INTERNATIONAL JOURNAL OF LEPROSY

Volume 48, Number 2
Printed in the U.S.A.

Organized Nerve Culture. I. A Technique to Study the Effect of *M. leprae* Infection[1]

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Leprosy is primarily a disease of the peripheral nerves of man. *Mycobacterium leprae* can be found in various components of the nerve such as the Schwann cells, perineural cells, endothelial cells, and occasionally in the axons of nerve biopsies taken from lepromatous leprosy patients ([8, 13, 18]) and even in nerve biopsies taken from very early cases of leprosy ([2, 11]). The mechanism of the association of bacilli to the various components of the nerve and the sequence of events that results in pathological changes in the host cells remain to be elucidated.

In vitro cultured nerves are readily accessible and can be studied at various stages of their growth, organization, and myelination ([12]). Such cultures can also be inoculated with *M. leprae* in the early premyelin secretory phase, and the effect of the long-term presence of bacilli on the subsequent organization and myelination of the nerves can be investigated.

Using organized nerve culture, we report here that *in vitro* Schwann cells in the premyelin secretory phase are phagocytic, and it is possible to infect them with *M. leprae*. Once infected, *M. leprae* interfere with further Schwann cell-axon association, interaction, and subsequent myelination. If this finding can be extended to the *in vivo* situation, it may be one of the important links in the chain of events leading to nerve damage in leprosy.

MATERIALS AND METHODS

The technique described by Murray ([12]) with a slight modification was used to culture the dorsal root ganglia of neonatal mice *in vitro*.

Sensory ganglia of 1–3 day old Swiss white mice were removed under sterile conditions. Nerve fibers, both incoming and outgoing, were cut at the edge of the ganglia and the capsule excised in several of them. These ganglia were then trypsinized with 0.25% trypsin in calcium and magnesium free phosphate buffered saline (PBS) and explanted on collagen coated glass coverslips. Collagen coating of these coverslips was done by the method described by Bornstein ([3]). Briefly, adult rat tail was treated with 1% acetic acid to extract collagen. This collagen-acetic acid mixture was dialyzed against distilled water, and 2 drops of dialyzed collagen solution were spread over the coverslip and polymerized by exposure to ammonia vapor for 5 min. The ammonia was removed by rinsing the coverslips several times with Hanks' Balanced Salt Solution (BSS). The coverslips were then transferred to plastic petri dishes (Falcon, U.S.A.), incubated in 100% humidity at 36°C, and fed on alternate days with a mixture of 70% Eagle's Minimum Essential Medium containing 600 mg% glucose, 20% fetal calf serum, 10% chick embryo extract (EE50), and penicillin (100 IU/ml).

The cultures were regularly viewed with an inverted microscope in the living state and terminated at desired intervals. Some of the cultures were exposed for 24 to 144 hr to 5–8 $\times$ 10^7 *M. leprae* isolated from fresh nodules of lepromatous leprosy patients by the method of Ambrose, *et al.* ([1]). Some of the cultures were terminated soon after inoculation to look for phagocytosis. Others were washed with BSS, fed with normal feeding mixture, maintained for a period of 2 to 4 weeks post infection, and then terminated. A few cultures were infected with heat killed *M. leprae*, ICRC-C44, and cultivable strains of acid-fast bacilli received from Professor L. Kato (Laval, Canada).

Terminated cultures were rinsed with BSS, fixed in formal-saline for 24 hr, and

[1] Received for publication on 1 November 1979; accepted for publication on 11 December 1979.

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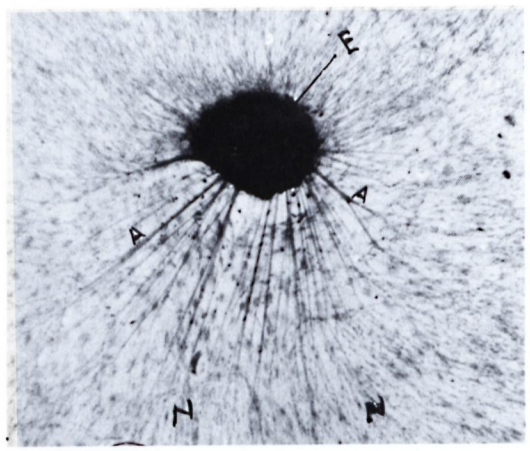

FIG. 1. Overall view of a dorsal root ganglion cultured for 10 days *in vitro*. E = explant region containing neuronal cell bodies, A = axon bundles, N = bed of non-neuronal cells consisting of invading nerve fibers, Schwann cells, and neurofibroblasts. (×29).

