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Organized Nerve Tissue Cultures Infected with *Mycobacterium leprae* and *Mycobacterium lepraemurium* [1, 2]

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A characteristic feature of leprosy is the involvement of the peripheral nervous system (PNS) extending as far as, and ceasing abruptly at, the dorsal root ganglion (DRG) ([8]). It has been suggested that this involvement represents a "special affinity" between *Mycobacterium leprae* and the Schwann cell, not found in any other mycobacterial or bacterial infection ([21]). In an attempt to elucidate further this infection of the PNS, organized tissue cultures of rat and mouse DRG were confronted with *M. leprae* (harvested from the mouse foot pad) and incubated *in vitro* for a period of 50 days.

A culture technic which permits the maturation and prolonged survival of all the cellular elements of the peripheral nerve (except the vascular tissue) has been developed by Murray and her colleagues ([10, 11]). An organotypic culture is obtained whose components establish and maintain the cellular interrelationships found *in vivo*. The morphologic fidelity of cultures prepared from both central and peripheral nervous tissue, as compared with the same tissues *in situ*, has been demonstrated with both the light and electron microscopes ([3]), and the physiological fidelity of these cultures has been demonstrated in electrophysiological studies ([6]). This culture technic has been used successfully to examine the pattern of demyelination and regeneration after, e.g., Wallerian degeneration, exposure to diptherial toxin and to heavy metals ([14]), and to pyridoxine and thiamine analogs ([23, 24]). It has also been used for demonstrating immunological responses, e.g., demyelinative action by serum globulin fractions IgG and IgM ([4]). Patterns of response in these various experiments are usually distinguishable from one another. There is now, therefore, considerable evidence to suggest that this type of culture provides a valid system for investigating *in vitro* the relationship between *M. leprae* and the PNS.

This communication reports a series of preliminary observations designed to explore the feasibility of combining the experimental plan outlined below with a model system of cultured nervous tissue for bio-assay. The experiments were controlled by 1) comparing the response of cells of PNS origin with those of central nervous system (CNS) origin (which are not ordinarily infected by *M. leprae in vivo*), by establishing explants of both on the same carrying coverslip, and 2) by simultaneous assessment of cultures inoculated with *M. lepraemurium* which does not infect nervous tissue of either type, and 3) by maintaining control, uninfected cultures of tissues from the same origins.

MATERIALS AND METHODS

Organotypic cultures of fetal (19 days *in utero*) rat and mouse cervical or lumbar DRG; and fetal (10 days *in utero*) mouse whole cross sections (somite, cord and ganglia) were explanted in Maximow double coverslip assemblies ([15]). The carrying coverslips were of glass or Aclar plastic ([9]) which had been coated previously with reconstituted rat-tail collagen. Explants were fed with one drop of medium consisting of equal parts Eagle's MEM (minimal essential medium), bovine serum ultrafiltrate, human placental serum and chick embryo extract; this formula was supplemented with 600 mg glucose per 100 ml. The cultures, incubated at 35° C, were washed twice weekly in Simms' balanced salt solution (BSS) and refed with one drop of fresh medium. They were used for experimentation after attaining mature organization *in vitro*, i.e., three to four weeks after explanation.

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The suspensions of mycobacteria were harvested by Dr. R.J.W. Rees (N.I.M.R., London) and dispatched to New York by air, packed in ice. *M. leprae* was harvested from both hind foot pads of a thymectomized, x-irradiated ([16, 17, 18]) mouse and yielded a count of 3.3×10^9 acid-fast bacilli (AFB) per ml. *M. lepraemurium* was obtained from the liver of an infected mouse and the suspension counted at 1.2×10^{11} AFB per ml. Methods used in the harvesting and counting of bacilli are given by Rees ([16]). The bacillary suspensions were checked for gross contamination by inoculation of full tissue culture medium which was then incubated overnight at 35°. Two cultures were also inoculated at the same time to check for immediate toxicity reactions and to assess the uptake of bacilli. No immediate toxicity was observed and an inoculation of 3.0×10^7 AFB gave a satisfactory distribution of bacilli in the culture. The cultures were subsequently inoculated with this count of AFB and incubated at 35°. After four days of incubation they were washed and refed with one drop of fresh medium.

