed bacilli.

using ^{3}H-thymidine uptake in cultures containing "live" bacilli as an indicator of DNA synthesis and compared with b) FDA/EB staining, where green fluorescing bacilli indicate the presence of an esterase activity associated with an integral membrane function and red bacilli reflect entry of ethidium bromide into nonviable organisms. In addition, bacilli from slit-skin smears of patients undergoing treatment were examined by the FDA/EB staining method.

Biopsies. ^{3}H-Thymidine uptake expressed as the incorporation index (mean cpm of cultures with "live" bacilli/mean cpm of cultures with dead bacilli) ranged from 0.5 to 7.5 in the 73 biopsies tested. Absolute numbers and percent of green fluorescing bacilli, ranging from 0.25 to 5 × 10^5 ml and 5% to 100%, were present in the bacillary extracts at the time of inoculation of the same isolates into macrophage cultures. Comparison of the two assays showed a linear relationship with a high significance value ($p < 0.001$). It may be pointed out that a good fit between the data from the two assays occurred when both absolute numbers per ml and percent of green bacilli were computed against the incorporation index. Data for the former are depicted in Figure 1. The number of green bacilli is higher than what can be accounted for by the cpms of ^{3}H-thymidine uptake. Therefore, it appears that all green bacilli in the inoculum may not undergo DNA synthesis during the limited period of the macrophage assay. Although the radiometric assay monitors a more complex biological phenomenon in the mycobacterial cell, it is of significance that the presence of the enzyme, esterase, correlated well with DNA synthesis.

Further studies were carried out on biopsies to evaluate the significance of FDA/EB staining as a monitoring test of treatment status. Forty-one patients who had received less or more than 1 year of treatment with a multidrug regimen of 100 mg daily of dapsone, 100 mg of clofazimine on alternate days, and 600 mg pulse therapy of rifampin were compared. In keeping with the above studies, it was found that when absolute numbers of green bacilli/ml were calculated, the group of patients treated for more than 1 year showed a significant decrease ($p < 0.01$) of green bacilli as compared to the 0–1 year treated group (Fig. 2A). However, a comparison of the percent of green fluorescing bacilli in the biopsies showed no significant differences in the early and long-term-treated groups of patients. Besides the differences in the number of green bacilli in the two groups of patients, notable differences were observed in the extent of ^{3}H-thymidine incorporation by the bacilli derived from them. Fifty-eight percent of the short-term-treated patients showed twofold or more of uptake of ^{3}H-thymidine as opposed to only 36% in the latter group.

Slit-skin smears. Since slit-skin smear examination would be operationally more feasible than obtaining skin biopsies, systematic efforts were made to standardize and evaluate the value of FDA/EB in the field. In preliminary studies on fresh bacilli extracted from stored armadillo tissue, 90% showed green fluorescence. Beaded and bi-

THE TABLE. *FDA-EB staining evaluation in longitudinal studies in treated patients.*

Patients	Duration of treatment[a] (mos.)					
	0		3		6	
	BI[b]	GB[c]	BI	GB	BI	GB
1	2+	73	2+	63	2+	42
2	3+	68	3+	61	2+	65
3	4+	72	4+	63	3+	39
4	4+	68	4+	62	3+	44
5	4+	86	ND[d]	ND	3+	60
6	3+	54	3+	52	1+	46
7	3+	68	2+	48	2+	45

[a] Received multidrug treatment 100 mg of dapsone/day, 100 mg of clofazimine on alternate days, and 600 mg of rifampin pulsed.
[b] Bacterial index.
[c] Ratio of green fluorescing bacilli seen in 10 microscopic fields/smear from four different sites expressed as percent.
[d] ND = not done.