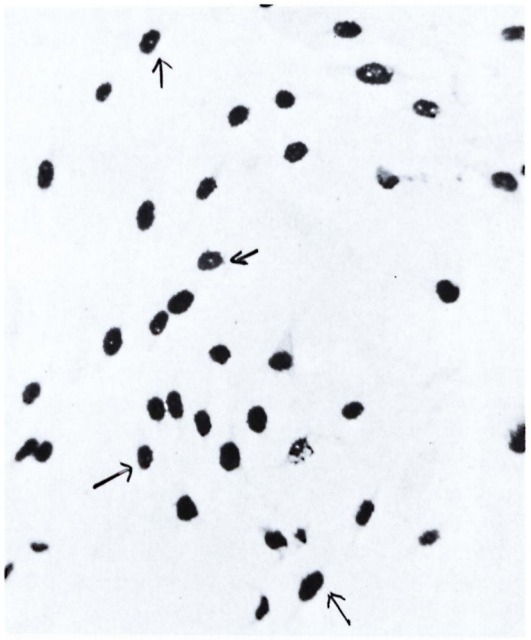

FIG. 2. Autoradiogram of 10 day old culture. Schwann cell nuclei are heavily labelled with ^{3}H-thymidine (arrows). (×200).

stained with Holmes' silver for axons, methylene blue for cellular organelles, Sudan black B for myelin, and Ziehl-Neelsen for acid-fast bacilli.

Some of the cultures in the pre-myelin secretory phase were pulsed with ^{3}H-thymidine (specific activity 8.9 Ci/mM) and ^{3}H-leucine (specific activity 7.9 Ci/mM) (Isotype Division, BARC, Bombay), 2 μCi/ml each for 24 hr, washed with BSS, and then fixed in formal-saline for 24 hr. These coverslips were then coated with Ilford K5 emulsion diluted 1:1 in distilled water. The emulsion was air dried, and the coverslips were stored in light proof boxes at 4°C. They were developed on the 10th or 12th day after coating and fixed and stained with the Ziehl-Neelsen stain.

RESULTS

About 500 cultures of dorsal root ganglia were cultivated *in vitro* for periods ranging from 7 to 90 days, from which the following observations were made:

General morphology. A typical culture cultivated for 10 days *in vitro* appears as in Fig. 1. From the explant, nerve fibers and non-neuronal cells radiate in all directions. Neuronal cell bodies of different sizes are seen in the explant. These cells have central large nuclei containing central neucleoli and are seen to be surrounded by 2 to several satellite cells, indicating their good health ([12]).

Vast numbers of nerve fibers can be seen already well organized into fascicles as they emerge. After a distance, these break up in the bed of non-neuronal cells. In the periphery, they reunite to form smaller fascicles. During this process, each nerve fiber branches several times and either ends on a fibroblast or lies free.

The non-neuronal cells which migrated out were morphologically 2 distinct types: 1) slender bipolar cells with oval nuclei, each nucleus containing 1 or 2 nucleoli and 2) relatively larger and flatter tri- or multipolar cells containing large flat nuclei with 2 to 7 nucleoli. These cells, as indicated autoradiographically by their thymidine uptake, seemed to have undergone mitosis (Fig. 2).

The first type of cell was identified as a Schwann cell because of its typical light microscopic morphology and its close association with nerve fibers (Fig. 3a). Some of these cells were sectioned and viewed under the electron microscope; they showed basement membrane, and their processes engulfed the neighboring nerve fibers (Fig.

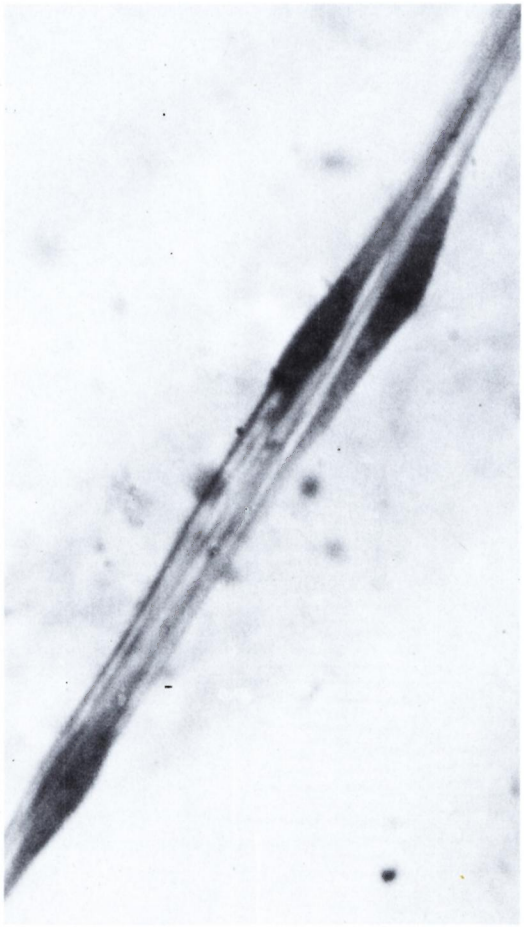

FIG. 3a. A bundle of nerve fibers closely associated with Schwann cells. Underlying is a large neurofibroblast. (×1225).