The three sets of cultures were inoculated as follows:

1. DRG of 19-day fetal mouse (two explants per coverslip culture); five cultures inoculated with *M. leprae,* five cultures inoculated with *M. lepraemurium.*

2. Whole cross sections of 10-day fetal mouse (two explants per coverslip); ten cultures inoculated with *M. leprae,* ten cultures inoculated with *M. lepraemurium* and five cultures uninoculated as controls.

3. DRG of 19-day fetal rat (two explants per coverslip [plastic]); seven cultures inoculated with *M. leprae,* seven cultures inoculated with *M. lepraemurium* and three cultures uninoculated as controls.

The culture models were followed in the living state with brightfield light microscopy. Prior to fixation and staining for light microscopy, cultures were rinsed free of feeding medium, using either Locke's solution or BSS. They were then fixed in 10% formol-Locke's solution and stained for AFB using the procedures and dyes recommended by Shepard ([19]), and for nervous tissue using a modification of the Holmes silver stain. The preparation of cultures for electron microscopy was as described by Bunge, Bunge and Peterson ([2]). All cultures were fixed in 2% OsO_4 in Veronal acetate buffer (containing 0.05% $CaCl_2$) for one hour at 4°C. Some were prefixed in glutaraldehyde (1.5% in 0.067M cacodylate buffer at pH 7.4 containing 1% sucrose) in an attempt to improve the preservation of muscle tissue. The tissue was embedded in Epon, cut on a Porter-Blum microtome and examined in a Philips 200 electron microscope.

RESULTS

The results reported here represent the data obtained from this initial set of experiments using one suspension of *M. leprae* and one suspension of *M. lepraemurium.*

The cultures were maintained for a total incubation time of 50 days after inoculation. During this period no obvious toxic reaction to the bacilli could be observed in any of the living cultures; there was no evidence of Wallerian degeneration or breakdown of existent sheaths, and myelin sheaths continued to form *de novo.* In the cultures containing fetal cord and somites, outgrown muscle fibers became striated and their activity progressed from a sporadic fibrillation to a more synchronized contraction which has been shown to be indicative in these cultures of the formation of neuromuscular junctions ([5, 20]). To maintain the activity of the muscle tissue and prevent vacuolation of the fibers, additional glucose (to give 900 mg per 100 ml) and more frequent feedings (three times weekly) were required. It was noted that loss of striation and vacuolation occurred more readily in cultures inoculated with *M. leprae,* but these changes were easily reversed by the more frequent feedings.

In cultures fixed and stained for AFB at various time intervals throughout the incubation period a marked difference was observed in the distribution of the two species of mycobacteria. In all instances cultures inoculated with *M. leprae* contained large numbers of bacilli, whereas in those inoculated with *M. lepraemurium,* organisms were rarely found. The difference became more striking in samples taken towards the end of the experimental period. The results are illustrated in Figures 1-6. *M. leprae* are generally arranged in the typical microcolonies or "globi" and the proportion of solid staining bacilli is high. In the cultures inoculated with *M. lepraemurium,* however, the bacilli, which occur singly or in small clumps, are mostly irregularly stained.

FIG. 1. Baseline sample, showing uptake of *M. leprae*; four days in culture, rat dorsal root ganglia. X 750.

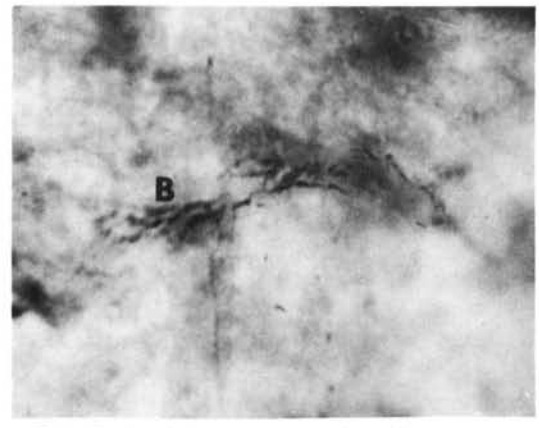

FIG. 2. *M. leprae*; 21 days in culture, mouse whole cross section. In duplicate set of cultures inoculated with *M. lepraemurium*, bacilli were rarely seen and depth of cultured mouse tissue made photographic recording impossible. X 750.