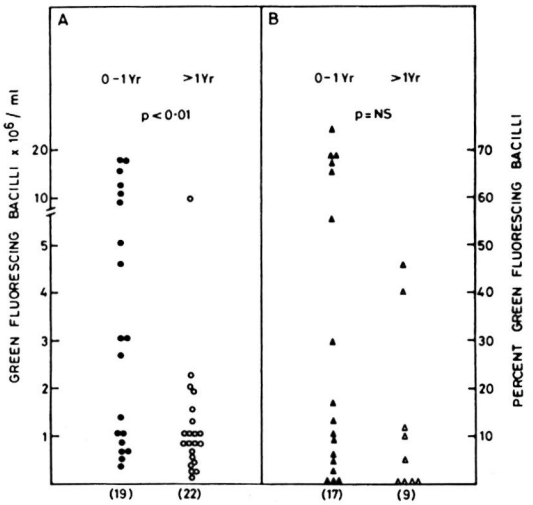

FIG. 2. Scattergram depicting the qualitative and quantitative effect of treatment on green bacilli as assessed by FDA/EB in individual bacilliferous leprosy patients' skin biopsies (A) and in slit-skin smears (B). Duration of treatment 0–1 year (●, ▲) and >1 year (○, △). Figures in parentheses indicate number of patients in each group.

polar staining as well as dual staining were routinely observed. Autoclaving at 15 lbs for 15 min showed only red fluorescence, indicative of death, and confirmed the earlier observations of Kvach and Veras ([5]).

Smears prepared from a single batch of armadillo-derived bacilli were exposed to various environmental stresses to optimize the field conditions required to obtain reproducible results. Smears were heat fixed by flaming once, or air dried and stored at room temperature (non-air-conditioned, 40°C) or in a refrigerator (4°–8°C). The smears were stained with the fluorogenic staining mixture on days 1, 4, 7, and 14. No statistical reduction in green bacilli was observed in the heat-fixed smears stored at 4°C over a 14-day period; whereas smears stored at the ambient tropical temperature of 40°C showed a significant reduction by day 7.

Further studies were undertaken using slit-skin smears for multibacillary patients from the endemic areas and Delhi clinics. The smears were heat fixed, stored at 4°–8°C, and stained with FDA/EB within 14 days (Fig. 2B). Since it was not possible to compute absolute numbers of bacilli in slit-skin smear samples, the data are expressed as a percent of green bacilli. Comparison of patient groups treated for <1 year or >1 year did not show statistical differences. However, a trend was observed with a number of samples with >60% green bacilli being seen in the former group and <50% in all of the long-term-treated group.

Longitudinal studies were carried out on seven patients attending the New Delhi hospital. Smears were made from these patients at 3-month intervals. The results showed a gradual decrease in the number of green bacilli in all patients (The Table). Three of these patients were available for follow up at 9, 12, and 18 months: Patients 2 and 4 showed no green bacilli by the 18th month associated with a fall in the bacterial index (BI) from 3–4+ to 1+; although no appreciable change in the BI was seen in patient 5, the number of green bacilli had fallen from 86% to 18% at the end of 18 months.

DISCUSSION

There is a significant concordance ($p < 0.001$) when percent and absolute numbers of green fluorescing bacilli stained by the FDA/EB method are compared concurrently with the ^{3}H-thymidine uptake in *M. leprae* resident macrophage cultures. It would appear that, in general, the presence of esterase in *M. leprae* correlated with the metabolic events leading to DNA synthesis. It may, however, be pointed out that esterase may still be active soon after *M. leprae*

are dead, and DNA synthesis is preceded by multiple, more complex biological events than a simple enzymic interaction. Moreover, the above comparison does not prove that every green bacillus is alive and capable of multiplication. The number of green bacilli were usually higher than the speculated level of DNA synthesis in the bacillary inoculum, as evidenced by the mean cpms of ^{3}H-thymidine uptake. Moreover, beaded and bipolar staining were also observed, indicating a lack of correlation between the morphological index (MI) and the presence of esterase. Interestingly, Odinsen, *et al.* ([11]) also reported an excellent comparison between a fluorescent cationic probe R123 and FDA/EB but none with the MI of the bacilli.

