INTERNATIONAL JOURNAL OF LEPROSY
Volume 73, Number 3
Printed in the U.S.A.
(ISSN 0148-916X)

Effects of Purification and Fluorescent Staining on Viability of *Mycobacterium leprae*[1]

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ABSTRACT

Over the years, researchers have carried out experiments with *Mycobacterium leprae* obtained from either human multibacillary lesions, or infected armadillo tissues, or infected footpad tissues of conventional mice as well as athymic nu/nu mice. In general, these sources of leprosy bacilli are satisfactory for most biochemical and mouse footpad studies, but less than satisfactory for studies in cell biology and immunology where contaminating host tissues pose a serious problem. We examined the utility of a procedure for eliminating mouse footpad tissue from *M. leprae* suspension using sodium hydroxide solution and its subsequent effect on the viability of the organism by determining the rate of palmitic acid oxidation, bacterial membrane integrity, and growth in the mouse footpad. We found that treating *M. leprae* suspension, obtained from infected nu/nu mouse footpad, with 0.1N NaOH for 3 min was sufficient to remove the majority of mouse tissue without adversely affecting the viability of the organism. This is a simple and rapid method to get suspensions of nu/nu footpad-derived viable *M. leprae* essentially free of host tissues, which can be a research reagent for studying the host-pathogen relationship in leprosy. We also report here a method for labeling *M. leprae* with the fluorescent dye PKH26, without compromising on the viability of the organism. This method may be useful in intracellular trafficking studies of *M. leprae* or in other cell biology studies that require tracking of the bacteria using fluorescent tag. We observed the staining to be stable *in vitro* over considerable lengths of time and did not affect the viability of the bacteria.

RÉSUMÉ

Depuis des années, les chercheurs ont mené des expériences à partir de *Mycobacterium leprae* issues de lésions multibacillaires humaines, de tissus infectés de tatous à neuf bandes ou bien de tissu de coussinets plantaires de souris tant conventionnelles que nues athymiques (nu/nu). Ces sources de bacilles de Hansen sont en général satisfaisantes pour la plupart des études biochimiques et d'inoculation au coussinet plantaire de souris, mais pas satisfaisantes pour les études de biologie cellulaire et d'immunologie, où les éléments contaminants provenant de l'hôte peuvent représenter un sérieux problème. Nous avons vérifié l'utilité d'une procédure à base de soude pour éliminer les tissus plantaires de souris contaminant les suspensions de *M. leprae*, en vérifiant son effet sur la viabilité de la bactérie par la détermination du taux d'oxydation de l'acide palmitique, de l'intégrité de la membrane de la bactérie et de la croissance dans le coussinet plantaire de souris. Nous avons trouvé que le traitement pendant 3 minutes avec 0.1 N NaOH, de suspensions de *M. leprae* obtenues à partir de coussinets plantaires de souris nu/nu, était suffisant pour enlever la majorité des tissus de souris sans pour autant affecter de façon adverse la viabilité de l'organisme. C'est une méthode simple et rapide, qui permet d'obtenir des suspensions viables de *M. leprae* à partir de lépromes de souris nu/nu dévolues presque entièrement de tissus de l'hôte, représentant de meilleurs réactifs de recherche pour étudier la relation hôte-pathogène de la lèpre. Nous rapportons également ici une méthode pour marquer les *M. leprae* avec le produit fluorescent PKH26, sans compromettre la viabilité du microorganisme. Cette méthode peut être utile pour étudier les mouvements et localisations intracellulaires de *M. leprae* ou bien des études de biologie cellulaire, où le marquage de la bactérie par un colorant fluorescent est requis, afin de pouvoir la suivre. Nous avons constaté que le marquage était stable *in vitro* pendant des temps importants et n'affectait pas la viabilité de la bactérie.

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[1] Received for publication on Feb. 28, 2005. Accepted for publication on May 15, 2005.