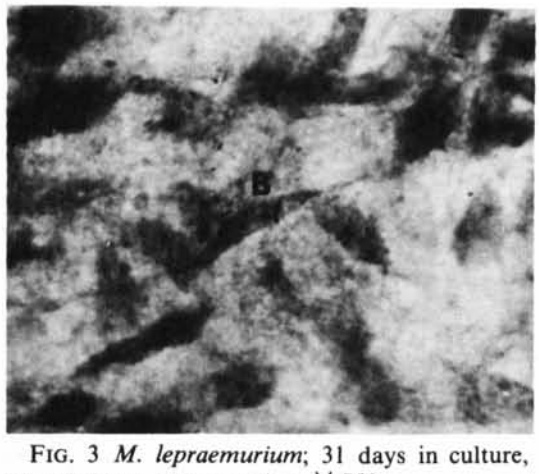

FIG. 3 *M. lepraemurium*; 31 days in culture, mouse dorsal root ganglion. X 750.

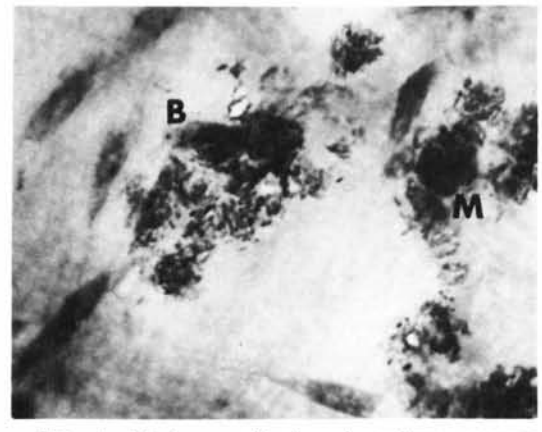

FIG. 4. *M. leprae*; 31 days in culture, mouse dorsal root ganglion. X 750.

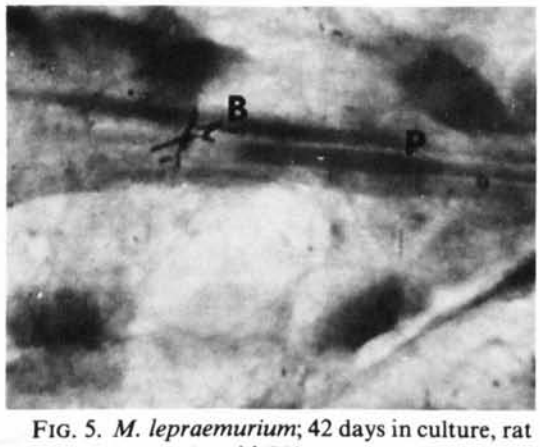

FIG. 5. *M. lepraemurium*; 42 days in culture, rat dorsal root ganglion. X 750.

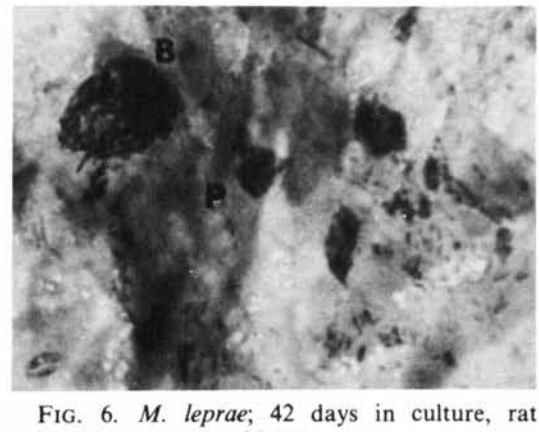

FIG. 6. *M. leprae*; 42 days in culture, rat dorsal root ganglion. X 750.
(B) Bacilli (S) Schwann cells
(P) Perineurial cells (M) Macrophage