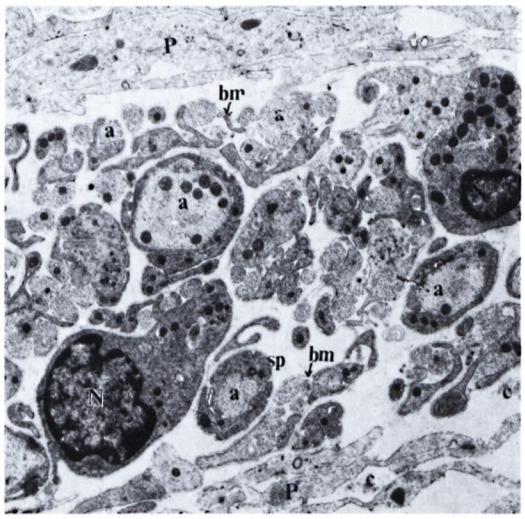

FIG. 3b. Electron micrograph showing large numbers of axons (a) either partially or completely ensheathed by the active Schwann processes (SP). There is an irregular secretion of basement membrane (bm) around the Schwann cell cytoplasm. Two Schwann neuclei (N) are also seen. The axons are enriched with neurotubules, neurofilaments, and large numbers of mitochondria. Longitudinal cell processes (P) are seen on either side, forming a compartment around axonal and Schwann cell processes. Minimal formation of collagen (c) is also seen (acetate and lead citrate stain).

3b). Our contention was further strengthened by the slow proliferative nature of these cells (their ^{3}H-thymidine labelling index peaked only in the second week of culture at 60%) and their subsequent myelin secretion as shown by Sudan black B staining.

The second type of cell was identified as a neurofibroblast destined to become the peri- or endoneural cell. These cells generally formed a layer under the Schwann cells and nerve fibers (Fig. 3a). In contrast to Schwann cells, these seemed to be rapidly dividing cells. They were minimized in many cultures by using antimitotic agents such as cytosine arabinoside and fluorodeoxyuridine at 10^{-5} M concentrations in the regimen suggested by Wood and Bunge ([18]).

Within 10 days *in vitro*, a single dorsal root ganglion gave rise to several organized bundles of nerve fibers along with their Schwann cells in various phases of association, ranging from partial attachment to complete merger, and neurofibroblasts. Myelination of these fibers, however, became evident only after 3–4 weeks *in vitro*.

Infection with *M. leprae* and related bacilli. One to 2 week old cultures were inoculated with *M. leprae*. Bacilli were seen in Schwann cells and fibroblasts but not in the neuronal cell body or axonal processes. In this paper, we restrict our observations to the Schwann cell only because of its important relation to myelin pathology in leprosy.

In the Schwann cells, bacilli were seen localized at the polar end of the cytoplasm and, in general, were few in number, i.e., 1 to 5. Rarely, globi of bacilli were seen (Fig. 4). *M. leprae* were phagocytosed slowly by these cells as indicated by their