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RESUMEN

A lo largo del tiempo los investigadores han realizado experimentos con *Mycobacterium leprae* obtenido de lesiones humanas multibacilares, de tejidos infectados de armadillo y de las almohadillas plantares de ratones convencionales y atímicos, *nu/nu*. En general, los bacilos de la lepra obtenidos de estas fuentes son satisfactorios para la mayoría de los estudios bioquímicos y para su inoculación en las almohadillas plantares del ratón, pero no son satisfactorios para estudios inmunológicos y de la biología celular donde los tejidos contaminantes del huésped representan un verdadero problema. Nosotros examinamos la utilidad de un procedimiento para eliminar el tejido de la almohadilla plantar de una suspensión de *M. leprae* usando una solución de hidróxido de sodio, y estudiamos el efecto de este tratamiento en la viabilidad del organismo (por oxidación del ácido palmítico), en la integridad de su membrana, y en su capacidad de replicación en la almohadilla plantar del ratón. Encontramos que el tratamiento de la suspensión de *M. leprae* obtenida de la almohadilla plantar de ratones *nu/nu* con NaOH 0.1N por 3 min fue suficiente para remover la mayoría del tejido de ratón sin afectar sensiblemente la viabilidad del microorganismo. Este es un método simple y rápido para obtener suspensiones viables del microorganismo esencialmente libres de tejido, a partir de la almohadilla plantar del ratón desnudo, que pueden usarse para estudiar las relaciones entre el bacilo y el hospedero. También reportamos aquí un método para marcar *M. leprae* con el colorante fluorescente PKH26 que puede ser de utilidad en los estudios del tráfico intracelular del microorganismo o en otros estudios de la biología celular donde se requiere localizar la bacteria usando una marca fluorescente. Observamos que la tinción es estable *in vitro* por periodos largos de tiempo y que no afecta la viabilidad de la bacteria.

Over one hundred thirty years after its discovery as the causative agent of leprosy, *Mycobacterium leprae* is yet to be cultured *in vitro*. This obstacle has not stymied experimentation with leprosy bacilli of human origin or from infected armadillos or the mouse footpad, although adequate numbers, purity, and questionable viability of *M. leprae* have affected experimental reproducibility and presented additional obstacles to researchers.

In order to have weekly access to large numbers of highly viable *M. leprae,* we maintain several isolates in serial passages in athymic nu/nu mice where growth in the footpad routinely produces a few billion organisms. We have adapted radiorespirometry (RR) procedures to measure oxidation of radiolabeled palmitic acid ([5]) to compare viability of different suspensions of *M. leprae* as defined by metabolic activity. RR was shown to correlate well with growth in the mouse footpad (MFP) ([22]). Recently, we have employed evaluation of the membrane integrity of individual *M. leprae* in a suspension with LIVE/DEAD BacLight Bacterial Viability Staining (VS) Kit® as an additional assay of bacterial viability on the assumption that the bacilli with damaged membrane are dead ([11]). However, even nu/nu MFP derived *M. leprae* suffer from one drawback and that is the presence of contaminating mouse tissue in the bacterial suspension. While, this contaminating host tissue does not affect subsequent passage to a new host or some *in vitro* investigations, it is absolutely not desirable in studies designed to observe immunological reactions, intracellular trafficking, and pathogenesis of the disease, which is markedly different from that of tuberculosis. Slow speed centrifugation removes larger pieces of mouse tissue from the suspension, but counterstaining of the acid-fast bacilli (AFB) gives clear evidence for unacceptable levels of remaining mouse tissue in the suspension.

In this study, we examined the utility of a procedure for eliminating mouse footpad tissue from *M. leprae* suspension using sodium hydroxide (NaOH) solution and its subsequent effect on the viability of the organism as defined by RR and VS. We also report here a method for labeling *M. leprae* with highly aliphatic reporter molecules containing fluorochrome groups, without compromising on the viability of the organism as defined by RR, VS and growth on the MFP.

