Intracellular Multiplication of Leprosy-Derived Mycobacteria in Schwann Cells of Dorsal Root Ganglion Cultures

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Organized nerve cultures of dorsal root ganglia from neonatal mice were infected with Mycobacterium leprae, the causative agent of leprosy. A significant multiplication of the acid-fast bacilli was observed within the Schwann cell component of the culture. The growth of these bacilli was sensitive to antileprosy drugs and was not observed directly in bacteriological media. These organisms were brightly stained with the monoclonal antibody to phenolic glycolipid-I, a M. leprae-specific marker. The antigenic, pathogenic, and biochemical characteristics of this mycobacterium are under investigation.

Mycobacterium leprae, the causative agent of leprosy, has not yet been cultivated in vitro. Hanks (20, 21) described it as an obligate intracellular parasite requiring a suitable cellular host for growth and multiplication. Consequently, attempts were made to cultivate this organism in several types of cultured cells, with only marginal results (8, 10, 19, 27). However, except for macrophages, the other cells used in these studies were not natural hosts for M. leprae. The possibility of cultivating M. leprae in Schwann cells, the other natural host, has not been explored, although there is considerable evidence that they are probably the target cells of M. leprae (2, 3, 5, 24, 39, 42). A histopathological study of the early lesions of both lepromatous leprosy and tuberculoid leprosy revealed the presence of acid-fast organisms primarily in the Schwann cells of the nerves (5). Dissemination of these acid-fast organisms to other tissues, such as the liver and spleen, is seen only at a later stage of excessive bacillary proliferation (2, 5). The bacteriological and morphological indices of the bacilli in nerves and Schwann cells are generally higher than those in the skin and other tissues (9). Schwann cells have also been known to harbor "persister" and "resister" organisms (4, 41). It can, therefore, be speculated that Schwann cells in tissue cultures could also serve as a host for the prolonged survival and multiplication of M. leprae.

Schwann cells can be cultured in vitro in isolation as a monolayer (11) or as a component of organized cultures of dorsal root ganglia (13) and can be easily identified (11, 13). The advantage of the latter model is that in this system, the Schwann cells express most of their functional properties, e.g., they associate with axons, acquire a basement membrane, secrete collagen, and synthesize myelin (12-14). These cells, therefore, are physiologically analogous to their in vivo counterparts and may be an appropriate host. Therefore, Schwann cells in organized nerve cultures were infected with M. leprae and maintained for extended periods to study the fate of the intracellular bacilli.

**MATERIALS AND METHODS**

Organized nerve cultures. The organized nerve cultures of the sensory ganglia of newborn Swiss white mice were grown on collagen-coated cover slips or in tissue culture flasks (25-cm² culture surface area) as described elsewhere (31).

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(i) Bacillary load of 200 Schwann cells sampled at several parts of each culture was determined.

(ii) Flask cultures. Cells from flask cultures were harvested with trypsin (0.25% [wt/vol])-EDTA (0.1% [wt/vol]), centrifuged at 92 x g for 10 min in a Sorvall RC-5B refrigerated superspeed centrifuge with an SS-34 rotor, suspended in phosphate-buffered saline, and homogenized in an all-glass homogenizer. The homogenate was centrifuged at 92 x g, and the supernatant thus obtained was centrifuged at 20,800 x g for 10 min. The pellet was suspended in isotonic saline with 1% (vol/vol) bovine serum albumin and counted by the method of Hart and Rees (22). Bacteria were counted on days 1, 7, 14, 21, 28, 35, and 42 postinfection.

Testing for mycobacterial contaminants. Mycobacteria inoculated into organized nerve cultures and harvested from them were inoculated into nutrient agar and into Lowenstein-Jensen, Dubos, and Sautons bacteriological media. The extractability of acid fastness with pyridine was tested by the technique of Convit and Pinaridi (15).

