Application of a viability-staining method for *Mycobacterium leprae* derived from the athymic (nu/nu) mouse foot pad

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Mycobacterium leprae cannot be cultured, so ascertaining viability of the organism remains a major obstacle, impeding many avenues of investigation. This study tested a two-colour, Syto9 and propidium iodide, fluorescence assay, which scores for membrane damage in individual bacilli, to determine if a rapid direct-count viability-staining technique can be reliably applied to *M. leprae*. A variety of experimental conditions were employed to validate this technique. This technique was also used to correlate the viability of *M. leprae* with the course of athymic mouse foot pad infection to optimize the provision of viable *M. leprae* as a research reagent. The data show that in untreated suspensions of *M. leprae* there is a good correlation between the metabolic activity of leprosy bacilli and their membrane damage. Fixation of *M. leprae* with ethanol, paraformaldehyde and glutaraldehyde completely suppressed their metabolic activity but showed little effect on their membrane integrity. The present study also showed that the metabolic activity of *M. leprae* declines more than the extent of membrane damage at 37 °C within 72 h, but that they are not significantly affected at 33 °C. Irradiation at 10⁴ Gy showed high numbers of dead bacilli by the staining method. The results show that the reliability of metabolic-activity data as well as viability-staining data is dependent on the method by which *M. leprae* is killed. This staining method helped us predict reliably that the smaller *M. leprae*-infected athymic mouse foot pad seen early in infection, between 4 and 5 months, yields markedly better quality leprosy bacilli than older, larger foot pad infections, as defined by their metabolic activity and membrane integrity.

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INTRODUCTION

The complete sequencing of the *Mycobacterium leprae* genome (Cole *et al.*, 2001) holds great promise for future progress in the study of the physiology, pathogenicity, epidemiology and genetics of the leprosy bacillus; however, the inability to culture *M. leprae in vitro* not only obstructs provision of a regular supply of fresh, viable leprosy bacilli, but also prevents our ability to easily distinguish between live and dead *M. leprae*. These two gaps in our knowledge are fundamental obstacles to the exploration of the unique interaction between *M. leprae* and its host, a relationship that underlies the pathogenicity of this poorly understood disease.

The only definitive measure of bacterial viability is the ability to replicate. The discovery by Shepard (1960) of the mouse foot pad (MFP) technique to demonstrate replication of *M. leprae* was a research milestone, permitting testing of new

anti-leprosy drugs, determination of drug-resistant strains of *M. leprae* and initial evaluation of vaccine protection. Variations of the MFP technique involving titration in large numbers of mice will detect differences in the relative viability of different suspensions of *M. leprae* (Welch *et al.*, 1980), but this labour-intensive, time-consuming, expensive technique is impractical for the study of interactions of *M. leprae* with its host cell *in vitro*.

Over the years our laboratory has employed alternatives to the MFP technique to provide a rapid, less-cumbersome, but indirect *in vitro* measure of *M. leprae* viability that would yield results in hours or days rather than months. We have applied ATP activity as well as various measurements of anabolic and catabolic metabolism to measure drug sensitivity of *M. leprae* (Franzblau *et al.*, 1992; Franzblau & Hastings, 1987; Harris *et al.*, 1988) and study the microbicidal role of activated macrophages against *M. leprae* (Ramasesh *et al.*, 1991).

In the course of these studies we and others found that a promising fluorescein diacetate-ethidium bromide (FDA-EB)-staining measurement of enzyme activity, although

Abbreviations: FCS, fetal calf serum; FDA-EB, fluorescein diacetate-ethidium bromide; IFN, interferon; MFP, mouse foot pad; PI, propidium iodide; RNI, reactive nitrogen intermediate; RR, radiorespirometry; RT, room temperature; VS, viability staining.

quite successful with other bacteria (Jayapal *et al.*, 1991; Kvach & Veras, 1982), was not readily applicable to *M. leprae* (Kvach *et al.*, 1984; Ramasesh *et al.*, 1991) isolated from host tissue. However, the concept of simple viability staining (VS) remains attractive, especially if, unlike metabolic assays, which require suspensions of large numbers of bacilli and hours or days to perform, a viability stain could be carried out immediately on individual organisms. Viability in *M. leprae* requires measurement by indirect methods. The present study attempts to improve on this definition of viability by developing a direct-count staining method of individual bacilli and correlating the results with metabolic activity of a suspension of organisms.