METHODS AND MATERIALS

Nude mouse-derived *M. leprae*. *Mycobacterium leprae* (isolate Thai-53) is maintained in serial passage in the footpads of athymic nu/nu mice (Harlan, Indianapolis, Indiana, U.S.A.). Mice were inoculated on the plantar surface of both hind feet with 5

× 10^7 fresh, viable nu/nu-derived *M. leprae*. When the mouse footpads became moderately enlarged (at ~6 months), they were harvested for intracellular *M. leprae* as described previously ([22]), washed by centrifugation (18,000 g for 30 min), resuspended in either medium 7H12 or RPMI-1640 (Gibco Invitrogen, Carlsbad, California, U.S.A.) + 10% (v/v) fetal calf serum [(FCS) Gibco Invitrogen, Carlsbad, California, U.S.A.], enumerated by direct count according to Shepard's method ([18]) and held overnight at 4°C, pending quality control testing for contamination. The bacterial suspension was passed 3 to 4 times through a 27G needle prior to counting in order to remove clumps. Freshly harvested bacilli were always employed in experiments (within 24 hr of harvest).

NaOH treatment of *M. leprae*. 1 × 10^9 fresh *M. leprae* were resuspended in 1.0 ml of the appropriate concentration of NaOH [0.1N–0.9N] (Sigma, St. Louis, Missouri, U.S.A.) and incubated for 3 min at room temperature, after which the bacteria were washed (10,000 g for 5 min at 4°C) thrice in 7H12 medium and finally resuspended in appropriate media. There was a 30 to 50% loss of bacteria in this process.

Scanning Electron Microscopy. Ten µl suspension (1 × 10^9/ml) of 0.1N NaOH treated or untreated *M. leprae* were spread on poly-lysine coated plastic cover slips, air dried, fixed, and washed prior to 1% Osmium tertraoxide treatment. Following which the cover slips were washed in deionized water and then dehydrated by several changes of 30% to 100% ethyl alcohol. After dehydration the samples were subject to critical point drying prior to sputter coating with gold and palladium. The samples were then visualized in a FEI Quanta 200 scanning electron microscope

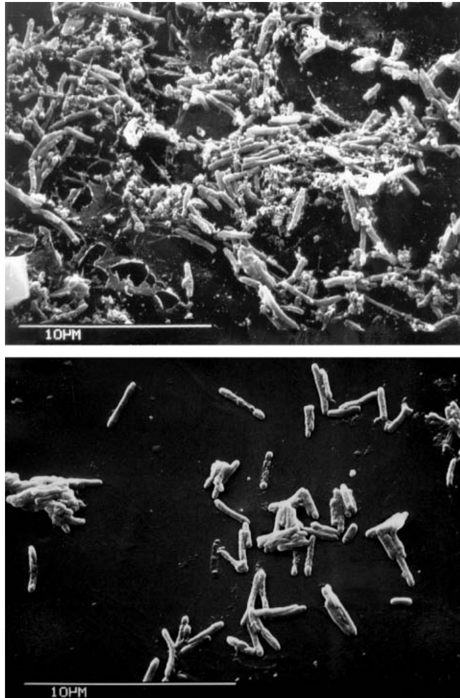
Radiorespirometry. 1 × 10^7 *M. leprae* were inoculated into 1.0 ml of BACTEC 7H12B media (Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.) containing ^{14}C-palmitic acid in a loosely capped vial which, in turn, was inserted into a wide mouth liquid scintillation vial lined with filter paper impregnated with NaOH, 2,5-diphenyloxazole (Sigma, St. Louis, Missouri, U.S.A.) and Concentrate I (Kodak, Rochester, New York, U.S.A.) and incubated at 33°C. When read daily, captured $^{14}CO_2$ determines the rate of ^{14}C-palmitic acid oxidation ([5]). In the present study, on the seventh day cumulative counts per minute (CPM) are reported.

Fluorescent staining for assessing bacterial membrane integrity. The membrane integrity of individual *M. leprae* in a suspension was evaluated with LIVE/DEAD BacLight Bacterial Viability Staining (VS) Kit® (Molecular Probes, Eugene, Oregon, U.S.A.) as described previously ([11]). Briefly, *M. leprae* were washed (10,000 g for 5 min) in normal saline and incubated for 15 min at room temperature with 6 µM Syto9 and 30 µM propidium iodide (PI). After staining the bacteria were resuspended in 10% (v/v) glycerol in normal saline, passed through 27G needle to dissociate clumps, and the percentage of dead and live bacteria in the suspension were enumerated by direct counting of fluorescent green and red bacilli using appropriate single bandpass filter sets.