Antileprosy drugs. Infected cultures from day 1 postinfection were incubated in the continuous presence of 1 μg of dapsone (DDS) or 5 μg of rifampin per ml. The feeding medium and the drugs were replaced twice a week. [3H]thymidine (1 μCi/ml) was added to each culture 7 days before termination. The cultures were terminated on day 7, 15, or 21 postinfection and processed for autoradiography.

FIG. 1. Portions of M. leprae-infected organized nerve cultures showing a gradual increase in the bacterial load (arrows) of Schwann cells on day 1 (A), day 14 (B), and day 28 (C) postinfection. Ziehl-Neelsen stain.

FIG. 2. Frequency distribution of AFB within Schwann cells of dorsal root ganglion cultures on days 1, 14, and 28 postinfection. Data are from 30 cultures, 10 per group.
The bacillary load and the number of silver grains on the bacilli of the control and drug-treated cultures were determined and compared.

**Immunofluorescence.** Cultures were washed with BSS, fixed for 10 min in 4% formalinized saline, rinsed several times with BSS containing 5% heat-inactivated horse serum (BSS-HS), and then incubated at room temperature in a moist chamber with the monoclonal antibody to phenolic glycolipid-I (supplied by B. R. Bloom) diluted 1:40 in BSS. After 40 min, the cover slips were washed with BSS-HS and incubated with fluorescein-conjugated goat anti-mouse immunoglobulin G (Cappel Laboratories) diluted 1:15 in BSS. After 30 min, the cover slips were rinsed with BSS-HS, mounted in 50% glycerol-phosphate-buffered saline, and sealed with nail varnish. These cultures were viewed under oil immersion with the 63× objective of a WL Zeiss microscope equipped with both phase-contrast and fluorescence optics and were photographed.

**RESULTS**

As reported earlier (31), acid-fast bacilli (AFB) were seen within Schwann cells and fibroblasts but not in neurons and axons. In this study, because the cultures were enriched for Schwann cells, the behavior of intracellular *M. leprae* cells observed was that of bacteria resident chiefly within Schwann cells.

The microorganisms contained within Schwann cells were rod-shaped AFB. No other forms of non-AFB or AFB were detected. Examination of the cover-slip cultures under a light microscope revealed bacilli in small, countable numbers localized at the polar end of 70% of the Schwann cells on day 1 postinfection, whereas on days 14 and 28 postinfection, the Schwann cells were loaded with significantly increased numbers of bacilli (Fig. 1A through C). The average number of bacilli per Schwann cell therefore showed a definite and gradual increase with the increase in the duration of the infection (Fig. 2). Over the course of the infection, the arrangement of intracellular bacilli also gradually changed from that of a few scattered AFB early in the infection (Fig. 1A) to more regular clumps and eventually globi after prolonged infection (Fig. 1C). Ultrastructural studies revealed that the organisms within Schwann cells were intraphagosomal and surrounded by electron-transparent zones (Fig. 3).

The increases in the numbers of intracellular bacilli observed in cover-slip cultures were corroborated by bacillary counts in flask cultures. The cumulative increases in the numbers of AFB within Schwann cells was gradual, peaked on day 28 postinfection, and declined thereafter (Fig. 4 and Table 1). The optimum increase over a period of 28 days was 9.34- to 16.82-fold, with a mean of 11.72-fold. The decrease

**FIG. 3.** Electron micrograph of a thin section of a *M. leprae*-infected dorsal root ganglion culture fixed in 2% glutaraldehyde and 1% osmium tetroxide, dehydrated, and embedded in araldite. Note the intracellular bacilli (b) enclosed within a phagosome (p) and surrounded by electron-transparent zones (z). Uranyl acetate-lead citrate stain.

**FIG. 4.** Growth curve of AFB cultured in Schwann cells. Each point on the graph represents the mean counts for 10 strains of bacilli.
in bacillary counts in cultures after 28 days postinfection was mainly caused by heavily infected cells floating on the substratum, leading to a fall in the cellular density of the culture.