Success in VS of different Gram-positive and Gram-negative bacteria (Boulos *et al.*, 1999) has been reported with the Molecular Probes' LIVE/DEAD BacLight method. The kit consists of two fluorescent dyes, the green Syto9 and the red propidium iodide (PI). Both dyes bind to bacterial nucleic acids, but while Syto9 can penetrate intact cell membranes, PI can only get into cells with damaged cell membranes. PI also has a higher affinity towards nucleic acids than Syto9 and hence reduces the intensity of the green Syto9 stain when both dyes are present. Thus, in this assay all bacterial cells stain green while those with damaged membranes also stain red.

In the present study we explored VS of *M. leprae* under different experimental conditions and re-examined the principal macrophage (MΦ) microbicidal mechanism, i.e. production of the reactive nitrogen intermediate (RNI) nitric oxide (Adams *et al.*, 1991), and the antimicrobial capacity of interferon (IFN)- γ activated MΦs (Ramasesh *et al.*, 1991). Finally, in order to optimize our provision of viable *M. leprae* as a research reagent, we employed the VS technique to correlate the relative viability of different passaged suspensions of *M. leprae* with the subsequent course of nu/nu MFP infection.

METHODS

Nude-mouse-derived *M. leprae*. The Thai-53 isolate of *M. leprae* is maintained in the foot pads of athymic nu/nu mice infected, 6–8 months previously, with 5×10^7 freshly harvested *M. leprae* in each hind foot pad. *M. leprae* were harvested from the foot pads as described previously (Truman & Krahenbuhl, 2001), washed by centrifugation (18 000 g for 30 min), resuspended in either medium 7H12 or RPMI 1640 (Gibco) plus 10% (v/v) fetal calf serum (FCS; Gibco), enumerated by direct count according to Shepard's method (Shepard & McRae, 1968) and held overnight at 4 °C pending quality control testing for contamination. Freshly harvested bacilli were always employed in experiments within 24 h of harvest.

Physical and chemical treatments of *M. leprae*. A variety of physical conditions and chemical methods were employed to determine their effects on *M. leprae* freshly harvested from nude mice foot pads. *M. leprae* killed by irradiation received 10^4 Gy from a cobalt source (Adams *et al.*, 2000b). Suspensions of *M. leprae* were held at different temperatures, 60 °C (30 min), 33 °C and 37 °C (72 h), or submitted to a single freeze/thaw cycle [–70 °C/room temperature (RT)]. The different chemical methods used and their duration/dose were: 70% (v/v) ethanol (Pharmco) at RT (1 h), 2% (w/v) paraformaldehyde

(Sigma) at RT (30 min), acetone (Sigma) at 4 °C (1 h) and 2% (v/v) glutaraldehyde (Sigma) at 4 °C (1 h). All treated bacilli were tested in parallel with untreated bacilli from the same harvest.

Macrophage culture. RPMI 1640 medium supplemented with 25 mM HEPES (Sigma), 50 μ g ampicillin ml^{–1} (Sigma), 2 mM glutamine (Sigma) and 10% (v/v) FCS was used throughout these studies. Resident peritoneal cells from Swiss mice were harvested and allowed to adhere for at least 6 h at 37 °C and 5% (v/v) CO₂ on LUX plastic cover slips (Miles Laboratory) in 24-well tissue culture plates (Corning) as previously described (Ramasesh *et al.*, 1991). After washing to remove non-adherent cells, the adherent cells were infected overnight at 33 °C with fresh nude mouse foot pad-derived *M. leprae* at an m.o.i. of 20:1. At the end of the incubation, extracellular *M. leprae* were removed by washing the cover slips, and the cells were either activated with 500 IU IFN- γ ml^{–1} (R & D Systems) and 5.0 ng LPS ml^{–1} (from *Escherichia coli* O111:B4, Sigma) or left as non-activated controls. Equal numbers of activated and control cells were lysed with 0.1 M NaOH (Sigma) at 0 h, 24 h, 48 h and 72 h, and the intracellular *M. leprae* were processed for radiorespirometry (RR; Ramasesh *et al.*, 1991) and VS.