At least 200 individual bacteria or 10 microscopic fields, whichever was more, were counted to evaluate the percentage of bacteria having membrane damage in the suspension.

Staining with PKH dyes. Freshly harvested *M. leprae* treated with 0.1N NaOH (1 × 10^9) were resuspended in 1.0 ml of the provided "diluent C" and then stained for 2 min at room temperature with a 1:250 dilution of either PKH26 (red) or PKH67 (green) dye (Sigma, St. Louis, Missouri, U.S.A.). After 2 min the staining was halted by adding an equal volume of FCS. The suspension was washed (10,000 g for 5 min) thrice in appropriate medium. The numbers of bacteria were recounted following staining by Shepard's direct count method ([18]).

Macrophage culture. Resident peritoneal cells from Swiss mice were harvested and allowed to adhere for at least 6 hr at 37°C and 5% (v/v) CO_2, on plastic cover slips in 24 well tissue culture plates (Corning, Corning, New York, U.S.A.) as previously described ([14]). After washing to remove non-adherent cells, the adherent cells were infected overnight at 33°C with PKH26 stained *M. leprae* at a multiplicity of infection of 20:1. At the end of the incubation extracellular *M. leprae* were removed by washing the cover slips.



a

b

FIG. 1. Scanning electron photomicrograph (6200×) showing nu/nu mouse footpad derived *M. leprae* suspension before (a) and after (b) treatment with 0.1N NaOH for 3 min. Most of the mouse tissue had been removed by the treatment and subsequent washings.

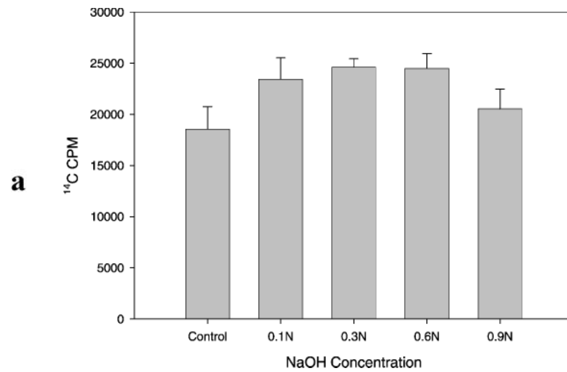


FIG. 2. Effect of different concentrations of NaOH treatment for 3 min on palmitic acid oxidation rate of *M. leprae*. Bargraph showing the cumulative seventh day radiorespirometry counts ± S.D. The data are representative of five independent experiments. *M. leprae* were obtained freshly from athymic (nu/nu) mouse footpads for each experiment.

Footpad growth of *M. leprae*. BALB/c mice, 5 in each group, were inoculated on the plantar surface of both hind feet with 1×10^4 PKH26 stained or control *M. leprae*. At 3 and 6 months both hind footpads were harvested, processed and the number of AFB enumerated using Shepard's technique.

Statistical analysis. The data are shown as means ± standard deviation (S.D.) from a representative of three to four experiments. The raw data were subjected to one-tailed or two-tailed Student's *t* test to determine whether the observed differences between the means were significant. $p < 0.05$ was taken as significant.

RESULTS

Scanning electron microscopy of NaOH treated *M. leprae*. To observe the effects of NaOH treatment on the appearance of a suspension of nu/nu footpad derived *M. leprae*, suspensions were treated for 3 min with 0.1N NaOH, washed and observed with the S.E.M. The results (Fig. 1) showed that treatment resulted in a marked clearance of mouse footpad tissues from the *M. leprae* suspension in comparison to untreated controls. We did not observe any significant improvement in the quality of the *M. leprae* suspension, by scanning electron microscopy, following treatment with 0.1N NaOH for a longer period or with higher concentrations of NaOH (data not shown).

