A New Model for Studying the Effects of *Mycobacterium leprae* on Schwann Cell and Neuron Interactions

Deanna A. Hagge, Sandra Oby Robinson, David Scollard, Gregory McCormick, and Diana L. Williams

Millions of patients with leprosy suffer from nerve damage resulting in disabilities as a consequence of *Mycobacterium leprae* infection. However, mechanisms of nerve damage have not been elucidated because of the lack of a model that maintains *M. leprae* viability and mimics disease conditions. A model was developed using viable *M. leprae*, rat Schwann cells, and Schwann cell–neuron cocultures incubated at 33°C. *M. leprae* retained 56% viability in Schwann cells for 3 weeks after infection at 33°C, compared with 3.6% viability at 37°C. Infected Schwann cells had altered morphology and expression of genes encoding cellular adhesion molecules at 33°C but were capable of interacting with and myelinating neurons. Cocultures, infected after myelination occurred, showed no morphological changes in myelin architecture after 1 month of incubation at 33°C, and *M. leprae* retained 53% viability. This article describes a new model for studying the effects of *M. leprae* on Schwann cells.

*Mycobacterium leprae* is an obligate intracellular parasite that is unique among bacterial pathogens in its ability to invade the peripheral nervous system and is the leading cause of non-traumatic neuropathy worldwide. Approximately 3 million patients with leprosy worldwide are physically disabled as a result of damage to peripheral nerves and the attendant sensorimotor loss [1]. One-fourth of the newly diagnosed patients each year will suffer from irreversible nerve damage and are at risk of developing the classic hand and foot deformities of leprosy and concomitant disabilities as a long-term consequence of infection with *M. leprae* [2, 3]. Despite this, the specific effects of *M. leprae* on nerves and the events that result in nerve destruction are not understood.

The Schwann cell, the principle support cell in the peripheral nervous system, appears to be the major target of *M. leprae* [4–6] in peripheral nerves. As a long-term consequence of infection and immunologic response, Schwann cells are ultimately destroyed or functionally impaired in damaged nerves [7]. Atrophy of myelinated fibers and secondary demyelination has been shown to occur as result of infection, sometimes even in the absence of evidence of inflammation or acid-fast bacilli [8, 9].

The mechanism by which *M. leprae* enter the nerve to reach Schwann cells is under investigation [10, 11], and a potential mechanism of binding of *M. leprae* to the Schwann cell has been elucidated [6, 12, 13]. Once *M. leprae* adhere to the Schwann cell surface, they are ingested [13], but the Schwann cell appears to be incapable of destroying this intracellular parasite [14].

Although the mechanisms of nerve injury are not well understood, it is conceivable that the intracellular presence of the bacilli could alter Schwann cell gene expression and thereby alter the Schwann cell’s functional relationship with an axon through altered myelin production or during nerve regeneration. There are conflicting reports of the effects of *M. leprae* infection on Schwann cell survival and function. In addition, no comprehensive study has been performed to determine the result of infection on *M. leprae* viability within Schwann cells. It is conceivable that long-term infection of and multiplication within Schwann cells may lead to damaging effects on these cells, resulting in loss of nerve function prior to or apart from stimulation of the immunologic response [7].

One potential reason for our lack of understanding is that no standardized model exists for these studies in vitro. *M. leprae* has been harvested from various sources and irradiated, frozen, and thawed or held at various temperatures prior to infection of Schwann cells, and all published studies have been conducted at 37°C [15-18]. It recently has been documented that the viability of *M. leprae* harvested from a variety of sources varies greatly and that many standard laboratory practices, such as incubation at 37°C, rapidly reduce the viability of *M. leprae* [9]. Storage of *M. leprae* at 33°C in Middlebrook 7H12 medium (Becton Dickinson) has been shown to maintain the viability of *M. leprae* for weeks [19].
These data suggest that an appropriate model for studying the effects of viable *M. leprae* on Schwann cells and Schwann cell–neuron interactions should include infection of Schwann cell cultures and Schwann cell–neuron cocultures with freshly harvested *M. leprae* and maintenance of these cultures at 33°C. Therefore, to develop a new model for these studies, the effect of incubation at 33°C on Schwann cell survival, gene expression, and function was analyzed. This model was then used to determine the effect of *M. leprae* infection on Schwann cells and Schwann cell–axon interactions and the effect of incubation temperature on the viability of *M. leprae* in Schwann cells. To accomplish this, the following 3 aspects of infection were evaluated: (1) the ability of infected Schwann cells to maintain monolayer morphology and to associate and interact with neurons to form myelinated cocultures (nerve regenerative response), (2) the effect of *M. leprae* infection on intact mature Schwann cell–axon units (intact nerve fiber response), and (3) the effect of incubation at 33°C on *M. leprae* viability within Schwann cells. The results of this study indicated that a new model for studying the effect of *M. leprae* infection on Schwann cells and Schwann cell–neuron interactions has been developed. In this model, *M. leprae* metabolic activity is retained apparently without the loss of Schwann cell and neuron function and survival.

**Materials and Methods**

**Bacteria.** *M. leprae* Thai-53 were passaged and purified from the hind footpads of BALB/c athymic nude mice (Hsd:athymic Nude-nu; Harlan), using a method described elsewhere [20]. These bacteria were suspended in Middlebrook 7H12 medium containing albumin, 50 μg/mL ampicillin (Sigma Chemical), and 50 μg/mL amphotericin B (Sigma) and were incubated for 3 h at 37°C. The bacteria were pelleted by centrifugation at 10,000 g for 5 min and resuspended in D-10 medium consisting of Dulbecco's MEM (Life Technologies) with 10% heat-inactivated fetal calf serum (Hyclone Laboratories) and 50 μg/mL gentamicin (Sigma). Bacterial counts were obtained using acid-fast staining (BBL7 TB Ziehl-Neelsen Kit, Becton Dickinson Microbiology Systems).