Radiorespirometry. The metabolism of a suspension of *M. leprae* was measured by evaluating the oxidation of ¹⁴C-palmitic acid to ¹⁴CO₂ by RR as described previously (Franzblau, 1988). Briefly, for experiments in axenic medium 1×10^7 *M. leprae* were suspended in 1.0 ml of commercially available BACTEC 7H12B medium (Becton Dickinson) in a 5 ml glass vial with a loosened cap, which in turn was inserted into a wide-mouth liquid scintillation vial lined with filter paper impregnated with NaOH, 2,5-diphenyloxazole (Sigma) and Concentrate I (Kodak). Four millilitres of BACTEC PZA medium (7H12B at pH 6.5, Becton Dickinson) was used in experiments where RR was employed on *M. leprae* obtained by NaOH lysis of macrophages. When read daily, captured ¹⁴CO₂ determines the rate of ¹⁴C-palmitic acid oxidation. In the present study the day 7 cumulative c.p.m. are reported.

Fluorescent staining for quantification of bacterial viability. The membrane integrity of individual *M. leprae* in a suspension was evaluated with a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes). *M. leprae* (2×10^7) were washed twice (10 000 g for 5 min) in normal saline and incubated for 15 min at room temperature with a final concentration of 6 μ M Syto9 and 30 μ M PI. The bacteria were washed twice in normal saline, the pellet was resuspended in 20 μ l of 10% (v/v) glycerol in normal saline and 5 μ l of the suspension was placed on a glass slide with an 18 mm² glass cover slip. The dead and live bacteria were enumerated by direct counting of fluorescent green and red bacilli under a fluorescence microscope using appropriate single bandpass filter sets. The excitation/emission maxima are 480 nm/500 nm for Syto9 and 490 nm/635 nm for PI.

Generation of NO *in vitro* by NaNO₂ in acidified media. Medium 7H12 was adjusted to either pH 6.5 or 5.5 with 0.1 M HCl. Freshly harvested *M. leprae* from nu/nu foot pads were added to these pH-adjusted media at a concentration of 1×10^7 ml^{–1}. A 1.0 ml quantity of these suspensions was dispensed in an appropriate number of microfuge tubes and the required amount of 1.0 M NaNO₂ (Sigma) solution was added such that the final NaNO₂ concentrations in quadruplicate tubes were 0, 0.6, 2.5 and 10 mM (Rhoades & Orme, 1997). All the tubes were incubated at 33 °C for 24 h, after which the *M. leprae* were washed twice with 7H12 medium and resuspended in 200 μ l of 7H12 medium for RR or VS.

Statistical analysis. The data are shown as means $\pm$ SD from a representative of three to five 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

Viability staining of *M. leprae*

In Fig. 1(a), is shown a typical microscopic field of freshly harvested, wet-mounted untreated *M. leprae* in which all the bacilli in the suspension stained with Syto9 (green), i.e. both those with intact membranes as well as those with damaged membranes. Viewing this field by phase-contrast microscopy confirmed that there were no unstained bacilli. A change of the filter set revealed in the same microscopic field (Fig. 1b) those bacilli (red) that had sustained sufficient cell membrane damage to admit the DNA stain PI.

Correlation of viability staining with radiorespirometry

In Fig. 2 are shown data in which the viability of freshly harvested *M. leprae* from 20 individual nu/nu MFP harvests were compared by VS and RR. These data show that viability does vary from harvest to harvest but there is a good correlation ($r^2 = 0.73$) between metabolic activity of a suspension of *M. leprae* and the cell wall integrity of individual bacilli as measured by VS.

Effects of physical and chemical treatments on the viability of extracellular *M. leprae*

In these experiments, in order to compare RR with VS, RR data were normalized where day 7 data from fresh untreated control samples represented 100 % metabolic activity. In-

Fig. 2. Correlation of VS with RR. Linear regression model comparing the cumulative day 7 RR counts with the percentage of the *M. leprae* that were live, as measured by the VS technique. The *M. leprae* were obtained freshly from individually harvested athymic (nu/nu) mouse foot pads ($n = 20$) and subjected to both assays.

cubating *M. leprae* at 33 °C for 72 h did not significantly decrease RR counts in comparison to the freshly harvested control ($P = 0.13$), nor did this treatment significantly affect the number of PI-stained bacteria ($P = 0.83$). However, incubation of *M. leprae* at 60 °C for 30 min or irradiation with 10^4 Gy almost totally inhibited metabolism (Fig. 3a), and also markedly reduced the percentage of bacilli stained as

Fig. 1. Photomicrographs showing a wetmount suspension of Syto9- (a) and PI- (b) stained *M. leprae*. Both (a) and (b) depict the same field taken with a change of filter. All the bacilli, irrespective of membrane damage, stain green while only the ones with membrane damage stain red.