Effects of NaOH treatment on metabolic activity of *M. leprae*. To determine the effects of NaOH treatment on sustained *in vitro* metabolic activity, *M. leprae* were treated with 0.1N, 0.3N, 0.6N, or 0.9N NaOH for 3 min at room temperature, washed in 7H12 medium and prepared for RR. The seventh day RR data (Fig. 2) showed no significant differences in the cumulative oxidation of radiolabeled palmitic acid between the control and the NaOH treated *M. leprae*. However, a significant fall in the RR was observed when the 0.6N or higher concentration NaOH treatment was carried out for longer than 7 min (data not shown).

Effects of NaOH treatment on membrane integrity of *M. leprae*. To assess the effects of different NaOH treatments on the membrane integrity of *M. leprae* BacLight fluorescent staining was done. In this assay all the bacilli in the suspension stain with Syto9 (green), i.e. both those with intact membranes as well as those with damaged membranes but the bacilli having damaged

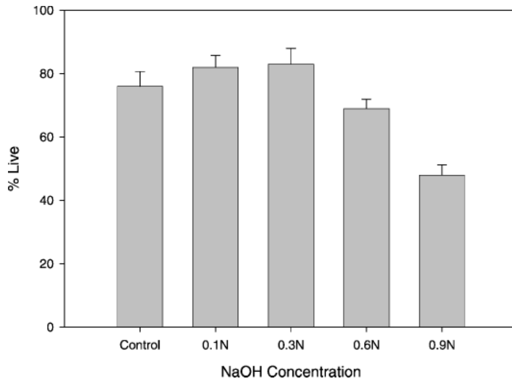


FIG. 3. Effect of different concentrations of NaOH treatment for 3 min on membrane integrity of *M. leprae*. Bargraph showing the percentage live *M. leprae* ± S.D. as determined by the viability staining. The data are representative of three independent experiments. *M. leprae* was obtained freshly from athymic (nu/nu) mouse footpads for each experiment.

membrane also stain with PI (red). This assay assumes that all the bacteria having damaged membrane (staining red) are non-viable or dead. The data (Fig. 3) clearly showed that 3 min treatment with 0.1N, 0.3N or 0.6N NaOH did not impart any significant membrane damage. However, treatment with 0.9N NaOH for the same duration resulted in a significant decrease ($p < 0.0001$) in the number of *M. leprae* having intact membrane.

Staining of *M. leprae* with PKH26 dye. The staining of *M. leprae* with PKH26 dye was done after treating the suspension of bacilli with NaOH. We chose the 3 min treatment with 0.1N NaOH as described above. A 1:250 dilution of PKH26 dye provided bright red fluorescent bacteria that maintained solid fluorescence for at least 15 days when held *in vitro* in dark at 4°C (data not shown). Similar findings were observed when the green (PKH67) dye was employed. We used 3 different dilutions of the dye (1:250, 1:500 and 1:1000) and followed the manufacturer's protocol for staining *M. leprae* and found no detrimental effects of PKH26 staining on subsequent palmitic acid metabolism as measured by RR (Fig. 4).

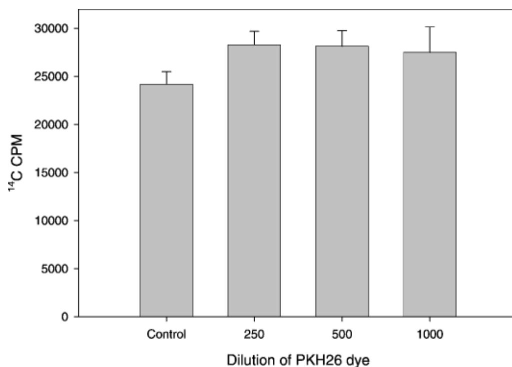


FIG. 4. Effect of PKH26 staining on palmitic acid oxidation rate of *M. leprae*. Bargraph showing the cumulative seventh day radiorespirometry counts ± S.D. The data were representative of three independent experiments. *M. leprae* were obtained freshly from athymic (nu/nu) mouse footpads for each experiment.

