Multiplication of Armadillo-derived *M. leprae* in Murine Dissociated Schwann Cell Cultures

TO THE EDITOR:

For as long as viability of *Mycobacterium leprae* is thought to be responsible for its pathogenesis, its *in vitro* cultivation holds an edge over the production of structural epitopes by recombinant technology. In 1985, Mukherjee and Antia reported a sustained multiplication of human-derived *M. leprae* within Schwann cells of an organized nerve culture derived from dorsal root ganglia (DRG) of newborn mice ([5]). Since the DRG culture technique is a relatively fastidious one and requires almost 3 weeks for cellular organization, it was decided to attempt pathogen growth in dissociated Schwann cell cultures whose characteristics are likely to be different from their organized counterparts. Besides achieving ease in maintenance, reducing the culture period, and acquiring homogenous cell populations, it was also anticipated that such trials might yield leads to the Schwann cell properties responsible for supporting the intracellular growth of *M. leprae*.

This communication describes the culture conditions found to be optimal for obtaining *M. leprae* growth in dissociated Schwann cells (DSC) and to emphasize the conditions that differ from those we reported for DRG cultures in 1985 ([5]).

Since a semi-quantitative count of intracellular bacilli on culture coverslips could be subjective and therefore erroneous, a quantitative petri plate culture method was used to evaluate growth. Dissociated Schwann cells were obtained from the sciatic and brachial nerve plexuses of 1-day-old, Swiss white mice by the method of Brockes, *et al.* ([2]) and cultured in Falcon petri plates. An equal number of petri dishes with the equivalent numbers of Schwann cells in each set was terminated at time periods of 2 weeks and 4 weeks. All of the cultures were used at a maturation period of 7–12 days after initiation. Since initial viability of the inoculum is a prime factor in the outcome, *M. leprae* derived by homogenization from infected armadillo spleen and liver tissues were used in all of the experiments. *M. leprae*-infected armadillo tissues were supplied by Dr. E. Storrs, Melbourne, Florida, U.S.A. The tissues were shipped frozen to Bombay where they were aliquoted and stored at −90°C until use. For use, the aliquoted tissues were thawed and homogenized in Hanks' balanced salt solution. Tissue contamination was reduced by differential centrifugation and the bacilli quantitated. Before use the suspensions were inoculated into sterile Dubos broth for detection of contam-

TABLE 1. *Acid-fast bacilli recovered from dissociated Schwann cell cultures inoculated with armadillo-derived* M. leprae.

Experiment no.	No. acid-fast bacilli × 10^6 (fold increase) at			Doubling time (days)
	Initial (4–6 hr)	2 weeks	4 weeks	
1	0.17	2.60 (14.4)	4.19 (23.8)	6.00
2	0.66	N.D.[a]	7.50 (12.5)	7.60
3	1.25	N.D.	12.02 (9.6)	8.60
4	1.29	8.45 (6.5)	14.70 (11.5)	7.95
5	0.11	1.18 (10.7)	1.90 (17.2)	6.82
6	0.27	2.69 (10.7)	3.79 (14.0)	7.36

[a] N.D. = not done.

inant cultivable mycobacteria and verified as *M. leprae* by staining with anti-phenolic glycolipid-I (PGL-I) antibody (46.7). A total of 12.5 × 10^6 acid-fast bacilli (AFB) was added to each petri dish, and phagocytosis was allowed to proceed for periods varying from 4 hr to 72 hr. The cultures were then washed, and the extracellular bacilli were counted and discarded. The intracellular AFB count of one set of petri plates was taken. This initial count was obtained by extensive washing of the monolayer with 2 mM EDTA and by harvesting the cells from the petri plates with trypsin EDTA. The intracellular bacteria were released by 3–5 cycles of freeze-thawing before enumeration on spot slides by staining with Ziehl-Neelsen carbol fuchsin.

The cultures were fed every other day. At the first feeding after injection, the bacteria released in the culture supernatants were counted and reinfected into naive Schwann cell cultures (NSC). A set of petri dishes for termination at any one time (either 2 weeks or 4 weeks) had its own NSC culture dish. On subsequent feedings, bacteria released in the culture from the NSC were counted and then discarded. Intracellular bacteria were harvested at days 14 and 28 as described above. Growth at 2 or 4 weeks was enumerated as:

$$\text{Intracellular AFB count of petri plates terminated at 2 or 4 weeks} + \text{that of NSC culture receiving bacteria from supernatant of equivalent petri plate} + \text{Sum of bacteria obtained in supernatant of NSC cultures}$$

Table 1 demonstrates the growth achieved over a period of 4 weeks in infected DSC cultures. An average of 15 ± fivefold growth was noted over the six sets of experiments performed. The mean doubling time of *M. leprae* was calculated to be 7.3 days ([4]), a value close to the 7.8 days reported by Mukherjee and Antia ([5]). No correlation was seen between the degree of growth obtained and the initial morphological index of the culture suspensions. This marked degree of multiplication was achieved through the inclusion of the following in the present culture protocol.