Since FDA/EB staining would be operationally feasible in field areas and can be employed on slit-skin smears, we made systematic efforts to optimize the conditions required for reproducible results. Heat fixation of smears with a single flaming was not deleterious, although autoclaving led to red fluorescence (death) of all bacilli. It was of interest that ambient temperatures of 40°C reduced the percent of green bacilli only after the first week, and maintenance of smears between 4°–8°C in a refrigerator up to 14 days had no effect. It would, thus, be possible to bring slit-skin smears from the field to the leprosy center where they could be stored in a refrigerator until further study. An added advantage of this method is that the same smears can be stained by Ziehl-Neelsen stain after the FDA/EB study is completed. Thus, the traditional BI and the FDA/EB staining can be undertaken without additional stress for the patient or the field worker.

The monitoring of chemotherapy using FDA/EB staining on slit-skin smears of 26 patients showed a trend but not a statistical significance between the patient groups who had <1 year and >1 year of treatment. This may be due to the small number of patients studied. Longitudinal studies are under way to evaluate the change in percent green bacilli in the same patients following multidrug therapy.

SUMMARY

Earlier studies from our laboratory reported that a radiometric *Mycobacterium leprae* resident macrophage assay was a useful *in vitro* indicator of bacillary viability with good correlation with the established mouse foot pad model. The present study compares our assay with the recently described fluorescein diacetate/ethidium bromide (FDA/EB) method. *M. leprae* extracted from the dermal lesions of 73 bacilliferous leprosy patients were tested concurrently by both techniques. Good correlation ($r = 0.52$, $p < 0.001$) was found between the radiometric assay evaluating DNA synthesis and the FDA/EB staining reflecting the presence of active esterase enzyme. In addition, the utility of the FDA/EB staining in the monitoring of therapy was established. Twenty-two patients treated for >1 year showed lower numbers of green fluorescing bacilli when compared to 19 untreated or short-term-treated individuals.

RESUMEN

Previamente hemos reportado que el ensayo radiométrico de los *Mycobacterium leprae* residentes en macrófagos era un útil indicador *in vitro* de la viabilidad bacilar, que mostraba una buena correlación con el bien establecido método de la almohadilla plantar del ratón. El presente estudio compara el ensayo radiométrico con el recientemente descrito método del diacetato de fluoresceína/bromuro de etídio (DAF/BE). Los *M. leprae* extraídos de las lesiones dérmicas de 73 pacientes lepromatosos bacilíferos se probaron simultáneamente por ambas técnicas. Se encontró una buena correlación ($r = 0.52$, $p < 0.001$) entre el ensayo radiométrico que evalúa la síntesis de DNA y el método del DAF/BE que refleja la presencia de una esterasa activa. Además, se estableció la utilidad de la tinción con DAF/BE en el seguimiento de los efectos terapéuticos. Comparados con 19 individuos sin tratamiento o con periodos cortos de tratamiento, 22 pacientes tratados por más de 1 año mostraron números más bajos de bacilos verdes fluorescentes (viables).

RÉSUMÉ

Des études antérieures menées dans ce laboratoire ont montré qu'une épreuve radiométrique des mycobactéries de la lèpre situées dans les macrophages, était utile comme indicateur *in vitro* de la viabilité bacillaire; de plus, cette épreuve montrait une bonne corrélation avec les résultats obtenus par le modèle classique du coussinet plantaire de la souris. Cette étude compare cette épreuve radiométrique avec la méthode récemment décrite au diacétate de fluorescéine et au bromure d'éthidium (FDA/EB). On a étudié en parallèle par ces deux techniques des bacilles *M. leprae* extraits de lésions dermiques chez 73 malades atteints de lèpre ba-

cillifère. Une bonne corrélation ($r = 0,52$, $p < 0,001$) a été trouvée entre l'épreuve radiométrique qui évalue la synthèse de l'ADN, et la coloration par la méthode FDA/EB, qui traduit la présence d'une estérase active. De plus, on a pu établir l'utilité de cette méthode de coloration FDA/EB pour le contrôle de la thérapeutique. Chez vingt-deux malades traités pendant plus d'un an, on a observé un nombre de bacilles, colorés en vert par la méthode fluorescente, plus faible que chez 19 malades non traités ou traités pendant peu de temps.

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