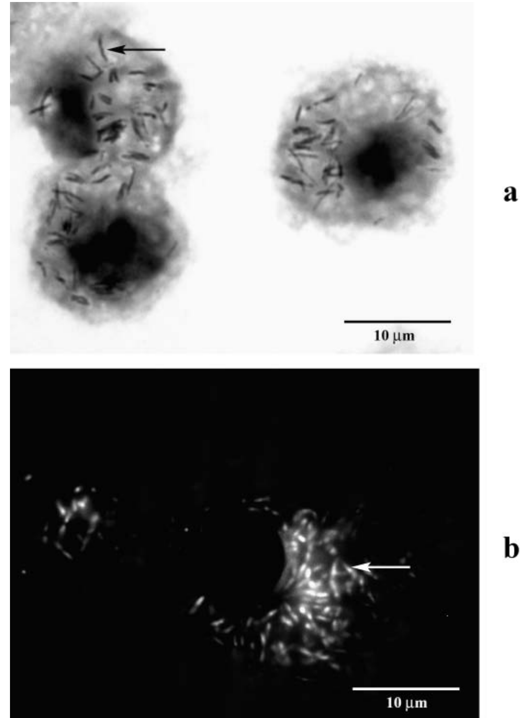


FIG. 5. Photomicrograph (400×) showing the uptake of unlabeled control (a) and PKH26 labeled (b) viable *M.leprae* by mouse peritoneal macrophages infected overnight at 33°C. Macrophages infected with unlabeled *M. leprae* (a) were fixed and stained by the Kinyoun acid-fast technique.

Uptake of PKH26 stained *M. leprae* by mouse peritoneal macrophages. To visualize intracellular fluorescent bacilli, adherent mouse peritoneal macrophages were infected *in vitro* with either PKH26 stained or unstained live *M. leprae* at a MOI of 20:1. We did not observe any difference in the up-

take of PKH26 stained *M. leprae* when compared to that of unstained control (Fig. 5).

Growth of PKH26 stained *M. leprae* in mouse footpads. One × 10^4 PKH26 stained or unstained *M. leprae* were used to infect each hind footpad of BALB/c mice. The footpads were harvested at 3 and 6 months and the total number of AFB per footpad were counted. The results (Fig. 6) indicate no significant difference between the growth kinetics of the PKH26 stained *M. leprae* ($3.3 \times 10^6 \pm 1.3 \times 10^6$AFB/ footpad at 6 months) to that of the control ($1.8 \times 10^6 \pm 0.7 \times 10^6$AFB/ footpad at 6 months).

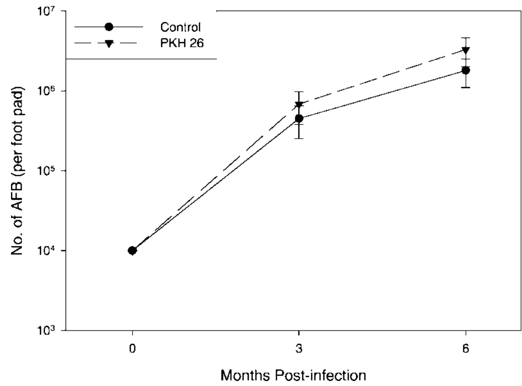


FIG. 6. Effect of PKH26 staining on the growth of *M. leprae* in the mouse footpad.

DISCUSSION

Over the years, researchers have carried out experiments with *M. leprae* obtained from a variety of sources, including the nodules or lesions from multibacillary (MB) patients, infected armadillo tissue, and infected footpads from conventional mice as well as immunocompromised neonatal thymectomized, lethally irradiated (NTLR) and nu/nu mice. In general, these sources of leprosy bacilli have proved satisfactory for MFP studies but less than satisfactory for *in vitro* experiments.