The growth of AFB was also evidenced by \(^3\)Hthymidine incorporation and hence DNA synthesis by bacilli in autoradiograms of infected cultures (Fig. 5). Silver grains were seen on individual bacilli and clumps of bacilli under both bright-field (Fig. 5A) and dark-field (Fig. 5B) illumination. Grain counts on intracellular bacilli were possible because of nonincorporation of \(^3\)Hthymidine by the nuclei of the host cells, an observation reported earlier (32). With the increase in the duration of the infection there was an increased incorporation of \(^3\)Hthymidine which paralleled the concomitant increases in the microscopic counts of intracellular bacilli (Fig. 5C). Both of these parameters were significantly reduced by the antileprosy drugs DDS and rifampin at doses of 1 and 5 \(\mu\)g/ml, respectively (Fig. 5C).

**TABLE 1. Counts of intracellular bacilli maintained in flask cultures of dorsal root ganglia**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bacillary count ((10^9)) on day postinfection*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>0.92</td>
</tr>
<tr>
<td>2</td>
<td>0.94</td>
</tr>
<tr>
<td>3</td>
<td>0.69</td>
</tr>
<tr>
<td>4</td>
<td>0.91</td>
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<tr>
<td>5</td>
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<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
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</tr>
<tr>
<td>8</td>
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</tr>
<tr>
<td>9</td>
<td>1.02</td>
</tr>
<tr>
<td>10</td>
<td>0.71</td>
</tr>
</tbody>
</table>

* The mean bacillary counts \(\times 10^6\) ± the standard deviation on days, 1, 7, 14, 21, 28, 35, and 42 postinfection were 0.90 ± 0.23, 1.69 ± 0.6, 4.17 ± 1.4, 7.55 ± 1.7, 10.87 ± 2.29, 10.48 ± 2.37, and 9.73 ± 2.35, respectively.

**TABLE 2. Counts of intracellular bacilli, between days 1 and 28 postinfection, subcultured in flask cultures of dorsal root ganglia**

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Bacillary counts ((10^9)) on day postinfection:</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>1</td>
<td>0.47</td>
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<tr>
<td>2</td>
<td>0.78</td>
<td>9.1</td>
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<tr>
<td>3</td>
<td>0.80</td>
<td>10.2</td>
</tr>
<tr>
<td>4</td>
<td>0.51</td>
<td>6.2</td>
</tr>
</tbody>
</table>

No increases in the numbers of bacilli were observed with heat-killed organisms. No growth of AFB was observed directly in the growth medium at the beginning or the end of the cultivation period. None of the strains of bacilli either before or after release from Schwann cells grew in Lowenstein-Jensen, Sauton, or Dubos bacteriological media. These bacilli also showed the property of extractability of acid fastness with pyridine.

The host Schwann cells showed no evidence of toxicity even up to 4 weeks postinfection. Good incorporation of \(^{14}\)Cacetate, \(^{3}\)Hleucine, and \(^{3}\)Hproline was seen as uniformly distributed silver grains over all the cells in autoradiograms of the infected cultures.

In the next set of experiments, *M. leprae* cells were passaged within Schwann cells in flask cultures by releasing the organisms from the host cells, counting them, and inoculating them at the original concentration of 5 \(\times 10^6\) ml into fresh flask cultures. Table 2 shows that the intracellular growth of AFB within Schwann cells was transferable and that the growth patterns of AFB in all four passages were similar, i.e., in each passage, the optimum increase of ca. 10- to 12-fold AFB occurred 28 days postinfection. The AFB obtained after passage 4 in Schwann cells had the same characteristics as the original AFB with respect to the extractability of acid fastness with pyridine and the inability...
to grow in bacteriological media. From the in vitro growth curve (Fig. 4), the generation time of *M. leprae* was calculated to be 7 days (25).

These organisms cultivated within Schwann cells showed marked fluorescence with the monoclonal antibody to phenolic glycolipid-I. The organisms from passages 1 through 4 were brightly stained (Fig. 6).