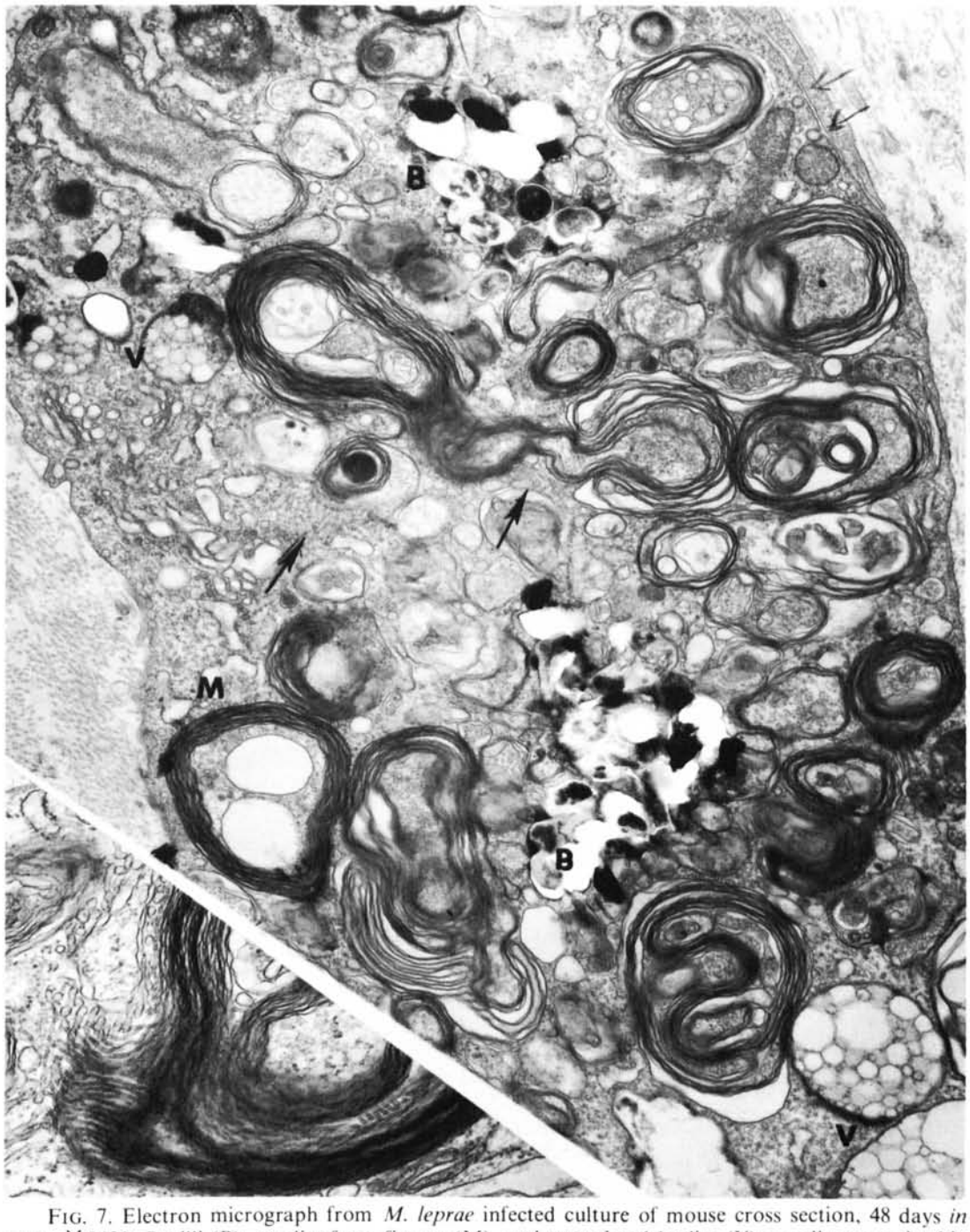

FIG. 7. Electron micrograph from *M. leprae* infected culture of mouse cross section, 48 days *in vitro*. X 8620. Bacilli (B), myelinoform figures (M), and vacuolated bodies (V) are all present in this cell. The tubular nature of the myelinoform material is evident here (arrowed) and can be seen more clearly in the enlarged insert, X 15,410.

With the electron microscope both species of bacilli are found intracellularly, and predominantly in macrophages and fibroblasts. They are surrounded by a large peribacillary space which frequently contains both granular and membranous material, and is separated from the cell cytoplasm by a membrane. The cells infected with *M. leprae*