*M. leprae* viability was determined by radiorepidentrometry, using the BACTEC 460 system (Becton Dickinson), by seeding 1 × 10^7 bacilli into BACTEC 12B medium (Becton Dickinson), consisting of Middlebrook 7H12 medium containing 14C palmitate, 50 μg/mL ampicillin, 0.04% citric acid, and 2 mg/mL amphotericin B, using a protocol described elsewhere [21]. All *M. leprae* preparations were analyzed for the presence of contaminants by culture on blood agar plates, culture on thioglycolate broth and trypticase soy broth media, and slant culture on Lowenstein-Jensen and 7H11 media (Invitrogen Life Technologies). *M. leprae* exposed to 1 × 10^6-rad gamma irradiation in a Shepherd Model 484 irradiator (J. L. Shepherd) were used as a “dead” control for all experiments [22].

**Schwann cell cultures.** Schwann cells were purified from the sciatic nerves of neonatal Holtzman outbred rats (HsdHot; Holtzman/J SD; Harlan), using a modified Brockes' method [23, 24]. In brief, sciatic nerves from 1–3-day-old rats were dissected and dissociated with 0.1% collagenase (Invitrogen Life Technologies) and 0.5% protease (dispase; Sigma) final concentration in L-15 medium (Life Technologies) and incubated for 30 min at 37°C. This preparation was then treated with a final concentration of 0.05% trypsin and 0.2% EDTA (trypsin-EDTA; both from Sigma), incubated at 37°C for 30 min with agitation, and resuspended in D-10 medium. The cell suspension was then passed through a 70-μm nylon filter (Becton Dickinson Labware) to remove remaining large tissue fragments and obtain a single-cell preparation. Schwann cells were enriched by differential adhesion, using a method described elsewhere [25], and further purified by incubation for 5–7 days in D-10 medium containing 10⁻³ M cytosine β-D-arabinofuranoside (AraC; Sigma).

Schwann cells were then dissociated from the tissue culture flask, using trypsin-EDTA in Hanks' balanced salt solution, and further enriched prior to replating, using complement-mediated lysis by incubation for 30 min each with anti–Thy 1.1 antibodies and sterile filtered rabbit complement (Accurate Chemical and Scientific) at 37°C with agitation [26]. Anti–Thy 1.1 antibodies were purified in our laboratory beforehand from a T11D7e2 mouse-mouse hybridoma cell line (ATCC TIB103), as specified by the American Type Culture Collection, and stored at −70°C until they were used. Schwann cells were subcultured for no more than 5 passages by plating in tissue culture flasks (Corning) coated with poly-L-lysine (PLL; Sigma) in D-10 medium at 37°C in 5% CO₂ until monolayers were confluent. To precoat tissue culture surfaces, PLL hydrobromide (Sigma) was diluted to 2 mM in sterile H₂O and incubated on the culture surface at room temperature for 30 min. The culturing surface (flask or well plate) was rinsed 2–3 times with sterile H₂O. Schwann cells were dissociated with trypsin-EDTA and re-plated onto PLL-coated 13-mm plastic ThermoXon cover slips (Nalge Nunc International) or in 24-well tissue culture dishes (Corning) at a density of 1 × 10⁶ cells/well in D-10 medium and incubated at 37°C in 5% CO₂ until they reached a confluent monolayer. Cultures were incubated for 24 h at either 33°C or 37°C in 5% CO₂ prior to infection with *M. leprae*.

To produce large numbers of Schwann cells for use in Schwann cell monolayer and established coculture experiments, Schwann cells were mitotically expanded using characterized Schwann cell mitogens. To accomplish this, Schwann cells were purified as described above and then plated into 25-cm² flasks with D-10 medium containing the Schwann cell mitogens β-hergulin (2.5 nM; a kind gift of Genetech), forskolin (2 μM; Sigma), and bovine pituitary extract (20 μg/mL; Biomedical Technologies). Cells were maintained until monolayers produced the typical “swirled” morphology. Four days before infection of Schwann cell monolayers with *M. leprae*, hergulin was removed from the medium; forskolin and bovine pituitary extract were maintained for the duration of the experiment. For established coculture experiments, all mitogens were removed from the Schwann cell monolayers, and cells were dissociated and seeded onto purified neurons.

**Purity and viability of Schwann cell cultures.** The purity of Schwann cell cultures was determined by immunostaining analysis for the presence of the Schwann cell marker, S100 cell-surface protein [27]. This was accomplished using anti-S100 antibody (IgG fraction of antisera developed in rabbit; Sigma), the Vectastain Elite ABC kit (Vector Laboratories), the AEC Chromagen Kit (Biogenex), and a protocol described elsewhere [28]. Cultures were considered to be...
pure when fibroblast contamination was <5% of the total cell population. Trypan blue dye exclusion (Life Technologies) was used to determine the viability of these cell preparations.

**Neuron cultures.** Neurons were dissociated and purified from dorsal root ganglia (DRG) harvested from day-15 embryonic Holtzman rats, using a protocol described elsewhere [24]. In brief, 1.5 DRG were suspended in 100 μL of Neurobasal medium (NB1; Life Technologies) containing 1% heat-inactivated fetal calf serum, B27 supplement (1 mL/50 mL in NB1; Life Technologies) and crude nerve-growth factor (10 μL/100 mL in NB1) [24]. Crude nerve-growth factor was produced in our laboratory by removing the salivary glands of 5 male mice and placing each gland in 10 mL of ice-cold sterile H2O. The glands were homogenized, and cellular debris was removed by centrifugation. The supernatant fluids were passed through a 22-μm sterile filter and used at a volume of 10 μL of supernatant/mL of NB1. This suspension was plated into 6-well tissue culture plates that were precoated with rat-tail collagen produced in our laboratory according to a protocol described elsewhere [24]. For some experiments, 0.5 DRG suspended in 25 μL of NB1 were plated onto 13-mm Thermonox coverslips precoated with rat-tail collagen in 24-well plates. Cultures were subjected to 3 weekly rounds of antimitotic treatment [24] using NB1 with 4 μM fluorodeoxyuridine (Sigma), after which the cultures were visually analyzed for purity, using phase-contrast inverted microscopy, and were considered to be pure if fibroblast contamination was <5%. All cultures were incubated at 37°C until axonal sprouts extended from neurons and thickened (3-4 weeks).