Fig. 3. Effects of physical treatment on viability of extracellular *M. leprae*. Bar graphs showing mean $\pm$ SD of cumulative day 7 RR counts (a), expressed as percentages of the control (40 516 c.p.m.), and mean $\pm$ SD of the percentage of the *M. leprae* that were live (b), as measured by the VS technique, after various physical treatments. *M. leprae* were obtained freshly from athymic (nu/nu) mouse foot pads for each experiment. The data are representative of three independent experiments. Bars: A, control; B, 60 °C for 30 min; C, freeze/thaw treatment; D, 37 °C for 72 h; E, 33 °C for 72 h; F, irradiation at 10^4 Gy.

viable in comparison to controls (Fig. 3b). A single freeze/thaw cycle at -70°C or incubation of the bacteria at 37°C for 72 h resulted in a marked reduction ($P < 0.001$ in both cases) in RR, but treatment at 37°C induced no significant increase ($P = 0.12$) in the number of PI-stained bacteria compared to the control. However, a single freeze/thaw cycle induced a modest but significant ($P = 0.02$) increase in the number of PI-stained bacteria as shown by VS (Fig. 3b).

All the different chemical treatments employed resulted in almost total inhibition of metabolism (Fig. 4a). The effects on VS were not as marked, although 70 % ethanol and 2 % glutaraldehyde (both for 1 h) treatments showed a significant decrease ($P = 0.003$ and $P < 0.001$, respectively) in the number of bacteria with intact membranes. Paraformaldehyde treatment (2 %) for 30 min did not show any significant increase ($P = 0.61$) in the number of PI-stained bacteria (Fig. 4b).

Effect of macrophage activation on the viability of intracellular *M. leprae*

To compare these two indicators of viability in a relevant experimental model, the antimicrobial effects of activated MΦs on *M. leprae* were measured by RR and VS. Infected MΦ monolayers were lysed and tested at 24, 48 or 72 h after infection. RR results showed a rapid effect of the enhanced

antimicrobial capacity of activated MΦs on the metabolic activity of intracellular *M. leprae*. Day 7 RR counts of intracellular *M. leprae* harvested from activated MΦs at 24 h were markedly lower ($P < 0.001$) than of those harvested from non-activated MΦs at 24 h (Fig. 5a), and by 48 h metabolism was virtually abolished. In contrast, no significant difference ($P = 0.14$) was found in the number of PI-stained *M. leprae* harvested from the activated and non-activated MΦs at 24 h, but at 48 h there was a significant increase ($P = 0.002$) in the number harvested from activated MΦs (Fig. 5b).

Effect of *in vitro*-generated NO on axenic *M. leprae*

In Fig. 6 are shown the results of an experiment in which the antimicrobial properties of NO for extracellular *M. leprae* were explored in 7H12 medium supplemented with various concentrations of NaNO_2 and acidified at either pH 6.5 or 5.5. It is quite clear from both RR and VS data that the lower pH alone has little effect on *M. leprae* viability. However, *M. leprae* viability, as measured by RR and VS, was markedly affected in a dose- and pH-dependant manner with increasing concentration of NaNO_2 . *M. leprae* viability after 24 h of

Fig. 4. Effects of chemical treatment on viability of extracellular *M. leprae*. Bar graphs showing mean $\pm$ SD of cumulative day 7 RR counts (a), expressed as percentages of the control (40 516 c.p.m.), and mean $\pm$ SD of the percentage of the *M. leprae* that were live (b), as measured by the VS technique, after different chemical treatments. *M. leprae* were obtained freshly from athymic (nu/nu) mouse foot pads for each experiment. The data are representative of three independent experiments. Bars: A, control; B, 70 % ethanol for 72 h; C, 2 % paraformaldehyde for 30 min; D, acetone for 1 h; E, 2 % glutaraldehyde for 1 h.