**DISCUSSION**

The inability to cultivate *M. leprae*, the causative agent of leprosy, in laboratories has to a great extent restricted research work on leprosy (21). Earlier attempts to cultivate it directly in vitro yielded either no growth or the growth of AFB not related to *M. leprae*, e.g., ICRC, H-57, and Corynebacterium spp. (7, 16, 38). Attempts to use several kinds of cultured cells have failed to yield any significant growth of the organism (19, 30, 33, 36). These findings suggest that *M. leprae* is an extremely fastidious organism requiring very specific conditions for its growth and multiplication, such as the available in vivo conditions within macrophages or Schwann cells. Attempts to culture *M. leprae* within macrophages have yielded limited multiplication (17, 34, 36, 40). The possibility of cultivating *M. leprae* in Schwann cell cultures, even though speculated by Lumsden (26), has not yet been explored.

The present study demonstrates that human-derived *M. leprae* cells undergo significant multiplication within Schwann cells in tissue cultures. Multiplication, as assessed by examining infected cultures under a light microscope as well as by making actual quantitative measurements, was consistent in 25 experiments. In flask cultures, although the original inoculum consisted of $2.5 \times 10^7$ organisms, the actual number of organisms phagocytosed at 72 h was less than or around $10^6$ (Table 1). The low level of selective phagocytosis of viable organisms has previously been reported (31). The remaining nonphagocytosed organisms from the original inoculum were carefully washed, and the growth medium was renewed twice a week, to ensure minimal repeated phagocytosis. These organisms exhibited several properties distinct from those of other leprosy-derived mycobacteria. The growth was strictly intracellular, inducing the inhibition of host cell proliferation, a feature reported earlier as being specific to *M. leprae* (32). Growth was inhibited by the antileprosy drugs DDS and rifampin. No growth was observed in the bacteriological media tested. Acid fastness was extractable with pyridine (15). Although these characteristics are common to *M. leprae*, there is still some reservation about their specificity.

Recent studies (23) have revealed that phenolic glycolipid-I, a part of the *M. leprae* capsule, is a highly specific molecule which differentiates *M. leprae* from all other mycobacteria and that the monoclonal antibody generated against phenolic glycolipid-I is a marker specific for *M. leprae* (28). The organisms cultivated by us in Schwann cells had the capacity to produce phenolic glycolipid-I. The other antigenic and pathogenic characteristics and cell wall composition of this cultivable organism are under investigation and will be reported shortly (manuscript in preparation).

The faster growth rate of the organisms cultivated within Schwann cells as compared with that in mouse foot pads (37) can be attributed either to the difference in the milieu and temperature of these two systems or to the inherent differences in the growth rates of these two types of organisms.

The retention of the growth potential and original characteristics after four passages demonstrates that within Schwann cells, *M. leprae* can be continuously subcultured without cross-contamination with any other species of mycobacteria.

The growth of *M. leprae* within Schwann cells is not entirely surprising. This culture system attempts to over-

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**FIG. 6.** Immunohistochemical staining with the monoclonal antibody to phenolic glycolipid-I of bacilli cultured in Schwann cells. (A and B) Phase-contrast (A) and fluorescence (B) microscopy of bacilli (arrows) after passage 1 in Schwann cells. Note the intracellular bacilli intensely stained with the antibody. (C) Same strain as in panels A and B but released from the host after passage 4 and brightly stained with the antibody.
come deficiencies described as inherent in direct in vitro culturing systems (21). M. leprae cells within Schwann cells are intraphagosomal and have capsules around them. Hence, these organisms are not leaky and are capable of retaining metabolic pools required for multiplication, as experimentally demonstrated by the DNA synthesis reported in this paper and the lipid synthesis to be reported later (manuscript in preparation). The possibility of host Schwann cells supplying metabolites for the growth and multiplication of M. leprae remains to be explored. At this stage, evidence in this direction is indirect but persuasive. Lipids are the important major cell wall components of M. leprae (29), and Schwann cells have the ability to synthesize in vitro bulk quantities of myelin components and other lipids that could be utilized by M. leprae (6, 13, 18, 43).

ACKNOWLEDGMENT
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LITERATURE CITED
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