M. leprae from infected human patients are difficult to obtain and there is no control of the investigator over the quality of these bacilli. Human derived bacilli were obtained from untreated cases if viable organisms were required ([4, 7]), but the quality (viability) of these bacilli was poor and human biopsies were an inconsistent source of organisms as it would be unethical to withhold treatment from an identified case of MB leprosy solely to provide a source of bacilli. The quality of bacilli obtained from passage of *M. leprae* in the conventional MFP model was more consistent than that from human origin. However, though conventional MFP model yielded adequate numbers of bacilli for a variety of additional MFP studies ([16]), these organisms were unsatisfactory for most *in vitro* studies as they were too few (maximum yield of ~1 × 10^6 per footpad) and consisted largely of footpad tissue. A single infected armadillo can yield tens of billions of bacteria ([10]), but the quality of bacilli in terms of viability remains poor (unpublished results). Hence, armadillo derived *M. leprae* are good for conducting certain biochemical studies but not for *in vitro* or cell culture studies where the viability of the bacterial inoculum can dictate the outcome of the experiments. For the latter kind of studies athymic nu/nu footpad-derived *M. leprae* is best, as a single mouse can yield a few billion highly viable bacteria.

The present report establishes a simple purification method for removing host tissue from suspensions of *M. leprae* without affecting the viability of the bacilli. Other procedures have been developed to purify tissue derived *M. leprae* on a large scale but the effects of these treatments on viability of the bacilli was never determined. Armadillo infected liver, spleen and lymph node tissue harbors billions of *M. leprae*([10]) and a two phase method devised by Draper for isolation of pure bacilli from large quantities of infected armadillo liver and spleen was developed([17]) and is used routinely for the provision of the enormous numbers of bacilli required to isolate and characterize cell wall and other *M. leprae* constituents ([8]). However, until very recently the infected tissues were irradiated with 2.5×10^6 rads([10]), a dose that kills the bacilli([1]) making the issue of viable, armadillo-derived *M. leprae* moot. In recent years these tissues have not been irradiated but our studies, employing both RR as a measure of metabolic activity and the BACTLIGHT stain for membrane integrity and MFP challenge show that the viability of even unirradiated armadillo-derived *M. leprae* is extremely low (unpublished results).

Another important consideration for the provision of *M. leprae* as a research reagent is that armadillos are too expensive to

maintain (1 to 2 years) as a source of bacilli for routine (weekly) experimentation. Armadillos are infected experimentally to provide maximum numbers of *M. leprae* upon harvest, a goal we have found to be inconsistent with providing highly viable organisms harvested during their log phase of growth([11]). *M. leprae*-infected athymic nu/nu mice on the other hand are readily available, far less expensive than armadillos to maintain and the infected footpads of individual mice can be harvested weekly to yield billions of bacilli in the crude homogenates of the infected footpad tissues.

Our laboratory is committed to characterizing nu/nu-derived *M. leprae* as a research resource and we have described their response to physical-chemical treatment including susceptibility to ionizing([1]) and UV([21]) radiation, effects of deep-freeze storage and incubation temperatures and response to various fixatives([22, 11]).

Previously we employed a Percoll density gradient separation of nu/nu derived *M. leprae* to yield pure suspensions of bacilli that were enriched for viability as defined by RR but losses of total bacilli were routinely >90%, an unacceptable yield. Treatment with 0.1N NaOH has been routinely employed to purify *M. leprae* of host tissue for studying the enzyme activity([13]) and isolation of bacterial components([9]). The present S.E.M. studies clearly show that brief treatment of nu/nu mouse footpad derived *M. leprae* with 0.1N NaOH eliminates mouse footpad tissue providing a pure suspension of bacilli for potential *in vitro* use as a leprosy research reagent. But a purified reagent is only a partial fulfillment of the needs of researchers; a pure and viable reagent is needed. For example, previous studies from this laboratory have described marked differences in afferent and efferent function of *M. leprae* infected macrophages, depending on whether infection was carried out with viable or non-viable bacilli([19]).

The present study investigated the effects of NaOH treatment on subsequent viability and shows that this method of purification does not affect their viability as defined *in vitro* by RR, a measure of metabolic activity that correlates well with growth in mouse footpad([22]). To further characterize viable *M. leprae* as a research reagent we have recently adapted the VS procedure to permit evaluation of the viability of individual leprosy bacilli as defined by membrane integrity([11]). Interestingly we found that a consistent, though not significant, increase in the cumulative seventh day RR counts and percentage live in VS assay was observed after treatment of bacterial suspension with 0.1N and 0.3N NaOH. These findings may be due to removal of some non-viable bacteria from the suspension by the NaOH treatment. The yield of bacteria after the NaOH treatment and subsequent washings was routinely between 30 and 50%.