**Schwann cell–neuron cocultures.** When the Schwann cell monolayers had a “swirled” appearance, they were considered to be ready for seeding onto purified neurons. The monolayers were dissociated using 0.05% trypsin-EDTA and resuspended in D-10/NB1 (1:1), and ~1 mL containing 5 × 10^6 Schwann cells was seeded onto purified neurons in each well of a 24-well plate. For some experiments, 5 × 10^5 cells/3 mL were seeded onto neurons in each well of a 6-well plate. Cocultures were then incubated at 33°C or 37°C in 5% CO2. After Schwann cells had attached to and proliferated along axons for ~12 days, myelination was induced in these cultures by addition of freshly prepared, filter-sterilized aqueous ascorbic acid (1% final concentration; Life Technologies), as described elsewhere [24].

**Infection of Schwann cells and cocultures.** To determine the effects of *M. leprae* infection on intact, myelinated Schwann cell–axon units were determined by shifting 2 myelinated cocultures to 33°C for 24 h before infection. Cocultures were then infected with *M. leprae* at MOIs of 100 and 300. Cultures were incubated for 48 h, washed with D-10 medium, and maintained at either temperature for up to 28 days after infection. Medium was changed 3 times/week. Cocultures were checked at various time points for microbial contamination by streaking culture supernatant fluids onto blood agar plates and incubating these plates at 37°C for up to 1 week.

*M. leprae* viability in Schwann cells. The viability of *M. leprae* in Schwann cells was determined indirectly using a modification of the Buddemeyer radiospirometry method [29]. In brief, medium was removed from wells containing infected Schwann cells, and cells were lysed by the addition of 400 μL of 0.1 N NaOH (Sigma) in sterile H2O. Next, 300 μL of the cell lysate was transferred to a Shorty vial (Wheaton Science Products) containing 4 mL of BACTEC 12B medium. Shorty vials were then placed in Poly-Q scintillation vials (Beckman Instruments) containing a 2 × 4-cm strip of Whatman #42 filter paper (Whatman International) that had been dipped into scintillation fluid mixture, as described elsewhere [29]. The vials were incubated at 33°C for 7 days and then analyzed for oxidation of palmitate by measuring the release of radiolabeled CO2, using a liquid scintillation counter (Beckman Scientific Instruments). Viability was reported as counts per minute.

**Acid-fast semithin sections and electron microscopy.** Adherent cells and monolayers were prepared for transmission electron microscopy (TEM) by fixation in fixative composed of 1.25% glutaraldehyde and 2% paraformaldehyde in 0.1 M Na-cacodylate buffer, postfixation in OsO4, and dehydration and embedding in Spurr medium, as described elsewhere [30, 31]. Thin sections were obtained from selected blocks, stained with uranyl acetate and lead citrate, and examined and photographed using a Philips 410 electron microscope. To determine the PI and the percentage of cells infected, semithin (1-μm) sections were prepared and stained for acid-fast organisms, using the method of Luderschmidt [32]. For scanning electron microscopy, Schwann cell monolayers and cocultures were fixed in 2% glutaraldehyde/0.1 M cacodylate buffer, postfixed in OsO4, and dehydrated and embedded in Spurr medium. Sections were treated with 1% aqueous aqueous ascorbic acid, embedding in Spurr medium, and placed in 1% OsO4 solution for 1 h. Sections were then dehydrated in ethanol and embedded in Spurr medium and allowed to polymerize for 24 h. Sections were rehydrated in ethanol and allowed to dry. Sections were then cut to 1-μm sections with a glass knife on a diamond knife using a Reichert Ultracut MT microtome and viewed using a Hitachi H-7500 transmission electron microscope.

**Purification of RNA and reverse-transcriptase polymerase chain reaction (RT-PCR).** To determine the effect of temperature and *M. leprae* infection on Schwann cell gene expression, the level of expression of several genes encoding markers typically expressed in short-term–cultured Schwann cells was analyzed by RT-PCR. To accomplish this, total RNA was purified from individual wells containing Schwann cell monolayers by removal of the medium and addition of 300 μL of guanidinium HCL (RNAgent Total RNA Isolation System; Promega). RNA was extracted from these cell lysates, using the manufacturer’s recommendations, and stored at −70°C until it was used.

Total RNA was converted to cDNA using the Advantage J cDNA Polymerase Mix and Advantage J RT-for-PCR Kit (Clontech) and oligo(dT) primer, as recommended by the manufacturer. Primer sequences and optimal PCR programs used in this study are shown in table 1. Primers and PCR parameters for N-cadherin,
intercellular adhesion molecule (ICAM), and L1 were initially designed, using Omiga 2.0 DNA and Protein Sequence Analysis software (Oxford Molecular LTD), from cDNA sequences obtained from GenBank (accession nos. NM_012967 and X59149). Primers for glial fibrillary acidic protein (GFAP) and neural cell adhesion molecule (NCAM) have been reported elsewhere [33], and primers and PCR parameters for transforming growth factor (TGF)-β1 and the housekeeping gene transcript, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), were obtained from a commercial manufacturer (Clontech).

All primer concentrations and PCR parameters were optimized initially using rat sciatic nerve cDNA to provide highly sensitive and reproducible assays for Schwann cell gene expression analysis. To accomplish this, total RNA was purified from the sciatic nerves of 17-day-old rats, cDNA was made using oligo(dT) primers and RT, and PCRs were optimized to detect 10-fold differences in template concentration, using 25–30 cycles of PCR. This cDNA pool was also used as a positive control for all PCR assays. To provide controls for DNA contamination, the total RNA from each sample was added to the RT reaction, but no enzyme was added.