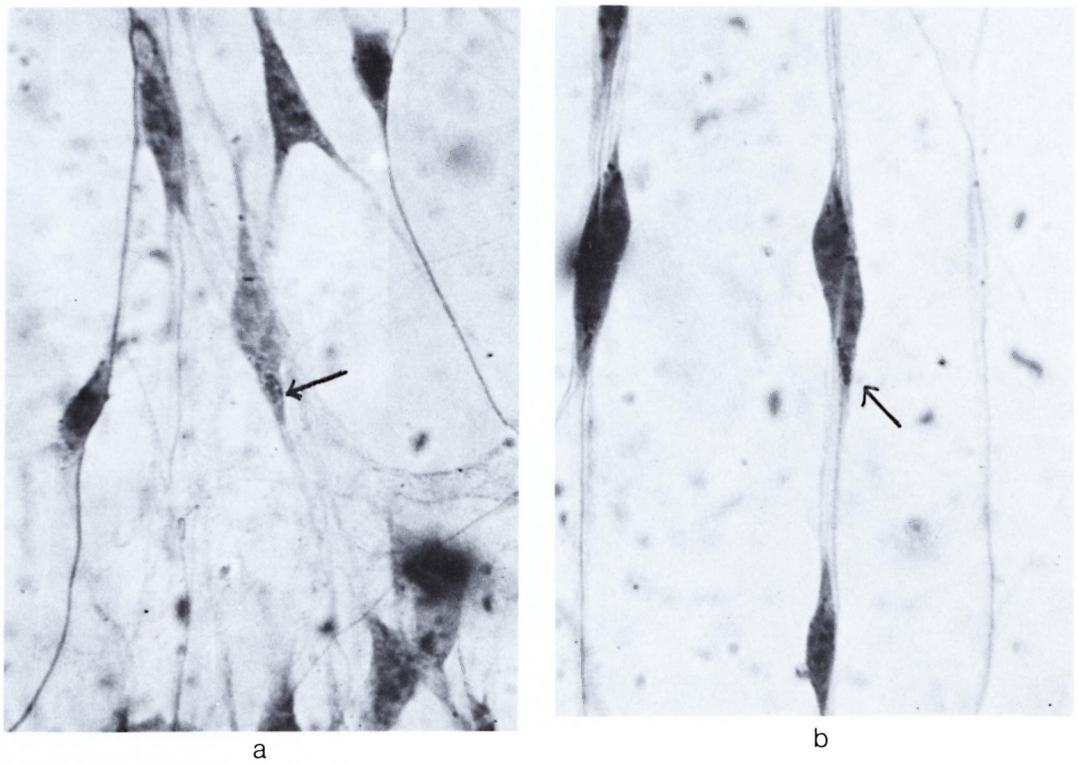

FIG. 4. Portion of 15 day old culture infected with *M. leprae* (5×10^7) to illustrate the uptake of bacilli by Schwann cells at different stages of association with nerve fibers. a) Bacilli located in the lower end of the cytoplasm of a free Schwann cell (arrow). (×979). b) Bacilli seen in the cytoplasm of well associated Schwann cells (very infrequent phenomenon). (×1123).

phagocytic index plotted against length of exposure to the bacilli. The phagocytic index increased from 15.9% at 24 hr to 67.2% at the end of 72 hr. In subsequent experiments, cultures were exposed to bacilli for 72 to 96 hr when the peak of phagocytosis was reached. This phenomenon is perhaps related to the viability of the bacilli. The uptake of heat killed *M. leprae* was very poor even after 144 hr of exposure, the phagocytic index being only 4.5%. Bacilli cultivated in the laboratory (ICRC-C44 and others), whose viability ranged from 60% to 90%, showed several fold higher phagocytic indexes than *M. leprae* with Morphological Indexes ranging from 3% to 5%. The former were engulfed to the extent of an index of 70%.

Phagocytosis of *M. leprae* by Schwann cells was also influenced by the state of their association with the nerve fibers. Proportionately, a greater number of Schwann cells lying free in the vicinity of the nerve fibers or in the early phase of axon association engulfed the bacilli as compared to cells in intimate association and completely physically merged with axons. These cells, after engulfing the bacilli, even when maintained for 3 to 4 weeks under normally favorable conditions, failed to effectively or intimately associate with nerve fibers. They were still seen in a free state or partly associated with nerve fibers but rarely completely associated or merged with the axons.

DISCUSSION

Organized nerves cultivated by us are morphologically comparable to those of Murray and co-workers ([4, 12, 15]). Nerve fibers and related cells were identifiable as early as the 3rd day, becoming quite distinct in their morphology and mutual relationships by a week *in vitro*. Schwann cells were always found in close association with nerves, the peri- and endoneural cells forming an underlayer. Similar findings have been reported by Varon, *et al.* ([17]) in their

culture studies of dissociated dorsal root ganglia of neonatal mice.

In our cultures, all the cellular components were functional and proliferating, as indicated by their ^{3}H-leucine and ^{3}H-thymidine uptake (except neurons which did not proliferate). The mitogenic stimulus for Schwann cells is thought to be provided by the associated nerve fibers ([18]). The cellular components of the organized nerve cultures engulfed *M. leprae* as early as 1 week *in vitro*, i.e., early pre-myelin secretory phase. Since Schwann cells seem to have a definite role in serving as host cells for *M. leprae* ([5, 8, 10]), their phagocytic characteristics were studied in detail. A large number of Schwann cells in this culture phagocytosed *M. leprae*.