Fig. 5. Effects of macrophage activation on the viability of intracellular *M. leprae*. Bar graphs showing mean $\pm$ SD of cumulative day 7 RR counts (a), expressed as percentages of the control (17 674 c.p.m.), and mean $\pm$ SD of the percentage of the *M. leprae* that were live (b), as measured by the VS technique. *M. leprae* harvested from athymic (nu/nu) mouse foot pads were used to infect mouse peritoneal macrophages, which were then either activated with LPS and IFN- γ or left as controls for the specified length of time. After the incubation (at 33°C) macrophages were lysed and the intracellular *M. leprae* were recovered to measure metabolic activity (RR) and membrane integrity (VS). The data are representative of five independent experiments. Bars: white, activated; black, control.

Fig. 6. Effects of *in vitro*-generated NO on extracellular *M. leprae*. Bar graphs showing mean $\pm$ SD of cumulative day 7 RR counts (a), expressed as percentages of the control (30 863 c.p.m. at pH 6.5 and 30 415 c.p.m. at pH 5.5), and mean $\pm$ SD of the percentage of the *M. leprae* that were live (b), as measured by the VS technique. *M. leprae* harvested from athymic (nu/nu) mouse foot pads were cultured (at 33 °C) in axenic 7H12 medium set at either pH 6.5 or 5.5 in the presence of different concentrations of NaNO₂ for 24 h. The data are representative of four independent experiments. Bars: A, B, C and D represent 0 mM, 0.5 mM, 2.5 mM and 10.0 mM NaNO₂, respectively; black, pH 6.5; white, pH 5.5.

exposure to 2.5 mM or 10 mM NaNO₂ at pH 5.5 or 6.5, was significantly lower at pH 5.5 by both RR ($P < 0.001$) and VS ($P < 0.001$).

Optimizing nu/nu MFP infection to obtain maximum *M. leprae* viability

The VS technique was employed in 61 consecutive harvests from seven different batches of *M. leprae*-infected nu/nu mice to determine its utility in prospectively estimating the relative viability of the leprosy bacilli obtained on the day of harvest. As mice were selected in the course of these studies, their infected feet were categorized according to size as 'small' (S), 'medium' (M) or 'large' (L) and the VS data from each suspension plotted (Fig. 7). It is important to point out that, in order to work rapidly and aseptically, only a portion of infected tissue was removed from each foot, i.e. no attempt could be made to harvest the total number of *M. leprae* from each foot. Nevertheless, the yield of *M. leprae* obtained (both feet) fell into discrete ranges: S, $2.0\text{--}7.0 \times 10^9$; M, $7.1\text{--}15 \times 10^9$; and L, $>15 \times 10^9$. More importantly, membrane integrity of the *M. leprae*, as determined by VS, differed significantly between the three groups (S vs M, $P < 0.0001$; M vs L, $P < 0.02$).

Fig. 7. Optimizing nu/nu MFP infection to obtain maximum *M. leprae* viability. (a) The relative sizes of the small, medium and large athymic (nu/nu) mouse foot pads used to harvest *M. leprae*. (b) Scattergram showing the percentage of the *M. leprae* that were live, as measured by VS, obtained from the small ($n = 18$, $85.4\% \pm 9.1$), medium ($n = 30$, $69.6\% \pm 12.7$) and large ($n = 13$, $55.7\% \pm 12.4$) foot pads.

DISCUSSION

As *M. leprae* cannot be cultured, differentiating between viable and non-viable *M. leprae* remains a major obstacle to the leprosy researcher, impeding many avenues of investigation. The MFP technique was invaluable for demonstrating anti-leprosy drug activity (Gelber *et al.*, 1995; Grosset *et al.*, 1985) and preliminary work on host resistance, including vaccine efficacy (Roche *et al.*, 2001; Shepard, 1983). It still remains a valuable tool as applied in transgenic and knock-out strains of mice, providing clues to the immunopathological spectrum that characterizes human leprosy (Adams *et al.*, 1997, 2002). However, the procedure is not readily applicable to studies of the intracellular relationship between the leprosy bacillus and its host cell, where a rapid, countable assay is desired. The present study compared two independent assays, RR and VS, which may be employed independently or in combination to measure *M. leprae* viability under a variety of experimental conditions.

RR has been used extensively in our laboratory since it was first described by Franzblau (1988), when large numbers of nu/nu-derived *M. leprae* became routinely available. RR was used to evaluate the susceptibility of *M. leprae* to different drugs (Franzblau *et al.*, 1992), UV (Truman & Gillis, 2000) and gamma radiation (Adams *et al.*, 2000b), as well as the effects of incubation and storage at different temperatures (Truman & Krahenbuhl, 2001). RR also lent itself well to the characterization of the role of activated MΦs in host resistance to leprosy (Ramasesh *et al.*, 1991) and was employed to identify a key mechanism of host defence, the production of inducible nitric oxide by the MΦs (Adams *et al.*, 1991).