Sample cDNA (5 µL) was added to reactions and amplified by PCR, and the resulting amplicons (15 µL) were separated by gel electrophoresis on 2% NuSieve/SeaKem (1:1) agarose gels (FMC BioProducts). Ethidium bromide–stained gels were visualized by UV transillumination, using a Gel-Doc 2000 (Bio-Rad Systems). Semi-quantitative analysis of the gene expression of various Schwann cell markers was performed by scanning photographic images of gels into a Bio Image System (Bio Image Products, Millipore), using a 3CX scanner (XRS X-ray Scanner). DNA band intensity was converted into integrated optical density (IOD), using Visage Electrophoresis Gel Analysis Whole Band Analysis software (Bio Image Products, Millipore). The data were normalized by dividing the IOD obtained for each template by the IOD of G3PDH, the housekeeping gene transcript, and reported as normalized IOD.

Statistical analysis. Experiments were performed in triplicate, and data were expressed as the mean ± SD of at least 3 replications per experiment. Data were analyzed either by Student’s t test or by SAS software (SAS Institute) analysis of variance in a factorial arrangement of treatment effects (temperature, live M. leprae, dead M. leprae, no infection). Post hoc analyses were conducted with pairwise Student’s t test comparisons of least square means, and all probabilities were considered to be significant at P < .05.

Results

Schwann cell monolayers. Schwann cells cultivated at 33°C appeared to survive and form typical “swirled” monolayers in a manner similar to those cultivated at 37°C (figure 1A and 1B). These cells maintained their viability and survived for several weeks in culture. In contrast, Schwann cells infected with viable M. leprae at 33°C appeared to retract from the surface of the flask and form large cellular aggregates, resulting in the loss of the typical “swirled” monolayer appearance (figure 1D). Very few cellular aggregates were observed in cultures containing irradiated bacteria incubated at 33°C or in cultures containing either viable or irradiated bacteria held at 37°C, even though tissue debris was observed in all of these cultures (figure 1C–1F and table 2). Higher magnification of monolayers demonstrated the differences between tissue debris (figure 2A) and cellular aggregates (figure 2B). TEM analysis of a cellular aggregate revealed that Schwann cells within this aggregate had notable microscopic Schwann cell–Schwann cell interaction alterations (figure 2C). The tightly packed, infected Schwann cells within the retracted areas exhibited more contact with neighboring cells than was observed in other, unaffected monolayers. However, individual Schwann cell–surface membranes remained visibly distinguishable, indicating that the mass of Schwann cells was not syncytial. The center of the clumps of Schwann cells contained amorphous material of unknown composition.

Schwann cell gene expression. When the level of expression of genes encoding typical Schwann cell markers was compared in uninfected Schwann cell cultures after 12 days of incubation, it was observed that expression of these markers was comparable at both temperatures (data not shown). When the expression of Schwann cell markers was evaluated in cells infected with M. leprae and held at 33°C, results indicated that the expression of several Schwann cell genes was altered in some cultures (table 3). Lower levels of GFAP mRNA were observed, regardless of whether cultures had been inoculated with viable or with irradiated M. leprae preparations. Significantly higher levels of NCAM mRNA and lower levels of N-cadherin mRNA were found in cultures infected with viable M. leprae than in

<table>
<thead>
<tr>
<th>Schwann cell marker</th>
<th>Forward</th>
<th>Primer sequence</th>
<th>Reverse</th>
<th>Optimal PCR annealing temperature, °C</th>
<th>Fragment size, bp</th>
</tr>
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<tbody>
<tr>
<td>GFAP</td>
<td>5'-ACTGCAGGGGCTGACGCTG-3'</td>
<td>5'-GATCACCCGCTCCGATTCACTG-3'</td>
<td>62</td>
<td>340</td>
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<tr>
<td>ICAM</td>
<td>5'-CTCATCTGCGTGGCCGGGT-3'</td>
<td>5'-CCGGGACGCCTGAGCCCTC-3'</td>
<td>67</td>
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<tr>
<td>L1</td>
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<td>5'-CATTACCTGAGTCAAGACAC-3'</td>
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<td>629</td>
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<tr>
<td>N-cadherin</td>
<td>5'-ATCTATGAGTCTCCATTCCG-3'</td>
<td>5'-TCGAAAGCCTGACCTCC-3'</td>
<td>55</td>
<td>759</td>
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<tr>
<td>NCAM</td>
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<td>5'-GCCCTAGAGGTGGTCAGGGT-3'</td>
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<td>286</td>
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<tr>
<td>G3PDH</td>
<td>5'-ACCAGACGTTCCATGCTACG-3'</td>
<td>5'-TCCACACCCCTTTGCTGTA-3'</td>
<td>64</td>
<td>452</td>
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<tr>
<td>TGF-β1</td>
<td>5'-CCTCAGCTCCACAGAGAGAAC-3'</td>
<td>5'-CACGATCATGTTGAACTGTC-3'</td>
<td>60</td>
<td>298</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. GFAP, glial fibrillary acidic protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM, intercellular adhesion molecule; NCAM, neural cell adhesion molecule; TGF, transforming growth factor.

* All PCRs were subjected to 94°C for 7 min, 30 cycles of a 3-step program consisting of 94°C for 30 s, the appropriate annealing temperature for 30 s, and 72°C for 30 s; and a final cycle of 72°C for 10 min.
uninfected cultures and cultures infected with irradiated *M. leprae*. TGF-β1 mRNA levels were not altered in cultures infected with viable *M. leprae* but were lower in the cultures exposed to dead *M. leprae*. L1 and ICAM transcript levels were not altered.