Most earlier work on the phagocytic properties of Schwann cells *in vitro* was done on neoplastic Schwann cells. Lumsden ([10]) in his experiments with schwannoma cells and *M. leprae* demonstrated vigorous phagocytic activity of schwannoma cells *in vitro*. Lalitha, *et al.* ([9]) reported identical results. In addition, they also observed an increase in the lysosomal activity of these cells. Our data corroborate the findings of Lumsden ([10]). His figures on the phagocytic index and numbers of bacilli per cell are similar to ours. In our cultures the phagocytic property of Schwann cells was influenced by a) the extent of the association of the Schwann cells with nerve fibers and b) the viability of the inoculated bacilli. Mainly, Schwann cells lying free or in the early phase of nerve fiber association engulfed the bacilli. Only a few Schwann cells physically merged with axons took up the bacilli, suggesting that in this state they are less phagocytic in nature, or as these cells acquire the myelin secretory function, they gradually lose their phagocytic activity. This hypothesis is supported by observations made in *in vitro* studies that normally the Schwann cell that has secreted myelin is a quiescent cell which becomes phagocytic only in the event of injury and degenerative changes in the nerve ([7, 14, 16]).

The viability of the bacilli inoculated is very important as demonstrated by the very poor uptake of heat killed *M. leprae*, modest engulfment of freshly isolated *M. leprae*, and massive uptake of the ICRC C-44 strain. This finding may explain the inability of Fildes ([6]) to satisfactorily infect Schwann cells where the viability of the *M. leprae* could have been affected due to transportation of the bacilli long distances.

The inability of most of these cells containing *M. leprae* over 3 to 4 weeks to intimately associate with nerve fibers and hence secrete myelin suggests that *M. leprae* interfere with the normal functioning of Schwann cells. It would be interesting to study the long term effect of the presence of *M. leprae* in them. At this stage, no obvious morphological effects were seen in the perineural cells under the light microscope.

SUMMARY

Neonatal dorsal root ganglia were cultivated *in vitro* by the technique of Murray. Within a week bundles of organized nerve fibers containing proliferating Schwann cells in different phases of axon association and fibroblast cells destined to become peri- or endoneural cells were obtained. Many of these nerve fibers were myelinated within 3–4 weeks.

Such 1 or 2 week old cultures were inoculated with *M. leprae*, and bacilli were found in the cytoplasm of Schwann cells and fibroblasts, demonstrating that these cells are phagocytic in nature and that it is possible to infect them with *M. leprae*. Schwann cells, mostly in the free or early association phase, engulfed the bacilli, and this affected their further interaction with the axons and subsequent myelin synthesis.

RESÚMEN

Usando la técnica de Murray se cultivó *in vitro* tejido nervioso de los ganglios de la raíz dorsal. En una semana se obtuvieron manojos de fibras nerviosas conteniendo células de Schwann proliferantes en diferentes fases de su asociación axónica y células fibroblásticas destinadas a ser células peri o endoneurales. Muchas de estas fibras nerviosas se mielinizaron en 3 a 4 semanas.

En los cultivos de una a dos semanas infectados con *M. leprae*, los bacilos se encontraron en el citoplásma de las células de Schwann y de los fibroblastos, demostrando que estas células son de naturaleza fagocítica y que es posible su infección con el *M. leprae*. Las células de Schwann, principalmente aquellas en la fase libre o de asociación temprana, endocitaron bacilos y esto afectó su interacción posterior con axones y la síntesis subsecuente de mielina.

RÉSUMÉ

On a cultivé *in vitro* des ganglions des racines dorsales de nouveau-nés par la technique de Murray. Dans la semaine, on obtient des faisceaux de fibres nerveuses organisées contenant des cellules de Schwann en prolifération, à différentes phases d'association axonique, de même que des fibroblastes destinés à se transformer en cellules périneurales ou endoneurales. La plupart de ces fibres nerveuses étaient myélinisées après 3 ou 4 semaines.

Ces cultures vieilles d'une ou deux semaines ont alors été soumises à *M. leprae*. On a trouvé des bacilles dans le cytoplasme des cellules de Schwann et des fibroblastes, ce qui démontre que ces cellules sont par nature phagocytaires, et qu'il est possible de les infecter avec *M. leprae*. Les cellules de Schwann, le plus souvent à l'état libre ou en association précoce, ont entouré les bacilles, ce qui a interféré avec toute interaction ultérieure avec les axones et avec la synthèse subséquente de myéline.

Acknowledgement. The authors are thankful to Professor E. J. Ambrose for his interest in the initial stages of this work.

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