Since RR measures the rate of oxidation of ^{14}C -palmitate by *M. leprae*, it is an indirect measure of viability. RR is dependent on enzymes that are assumed to be essential for the survival of the bacilli. Our previous studies have shown a good correlation between the RR results and growth in the MFP (Truman & Krahenbuhl, 2001). However, it is not clear how long these enzymes remain functional in non-viable bacilli. We have shown that residual RR activity continues for several days after lethal gamma irradiation of *M. leprae* (Adams *et al.*, 2000b). However, this same study showed radiation-induced blebbing and protrusions in *M. leprae* surface, suggestive of membrane damage.

There are two obvious differences in the RR and VS assays. First, RR measures the relative metabolic activity of a suspension of *M. leprae*, ideally 1×10^7 bacilli but adaptable to 1×10^6 , while VS records the membrane integrity of single leprosy bacilli. Second, RR data reflect 7 days worth of metabolic activity, or lack thereof, while VS readings are instantaneous, i.e. read immediately after experimental treatment. With *M. leprae*, the principle that green but not red bacilli are viable cannot be confirmed by determining colony-forming units. Nonetheless, our data showed a good correlation between membrane integrity (VS) and metabolic activity (RR) in bacilli freshly harvested from the nu/nu MFP, which in turn has been shown to correlate with MFP growth (Truman & Krahenbuhl, 2001). This operational definition of viability in freshly harvested *M. leprae* appeared to fit the description of suspensions as high, medium and low viability when we examined the growth rate in the nu/nu foot pad in routine passages. When inoculated with 80–90 % (high) viable inoculum, the foot pads were suitable for harvest at 4–5 months, yielding $3\text{--}5 \times 10^9$ bacilli, when inoculated with 80–90 % (high) viable inoculum rather than at 6–8 months or more when inoculated with 30–50 % (low) viable inoculum. However, in the present studies there were discrepancies under experimental conditions, underscoring differences in the nature of the two assays for estimating the viability of *M. leprae*.

It is noteworthy that centrifuged *M. leprae*, dispersed as described here, yielded a suspension of leprosy bacilli in which fluorescent single bacilli were easily examined. Treatments of *M. leprae* with ethanol, paraformaldehyde and glutaraldehyde completely suppressed RR, which is expected given the denaturing effect of the fixatives on enzymes and their function, but had little effect on VS. Treatments with all the different fixatives yielded very low day 7 RR counts, ranging from 7.1 % to 2.6 %. Incubation with 70 % ethanol for 1 h resulted in only 37 % PI-stained *M. leprae*, compared with 16 % in controls. No comparable studies have been done with other mycobacteria, due primarily to their propensity to clump making quantification difficult, but 70 % ethanol treatment led to >90 % PI staining in *E. coli* (unpublished results). The mycobacterial membrane is quite different from the membrane of Gram-negative bacteria and it appears that these fixatives are unable to induce sufficient membrane damage for PI to enter the cells.

The present results show that although there was a striking decline in the RR of *M. leprae* after incubation at 37 °C for 72 h, there was no corresponding marked increase in the number of PI-stained bacteria. These findings underscore the differences in the two assays, normal body temperature does not affect membrane integrity of the leprosy bacilli but the optimum metabolic activity of *M. leprae* is definitely not 37 °C. Although the enzymes of *M. leprae* responsible for oxidation of palmitic acid have not been characterized they appear to function optimally at 30–34 °C (Fukutomi *et al.*, 2004), and a relatively short-term incubation at 37 °C has clear deleterious consequences (Truman & Krahenbuhl, 2001).

A single freeze/thaw cycle reduced *M. leprae* RR by >90 % and increased PI staining to ~50 %, deleterious effects confirmed by poor recovery of thawed organisms in the MFP model (Colston & Hilson, 1979; Levy, 1971; Shepard & McRae, 1965). Thus, our results show that the reliability of RR as well as VS data is dependent on the method by which the *M. leprae* were killed. Careful studies with *E. coli* revealed that all organisms stained only with Syto9 (green) formed colony-forming units, while PI-stained *E. coli* with damaged membranes could not be resuscitated (Ericsson *et al.*, 2000). At present we are not able to confirm that the green-only staining *M. leprae* are indeed viable by demonstration of colony-forming units. However, it seems to us a safe assumption that if red-stained *E. coli* cannot repair their membrane and multiply, it is likely that *M. leprae* with damaged membranes cannot do so either and are indeed non-viable.