**Schwann cell regenerative response.** To determine whether Schwann cells could appropriately interact with axons and thereby potentially participate in a nerve regenerative response at 33°C, Schwann cells were incubated at 33°C for 12 days before being seeded onto the axons of embryonic neurons. These cells were able to attach to, align with, and proliferate along axons in a manner comparable to that of cells held at 37°C (figure 3A and 3B). To determine whether *M. leprae* infection altered the Schwann cell’s ability to interact with axons, infected Schwann cells were incubated at 33°C or 37°C for 12 days before being seeded onto the axons of embryonic neurons. The results of these experiments demonstrated that Schwann cells that were infected with viable or irradiated *M. leprae* were

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**Figure 1.** Scanning electron micrographs of purified primary rat Schwann cell monolayers. *A*, Monolayer held at 37°C. *B*, Monolayer held at 33°C. *C*, Monolayer infected with *Mycobacterium leprae* and held at 37°C. *D*, Monolayer infected with *M. leprae* and held at 33°C. *E*, Monolayer exposed to irradiated *M. leprae* and held at 37°C. *F*, Monolayer exposed to irradiated *M. leprae* and held at 33°C. All infected monolayers were exposed to *M. leprae* at an MOI of 100 for 48 h, and all cultures were maintained for 21 days after infection.
also able to perform these functions at both temperatures in a manner similar to that of uninfected cells (figure 3C–F).

When these Schwann cell–neuron cocultures were stimulated to myelinate in medium containing L-ascorbic acid, myelinated and non–myelin-producing Schwann cells were observed in both uninfected (figure 4A and 4B) and infected (figure 4C and 4D) cocultures at 33°C. Myelin sheath architecture, as well as Schwann cell–surface membranes, mitochondria, organelles, and nuclear membranes, were similar in all cocultures. Axons and neural cell bodies in these cocultures exhibited no apparent differences and were not infected.

Mitogen-expanded Schwann cells. All mitogen-expanded Schwann cells maintained their ability to “swirl” in culture and associate with, proliferate along, and myelinate axons in culture in a manner comparable to that of unexpanded Schwann cells at both temperatures. Mitotically expanded Schwann cells infected with M. leprae at 33°C also lost the “swirled” monolayer appearance, although large cell aggregates were not observed in these cultures (data not shown). These cells were capable of interacting with axons in a manner comparable to that of uninfected cells and supported the viability of M. leprae in a manner comparable to that of cells without mitogens (data not shown).

Established myelinating Schwann cell–neuron cocultures. Established myelinated cocultures originally produced at 37°C and shifted to 33°C showed no alterations in Schwann cell or axonal appearance after being held for up to 1 month at this temperature (figure 5A). M. leprae were phagocytized by myelinating and non–myelin-forming Schwann cells in these cocultures but not by neurons (figure 5B). Some cells contained large numbers of M. leprae (>15 bacteria/cell). There were no observed morphological differences in myelin architecture when 100 cells from infected cocultures were compared with the same number of their uninfected counterparts after 30 days of exposure to M. leprae (data not shown).

M. leprae viability. Purified M. leprae preparations harvested from the footpads of athymic nude mice and used for infection experiments had a mean (±SD) BACTEC growth index of 152 ± 8, and exposure of Schwann cell monolayers to these bacteria at an MOI of 100 for 48 h resulted in a mean (±SD) PI of 20 ± 5 M. leprae organisms/Schwann cell and >99% infection (data not shown). No differences were observed between PIs and the percentage of infection in any of the cultures. Buddemeyer radiospirometry analysis, used to monitor M. leprae viability in Schwann cell cultures, demonstrated that M. leprae retained 56% of their initial viability up to 21 days after infection in Schwann cells monolayers maintained at 33°C, compared with only 3.6% at 37°C (figure 6). In a similar experiment that was conducted for 28 days, a 5% increase in the mean counts per minute was observed between days 21 and 28 when cultures were held at 33°C (data not shown). When M. leprae viability was monitored in mature myelinating Schwann cell–neuron cocultures that were subsequently infected with M. leprae, it was found that bacteria retained 53% of their original viability after 30 days at 33°C, compared with a total loss of viability at 37°C (data not shown).

### Discussion

Most current working hypotheses of nerve damage in leprosy propose direct effects of M. leprae infection in multibacillary leprosy or an aggressive immune response to nerve-associated M. leprae or its antigens in paucibacillary leprosy [14, 34]. Histopathological and molecular evidence have demonstrated that the Schwann cell serves not only as the natural host for the growth and replication of M. leprae in the peripheral nerve but that Schwann cells protect this obligate intracellular organism from immune responses and exposure to therapeutic agents via the blood-nerve barrier. However, despite advances in neurobiology and neuroimmunology and numerous studies to define the specific effects of M. leprae infection on Schwann cells, the mechanisms underlying nerve damage in leprosy have not been fully elucidated. One plausible reason for this is the lack of a standardized experimental tissue culture model that sustains M. leprae viability and mimics in vivo disease conditions.

Previous studies that investigated M. leprae–Schwann cell interactions used M. leprae harvested from a variety of sources, including human skin biopsy specimens [35–37], conventional BALB/c mouse footpads [38], and armadillo tissue [6, 13, 39, 40], but no viability analysis was performed on these preparations. Recently, it was demonstrated that M. leprae obtained from a number of these sources have variable viability and that M. leprae carefully propagated in nude mouse footpads are the best source of highly viable bacteria [19]. Results from the present study confirm that M. leprae propagated in athymic nude mouse footpads produce highly viable bacterial preparations and that these preparations should be considered as a source for all future experiments that use viable M. leprae.