Short phagocytic pulse of not more than 6 hr. Significant *M. leprae* growth was not achieved when the phagocytic pulse was of 72 hr. In contrast, an ingestion period of 72

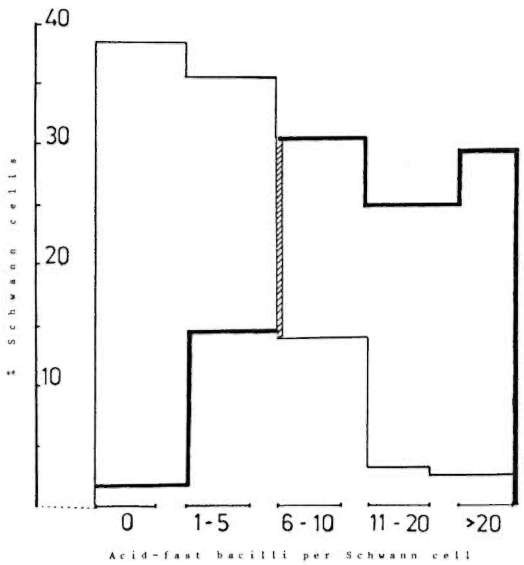
TABLE 2. *Fc receptor expression of mouse peritoneal macrophages on infection by* M. leprae *derived from supernatants of infected murine dissociated Schwann cell cultures.*[a]

	% EA rosetting[b]	
	Experiment no.	
	1	2
Uninfected macrophages	66[c]	78
Macrophages infected with *M. leprae* obtained from DSC culture supernatants after a pulse of 4–6 hr	69	92
Macrophages infected with *M. leprae* from same batch as above but not fed to DSC cultures	28	50

[a] A reduction of >10% from control values of uninfected macrophages is interpreted as activity of metabolically active *M. leprae*. Fc receptor expression by macrophages is assayed by the erythrocyte amboceptor (EA) rosetting method.

[b] Normal range of EA rosetting in mouse peritoneal macrophages is 50–90%.

[c] Each value is an average of duplicates.



THE FIGURE. Percentage of Schwann cells bearing varying intracellular loads of *M. leprae* 4–6 hours (——) and at day 14 (▬▬) after infection of cultures; (▨) = area common to both time periods

hr was found to be optimal for *M. leprae* growth within Schwann cells of DRG cultures ([5]). This was thought to be due to the overcrowding of dissociated Schwann cells with dead or metabolically inactive bacteria which managed to enter the Schwann cells over a period of 72 hr. The short phagocytic pulse that was successful in demonstrating growth may, therefore, be functional in enriching the Schwann cell for either live or metabolically active bacilli. The concept that live bacteria may gain a quicker entry into Schwann cells than dead or metabolically inactive bacteria gains support from experiments where viability of those organisms remaining uningested by Schwann cells at 4–6 hr was compared with that of the whole inoculum. Viability or metabolic activity was assessed by means of the macrophage Fc receptor expression assay ([1]). Uningested bacteria were unable to lower the Fc receptor expression of murine peritoneal macrophages from control values but the whole inoculum was able to do so rather strikingly (Table 2). In another series of experiments, it was noted that rifampin-treated bacteria were unable to gain entry into Schwann cells at 8 hr. However, at 72 hr they were found within Schwann cells in numbers comparable to untreated organisms (Choudhury, *et al.*, unpublished data).

Recycling of bacteria released from culture supernatants into NSC cultures. Semiquantitative counts made between days 14 and 28 of the culture period after infection revealed a marked increase in the proportion of heavily bacillated cells (The Figure). It was therefore considered futile to recycle the bacteria harvested from the culture supernatants back into autologous cells. Instead, they were fed into replicate Schwann cell cultures.

Short time interval (48 hr) between subsequent feeds and harvest of bacteria from supernatants. This was incorporated into the protocol to minimize the time period that supernatant bacteria would have to survive extracellularly. During the course of the experiments, it was noted that as the time interval was reduced, the uptake of bacteria by the NSC cultures was more efficient (data not shown).

Bacteria obtained from DSC cultures were identified as *M. leprae* on the basis of nongrowth in Dubos' medium and by positive staining of the organisms with anti-PGL-I (antibody 46.7 supplied by Dr. Barry Bloom). Viability of bacteria by the ethidium bromide-fluorescein diacetate method ([3]) was found to be consistently greater than 89%. Observations with the mouse foot pad are awaited.

The observations indicate an equivalent capacity of both dissociated and organized Schwann cells to support the intracellular growth of *M. leprae*. However, changes in the protocol were necessitated to facilitate the higher ingestion capacity of dissociated Schwann cells. The 15-fold increase in the number of *M. leprae* observed in DSC compared to the 10.76-fold increase reported earlier ([5]) could be artefactual due to the small number of experiments presented here, or due to the considerably higher degree of activity of armadillo-derived *M. leprae* compared to the patient-derived organisms that were used in the previous study. Alternatively, it may argue for differing metabolic properties of the somewhat "dedifferentiated" Schwann cells in dissociated cultures.

Nevertheless, growth of *M. leprae* does not necessarily mean its cultivation. The ability of neuroglial cell lines to support continuous multiplication of *M. leprae* is currently under investigation.

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Acknowledgments. This work was supported by a grant from WHO-THELEP. We gratefully acknowledge Dr. E. Storrs and LEPRA for providing us with infected armadillo material and Dr. Barry Bloom for a generous supply of the monoclonal antibody against phenolic glycolipid-I.

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