As shown by titration in MFP immunotherapy experiments (Krahenbuhl *et al.*, 1982), and by a variety of *in vitro* procedures that included ATP activity, PGL-1 (phenolic glycolipid 1) synthesis and RR (Ramasesh *et al.*, 1991), activated MΦs with an enhanced microbicidal capacity have a clear deleterious effect on *M. leprae*. Our laboratory has reported that IFN- γ - and LPS-activated mouse peritoneal MΦs have a deleterious effect on intracellular *Mycobacterium tuberculosis* and *M. leprae* in a model underscoring the key role for reactive nitrogen intermediates (RNIs) while failing to show a role for reactive oxygen intermediates (Adams *et al.*, 1997, 2000a). Actual microbicidal activity was shown by a colony-forming unit approach with *M. tuberculosis* (Rhoades & Orme, 1997), while the leprosy model revealed marked inhibition of metabolism as demonstrated by RR (Adams *et al.*, 1991). Our present study confirms and extends these findings by suggesting that in the case of intracellular anti-microbial effects on *M. leprae*, loss of metabolic activity precedes membrane damage as very low RR counts were obtained after 24 h of MΦ activation, while a significant increase in the number of PI-stained bacteria was only reported after 48 h. This MΦ model may also be employed for intracellular drug studies with *M. leprae* (unpublished results).

Finally, the VS technique brings an important quality-control tool to the provision of fresh, viable nu/nu mouse-

derived *M. leprae*, a weekly service with a high priority in our laboratory. As shown especially by VS (Fig. 7) and supplemented by RR (Truman & Krahenbuhl, 2001), we can now reliably predict that the smaller *M. leprae*-infected nu/nu foot pads, seen early in infection (4–5 months), yield greater numbers of metabolically active and morphologically intact leprosy bacilli, than those harvested from older, larger foot pads. We are now able to provide suspensions of *M. leprae* that are 80–90 % viable as determined by VS, an essential research reagent for workers attempting to advance our understanding of the unique relationship between the leprosy bacillus and its host cell.

The present studies confirm previous work from our laboratory regarding the predilection of the leprosy bacillus for cooler temperatures. These findings underscore the observation that, in man, the cooler sites in the body, the skin and mucous membranes of the upper respiratory tract are the preferred site for *M. leprae*, while in mice it is the cooler foot pads that support *M. leprae* growth. This preference of *M. leprae* for cooler temperatures likely defines the permissive nature of *Dasypus novemcinctus*, the nine-banded armadillo, which has a core body temperature of 33 °C, to support massive systemic growth of *M. leprae*. Thus the present study strongly validates the recommendations to other workers that accompany viable *M. leprae* provided by our laboratory for use *in vitro*. If viable bacilli are to be studied, the organisms must be employed while fresh (< 3 days out of the nu/nu mice) and experiments should be run at 33 °C or lower.

More studies are needed to characterize viable *M. leprae* as a research resource. A number of attempts have been made to define a rapid *in vitro* viability assay for *M. leprae*, including the subjective determination of a morphological index (Sathish *et al.*, 1982; Silva *et al.*, 1984), originally described by Rees (Waters & Rees, 1962), and staining for viability with FDA-EB, in which host-tissue esterases confuse interpretation (Kvach *et al.*, 1984). More quantitative assays included ATP activity (Franzblau & Hastings, 1987; Katoch *et al.*, 1988), uptake of radiolabelled protein or nucleic-acid precursors (Harshan *et al.*, 1990; Sathish *et al.*, 1982) and synthesis of the *M. leprae*-specific outer-coat phenolic glycolipid 1 (PGL-1; Chanteau *et al.*, 1990; Harris *et al.*, 1988). Drawbacks to the application of these techniques in the past included the employment of *M. leprae* of low viability (Truman & Krahenbuhl, 2001), i.e. frozen armadillo tissue, human biopsies or, as shown here, large, late-stage infected nu/nu mouse foot pads. It will be of great interest to re-evaluate and compare these objective assays using the fresh viable *M. leprae* as described in this report.

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