Regardless of the source, M. leprae preparations used for the

<table>
<thead>
<tr>
<th>Temperature, infection type</th>
<th>No. of cell aggregates</th>
<th>No. of wells with aggregates (n = 19)</th>
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<tr>
<td>33°C</td>
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<tr>
<td>No infection</td>
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</tr>
<tr>
<td>Viable M. leprae</td>
<td>79</td>
<td>15</td>
</tr>
<tr>
<td>Irradiated M. leprae</td>
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<td>8</td>
</tr>
<tr>
<td>37°C</td>
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<tr>
<td>No infection</td>
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</tr>
<tr>
<td>Viable M. leprae</td>
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<td>0</td>
</tr>
<tr>
<td>Irradiated M. leprae</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

* Schwann cell monolayers were analyzed for morphological differences using an inverted-phase microscope. Cell aggregates were identified as large clumps of cells that were distinctly different from debris.

* Schwann cells were infected with athymic nude mouse–derived M. leprae at an MOI of 100 for 48 h and held for 12 days.

* Schwann cells were exposed to irradiated athymic nude mouse–derived M. leprae at an MOI of 100 for 48 h and held for 12 days.
Figure 2. Scanning electron and transmission electron micrographs of Schwann cell (sc) monolayers exposed to *Mycobacterium leprae*. A. Tissue debris within an sc monolayer exposed to irradiated *M. leprae*. B. Cell aggregate in an sc monolayer exposed to *M. leprae*. C. Collage of several transmission electron micrographs of an sc aggregate in an sc monolayer exposed to *M. leprae*. All cultures were exposed to *M. leprae* at an MOI of 100 for 48 h and held at 33°C for 21 days after infection.
Table 3. Expression of Schwann cell genes in cells infected by Mycobacterium leprae after 12 days of incubation at 33°C.

<table>
<thead>
<tr>
<th>Schwann cell marker</th>
<th>Uninfected&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Infected with viable M. leprae&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Infected with irradiated M. leprae&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>1.52 ± 0.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.06 ± 0.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.17 ± 0.15&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1.52 ± 0.18</td>
<td>1.25 ± 0.18</td>
<td>0.86 ± 0.18&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>NCAM</td>
<td>0.67 ± 0.22</td>
<td>1.39 ± 0.22&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td>ICAM</td>
<td>0.33 ± 0.08</td>
<td>0.57 ± 0.08</td>
<td>0.32 ± 0.08</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>2.45 ± 0.50</td>
<td>0.91 ± 0.50&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.20 ± 0.05</td>
</tr>
<tr>
<td>L1</td>
<td>0.81 ± 0.26</td>
<td>0.49 ± 0.26</td>
<td>0.65 ± 0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup> nIOD of Schwann cells, mean ± SD
<sup>b</sup> Schwann cells infected with an MOI of 100 viable M. leprae organisms and maintained at 33°C
<sup>c</sup> Schwann cells infected with an MOI of 100 M. leprae exposed to 5 × 10<sup>9</sup> rad of cobalt 60 and maintained at 33°C

NOTE. Gene expression was measured by semiquantitative reverse-transcriptase polymerase chain reaction (PCR) using cDNA produced from total RNA by means of oligo(dT) primers (Promega) and PCR using gene-specific primer sets (table 1). The experiment was performed in triplicate. GFAP, glial fibrillary acidic protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM, intercellular adhesion molecule; NCAM, neural cell adhesion molecule; nIOD, normalized integrated optical density; TGF, transforming growth factor.

The majority of previous studies were either frozen and thawed [38, 41–43], irradiated [34], or stored at various temperatures prior to experimentation, and all experiments were conducted at 37°C [6, 13, 35–38, 41–44]. Recently, these standard laboratory practices have been shown to rapidly reduce M. leprae viability [19]. In addition, results from the present study confirm that incubation of M. leprae at 37°C in Schwann cell cultures and Schwann cell–neuron cocultures leads to a rapid loss of viability of this organism. In contrast, it was demonstrated that >50% viability was maintained within these cultures for at least 28 days when cultures were held at 33°C, and preliminary results suggest that M. leprae may be replicating in these cells, as indicated by an increase in metabolic activity within these cells. These data are the first demonstration, to our knowledge, of the positive effects of temperature on the viability of M. leprae in Schwann cells and suggest that conditions can, perhaps, be further modified to provide an in vitro method for cultivation of M. leprae.

Because M. leprae preferentially infects Schwann cells of the peripheral nerves in cooler regions of the body and is an obligate intracellular parasite of this cell type in vivo, it was not surprising that M. leprae would retain metabolic activity for longer periods within Schwann cells held at a temperature <37°C (core body temperature). In addition, these results suggest that incubation of cultures at 33°C is essential for evaluating the effects of viable M. leprae on Schwann cells.

Primary human, rat, and mouse Schwann cells and mitogen-expanded Schwann cells have been used to study the basic molecular biology of Schwann cells and Schwann cell–neuron interactions [45–48]. Long-term rat Schwann cell–neuron cocultures have been used to elucidate a possible mechanism of the binding of M. leprae to Schwann cells [6, 13]. However, all previously published studies have been conducted at 37°C, a temperature that may be ideal and is traditional for Schwann cell culture but clearly is not ideal for M. leprae. In the present study, rat Schwann cells and their mitogen-expanded counterparts survived and had similar levels of expression of cellular markers at 33°C and 37°C. In addition, rat Schwann cells readily aligned with axons, proliferated along the axons, and produced myelin on stimulation. Both myelinated and non–myelin-producing Schwann cells were observed in these cocultures, and incubation temperature had no apparent effect on Schwann cell or axonal structure when these factors were analyzed by electron microscopy. No aberrations or apparent differences were observed between Schwann cell or axon membrane interfaces, membrane integrity, or Schwann cell organelles. Myelin sheath formation and architecture were comparable and seemed to vary, as expected, with the diameter of the axon enshathed by the Schwann cell. Therefore, it appears that rat Schwann cells and mitogen-expanded Schwann cells were functionally as competent when held at 33°C as their counterparts held at 37°C.