Combining RR analysis with VS has allowed us to adjust our routine passage of *M. leprae* in the nu/nu mouse to maximize the viability of a harvested suspension and minimize the duration of the infection([22, 11]). In the infected nude mouse the footpad increases in size as bacillary numbers increase markedly and host cells become gorged with *M. leprae*. Viability as measured by RR correlated with MFP growth and was significantly correlated with time in tissue and the number of bacilli per gram of granuloma([22]). Very large footpads with high numbers of *M. leprae* per gram of tissue yield less viable bacilli. Highest viability for nude mouse derived *M. leprae* is associated with short to moderate periods *in vivo*. Thus routine short term passage is the best means to assure plentiful, viable stocks of *M. leprae*. The present study extends our interests in defining the properties of viable *M. leprae* as a research reagent.

Access to a reliable source of large numbers of pure, viable *M. leprae* would be an important research resource for today's leprosy researcher especially those interested in pursuing the cell biology of intracellular infection with *M. leprae* and the unique relationship between the leprosy bacillus and its host cell. A major tool became available to cell biologists a dozen or so years ago with the development of fluorescent tracker dyes which allowed the stable labeling of mammalian cells([20]). The technology was based on the incorporation of highly aliphatic reporter molecules containing fluorochrome groups into the lipid bilayers of cytoplasmic membranes. A key feature of these dyes is their retention. Once incorporated, they are trapped in the membrane by

virtue of their inherent insolubility in aqueous solutions. In a variety of eukaryote cells these dyes have been shown to be stable and non-toxic, permitting tracking of adoptively transferred cells *in vivo* without interfering with their function, for example cytotoxicity([12]). The tracking dyes do not interfere with doubling times of labeled cells and the dye appears to be equally partitioned between daughter cells when a labeled cell divides([6]). Similar dyes have been employed as fluorescent trackers to label prokaryote cells such as protozoa([15]) and bacteria([3]).

In the present studies, the short term effects of staining with PKH26 tracker dye on the metabolic activity of *M. leprae* was determined *in vitro* in axenic media. The growth of stained bacteria in MFP was also determined. Notably, all the PKH dye labeling of bacilli reported in this study was done subsequent to NaOH treatment to remove the contaminating mouse tissues which would also label with the PKH dye. We have also observed that *M. leprae* did not label uniformly with PKH dye if mouse tissue was present in the suspension. Therefore, these studies also demonstrated that neither NaOH treatment nor PKH26 labeling of *M. leprae* affected the viability of *M. leprae* as defined by RR, VS and growth in the mouse footpad, the "gold standard" measurement of the ability of the leprosy bacillus to survive and multiply *in vivo*. The PKH labeled bacteria can be visualized inside mouse peritoneal macrophages using either fluorescence or confocal microscope. It should be noted, that in cultures where the PKH26 stained *M. leprae* were maintained in peritoneal macrophages for more than 7 days there was slight diffusion of the PKH26 dye into the macrophage cytosolic compartments (data not shown).

The NaOH treatment reported here is an easy and fast method to obtain suspensions of nu/nu MFP derived viable *M. leprae* essentially free of host tissue, a valuable research reagent required for studying the host pathogen relationship in leprosy. The other research reagent described here is the fluorescently labeled viable *M. leprae* which can be utilized in intracellular trafficking studies of *M. leprae*([2]) or in other cell biology studies that require tracking of the bacteria using a fluorescent tag. We observed the staining to be stable *in vitro* over a considerable length of time and did not affect the viability of the bacteria. MFP studies that will explore in more detail the PKH-staining characteristics of multiplying *M. leprae* are underway.

Acknowledgement. The authors gratefully acknowledge the expertise of Mr. Greg McCormick in the preparation of S.E.M. photos of *M. leprae*. These studies were supported by a grant from The American Leprosy Missions, Greenville, SC.

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