When this model was used to examine the effects of M. leprae infection on Schwann cells, it was found that Schwann cells in monolayer cultures and in cocultures phagocytized M. leprae within 48 h, as was made evident by the presence of M. leprae within Schwann cells in acid-fast semithin sections and in ultrathin sections analyzed by TEM. No differences were observed between the PIs of cells infected with live or irradiated M. leprae at either temperature, which indicates that this was a passive process. These results are in contrast to those of a previous report that suggested that heat-killed bacteria were not phagocytized at the same rate as live bacteria at 37°C [35]. A plausible explanation for this is that cell-surface molecules, which are required for efficient phagocytosis, may have been removed during heat treatment. Irradiation, on the other hand, effectively kills M. leprae while preserving cell-surface components [22].

M. leprae–infected Schwann cell monolayers exhibited alterations in monolayer morphology within 4–12 days after infection when held at 33°C. Large aggregates of Schwann cells that appeared to have retracted from the monolayer were observed, and thin-section acid-fast and electron microscopic analysis revealed that the aggregates consisted of tightly packed infected Schwann cells surrounding unidentified amorphous material. This amorphous material may have been dead, degenerated Schwann cells or mouse tissue debris that remained from the M. leprae harvest suspension. Although residual elements of mouse tissue were present in cultures exposed to live M. leprae and those exposed to irradiated M. leprae at both temperatures, cells only retracted from the monolayer in cultures held at 33°C and consisted primarily of cells infected with viable M. leprae.

The large aggregates of Schwann cells may be indicative of altered Schwann cell–Schwann cell interactions. To further analyze this, infected cell monolayers were subjected to TEM analysis; the results showed that Schwann cells were tightly...
Figure 3. Scanning electron micrographs of Schwann cells (scs) seeded onto purified neurons for 24 h. A, scs expanded at 37°C, seeded onto neurons, and held at 37°C. B, scs expanded at 33°C, seeded onto neurons, and held at 33°C. C, scs infected with *Mycobacterium leprae* at an MOI of 100 for 48 h and held at 37°C for 12 days, seeded onto neurons, and then held at 37°C. D, scs infected with *M. leprae* at an MOI of 100 for 48 h and held at 33°C for 12 days, seeded onto neurons, and then held at 33°C. E, scs exposed to irradiated *M. leprae* at an MOI of 100 for 48 h and held at 37°C for 12 days, seeded onto neurons, and then held at 37°C. F, scs infected with irradiated *M. leprae* and held at 37°C for 12 days, seeded onto neurons, and held at 33°C.

packed within the aggregates, suggesting that these cells had more cell-surface contact with neighboring cells than did cells from the other experimental sets. Therefore, infection may have altered adhesion molecules on the surface of the Schwann cell, which resulted in increased binding of these cells to each other.

In contrast to these results, previous studies showed no apparent morphological or functional effects of infection on Schwann cell cultures at up to 6 weeks after infection [17, 35, 36]. A plausible reason for this is that previous experiments were conducted at 37°C, a conducive temperature for *M. leprae* growth and metabolism, and, therefore, the results from these studies most likely reflect the effect of dead bacteria on Schwann cells in culture. Taken together, these results indicate that Schwann cells respond differently to viable *M. leprae* when held at 33°C instead of 37°C.

When the expression of cellular adhesion molecules in Schwann cells infected with *M. leprae* was studied by RT-PCR, it was found that elevated levels of NCAM transcripts and
decreased levels of GFAP transcripts were observed. These molecules are both important mediators of Schwann cell–Schwann cell and Schwann cell–axon interactions [45, 47]. In addition, NCAM is an important adhesion molecule in the development and maintenance of myelin sheaths. GFAP has been shown to be important for glial cell process formation in the central nervous system [49] and most likely has the same function in the Schwann cells of the peripheral nerves. TGF-β1 gene expression was not significantly altered by intracellular viable *M. leprae*. However, the presence of dead bacilli was associated with lower levels of TGF-β1 expression. Lower levels of TGF-β1 mRNA have also been observed in cultured human monocytes exposed to irradiated *M. leprae* [50]. Because TGF-β1 regulates many Schwann cell functions, including the ability to mediate response to nerve trauma or damage, these data suggest that Schwann cells containing dead *M. leprae* may not be able to assist with nerve regeneration processes [13, 15]. Therefore, dead bacilli in lesions may also contribute to the neuropathology associated with leprosy and demonstrate the need for the use of both viable and dead *M. leprae* in the standardized infection model.

N-cadherin gene transcript levels were lower in *M. leprae*-infected Schwann cells held at 33°C for 12 days. Altered expression of this molecule may lead to altered Schwann cell–Schwann cell and Schwann cell–neuron interactions. N-cadherin has been shown to be localized on the plasma membranes at points of contact between both axons and Schwann cells but not basal lamina [51].

L1, ICAM, and NCAM are highly expressed during development by both the neuron and the Schwann cell, down-regulated postnatally, and reexpressed during axonal regeneration after injury in vivo. In the present study, at 12 days after in-

Figure 4. Transmission electron micrographs of Schwann cell (sc)–neuron cocultures. Infected cocultures were exposed to *Mycobacterium leprae* at an MOI of 100 for 48 h and held for 12 days at 33°C prior to seeding onto embryonic neurons. Cultures were induced to myelinate and then were maintained for 30 days at 33°C. *A*, Myelinated sc. *B*, Non–myelin-forming sc. *C*, Myelinated sc infected with *M. leprae*. *D*, Non–myelin-forming scs infected with *M. leprae*. 
infection with viable *M. leprae*, L1 gene expression levels were not altered, whereas NCAM gene expression levels were significantly increased. These neural cell adhesion molecules interact with similar molecules on associated Schwann cells and neurons [45], and both have critical roles in the Schwann cell and axon relationships. Zhang et al. [46] showed that in vitro L1 gene expression within neurons is not increased after injury. Martini and Schachner [47] demonstrated that both molecules are up-regulated on the surface of Schwann cell–Schwann cell and Schwann cell–axon interfaces during regeneration.

Adhesion molecules have also been shown to play a critical role in the proper relationship between the axon and Schwann cell, which is mandatory for the production and maintenance of myelin [52]. Any modification of this relationship could alter the Schwann cell’s ability to produce or maintain myelin sheath architecture, potentially resulting in demyelination and subsequent nerve damage and interfering with regeneration.

If altered transcription of Schwann cell markers is indicative of altered protein concentrations in infected Schwann cells, this may be at least somewhat responsible for the changes observed in Schwann cell monolayer morphology. However, even though significant changes in gene transcript levels may indicate potential changes in the production of these molecules, the functional aspects of the ability of *M. leprae*–infected Schwann cells to properly relate with axons and produce myelin sheaths did not appear to be affected. This was determined by seeding infected cells onto cultured neurons and assessing the ability of these cells to associate with, proliferate along, and myelinate axons. Results demonstrated that infected cells could perform all of these functions in a manner comparable to that of uninfected cells. In contrast, Mukherjee et al. [36] showed that infection leads to the inability of infected Schwann cells in culture with neurons to synthesize DNA and to associate with axons of neurons in nerve-explant cultures. However, no specific assays were used to definitively exclude the possibility of fibroblasts in these cultures. Cells incapable of adhering to axons in these studies were most likely not Schwann cells.

To simulate the long-term effects of *M. leprae* infection on intact myelinated axons, myelinated Schwann cell–neuron cocultures (which had been held for 3 weeks on myelinating feed) were infected with *M. leprae*, incubated for up to 30 days, and analyzed by microscopy. Under these conditions, both myelinated and non–myelin-forming cells became infected. Higher numbers of *M. leprae* were typically observed in non–myelin-forming Schwann cells; however, some myelinated Schwann cells contained numerous bacilli. There were no apparent ultrastructural changes in myelin architecture or in the number of cells that contained intact myelin sheaths between infected and uninfected cultures held at either temperature, even though some of these cells contained numerous bacilli.

These observations are in direct contrast to a recent report that concluded that *M. leprae* only infects non–myelin-forming Schwann cells and that the short-term exposure (30 min) of the...
surface of the Schwann cell–neuron cocultures to viable or dead *M. leprae*. *M. leprae* cell-wall extracts, or purified phosphoglycolipid I of *M. leprae* leads to demyelination within 24 h, resulting in axonal destruction after 72 h [53]. One plausible explanation for the conflict in the results of these studies is that there were several technical differences. The previous study used an MOI of 30 and an exposure time of 30 min for infection of cocultures, whereas the present study used an MOI of 100 and an exposure time of 48 h. Preliminary experiments in our laboratory have demonstrated that MOIs <100 and exposure times <48 h resulted in a lower percentage of infection in Schwann cells and lower PIs, particularly in the myelinated Schwann cell phenotype (data not shown). Previous histopathological observations from several laboratories have shown that, even though *M. leprae* preferentially infect non–myelin-forming Schwann cells, infected myelinated Schwann cells are routinely found in human nerve biopsy samples from patients with multibacillary leprosy [5, 7, 54]. Therefore, these data suggest that the experimental conditions used for the previous study were not optimal for successful infection of the myelinating Schwann cell phenotype and that the infection model described in the present study more closely mimics in vivo conditions.

One explanation for the differences observed in myelin architecture in these studies is that the previous study used very sensitive immunofluorescence techniques to detect markers associated with myelin in Schwann cells and TEM to analyze the early (24–72 h) effects of infection on these cells, whereas the present study used semithin-section acid-fast stain microscopic and inverted-phase microscopic techniques to study these early events. However, the latter techniques did not indicate that exposure to *M. leprae* leads to massive demyelination or axonal destruction. In addition, TEM of 30-day cultures showed no differences in the number of Schwann cells that contained intact myelin sheaths and no apparent differences in myelin architecture between infected and uninfected cultures.

It is feasible that if demyelination went unnoticed in the early cocultures in the present study, demyelinated Schwann cells in these cocultures repopulated axons and remyelinated axons during the 30-day time interval. We have shown that *M. leprae*–infected Schwann cells are capable of attaching to, proliferating along, and myelinating axons of cultured embryonic neurons.

In summary, the results of the present study clearly demonstrated that Schwann cells cultured at 33°C appear to survive and function in a fashion comparable to that of cells incubated at 37°C and that the metabolic activity of *M. leprae* is maintained longer within Schwann cells at 33°C than it is at 37°C. A new model for studying *M. leprae*–Schwann cell and Schwann cell–neuron interactions has been developed; this model uses highly viable, athymic nude mouse footpad–derived *M. leprae* and purified primary Schwann cells and Schwann cell–neuron cocultures and maintains these cultures at 33°C prior to and during infection studies. We have also demonstrated the usefulness of mitogen-expanded Schwann cells in the improved infection model and shown that their response to infection is similar to that of unexpanded cells. One of the main benefits of using mitogen-expanded Schwann cells is that they replicate every 2–5 days instead of every 7–8 days. Therefore, more cells are available in a shorter period of time for use in experiments. In addition, because they replicate so rapidly, they outgrow the low levels of fibroblasts in the population and thereby typically produce cultures with higher Schwann cell purity. Mitogen-expanded primary Schwann cells have been shown to retain many of the important primary Schwann cell characteristics and are capable of interacting with axons in a manner similar to that of unexpanded cells in vitro [48, 55]. Our data also support these conclusions. It is anticipated, therefore, that mitogen-expanded Schwann cells will be very useful as an integral part of a new model to study *M. leprae*–Schwann cell interactions.

In conclusion, it is anticipated that this new model will be useful for identifying the additional effects of *M. leprae* infection on Schwann cells and Schwann cell–neuron interactions. A better understanding of the direct effects of *M. leprae* infection on Schwann cells may identify factors that contribute to leprosy neuropathy. In addition, this standardized model will also be useful in defining the role that specific cytokines and immune cells have in the destruction of nerves in patients with leprosy.

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