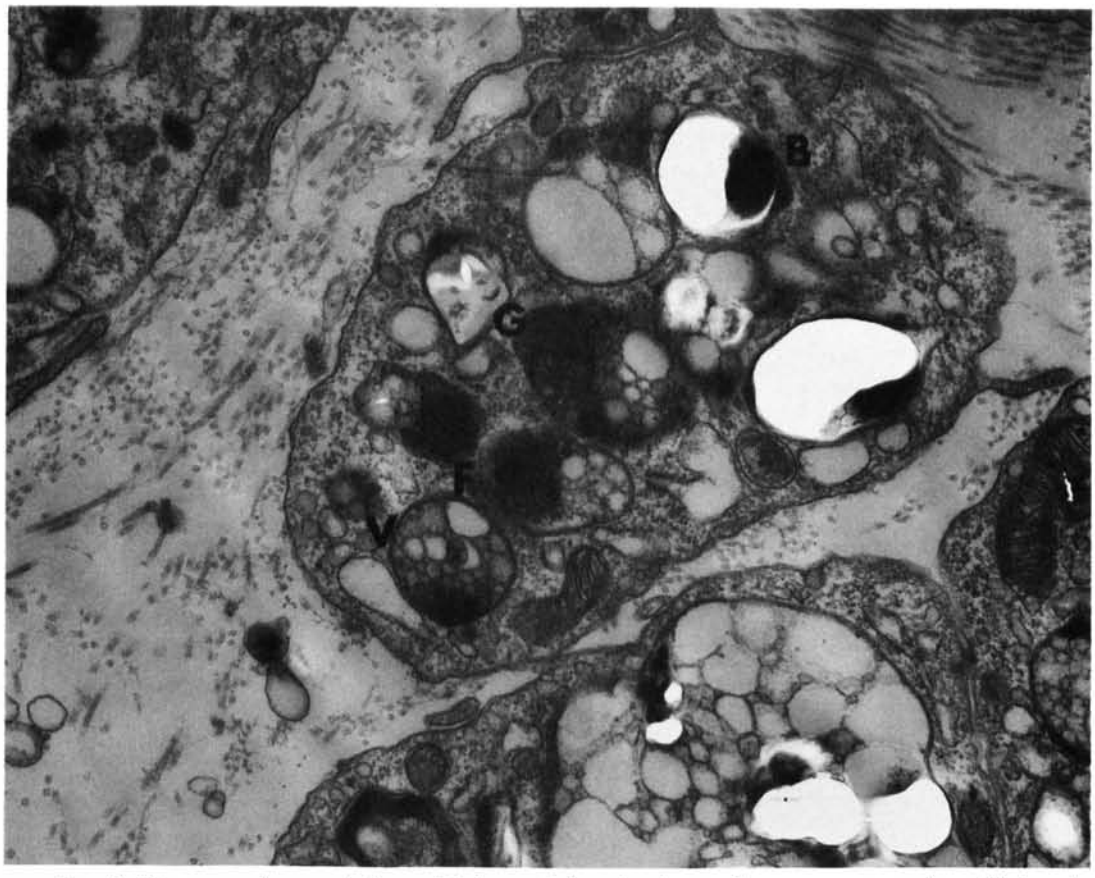

FIG. 8. Electron micrograph from *M. leprae* infected culture of mouse cross section, 48 days *in vitro*. X15,410. Extensive formation of vacuolated bodies gives these cells the characteristic foamy cell appearance. The bodies are bounded by a double membrane and also contain areas of granular (G) and fibrillar (F) material.

also contain large vacuolated inclusions (Figs. 7 and 8) which resemble the inclusions found in the characteristic Virchow or foamy cell seen in biopsy material from infected patients, and extensive areas of myelinoform whorled membranes.

Although no obvious toxic effect could be observed with the light microscope, there is some evidence of tissue reaction to the bacilli at the electron microscope level. Giant mitochondria were found in some Schwann cells, and in the infected cells there was an increase in the number of mitochondria, coated vesicles and lysosome-like dense bodies.

DISCUSSION

These results indicate that a distinct difference can be detected between tissue culture preparations of the PNS inoculated with *M. leprae* and those inoculated with *M. lepraemurium*. These differences are apparent with both the light and electron microscopes. In the light microscope the cultures inoculated with *M. leprae* still contain large numbers of AFB at the end of the maximum culture period (50 days), whereas those inoculated with *M. lepraemurium* contain only isolated AFB. In these preliminary experiments no absolute control of the numbers of bacilli present at the beginning and end of the experimental period was attempted. Therefore, no claim can be made that *M. leprae* is multiplying in this experimental system. The results may merely reflect a difference in the clearing capacity of the macrophages for the two species of bacilli. A similar lack of clearance of *M. leprae* (dead or alive) from the site of inoculation has been reported *in vivo* (7). The electron micrographs of the cultures inoculated with *M. leprae* suggest that this reten-

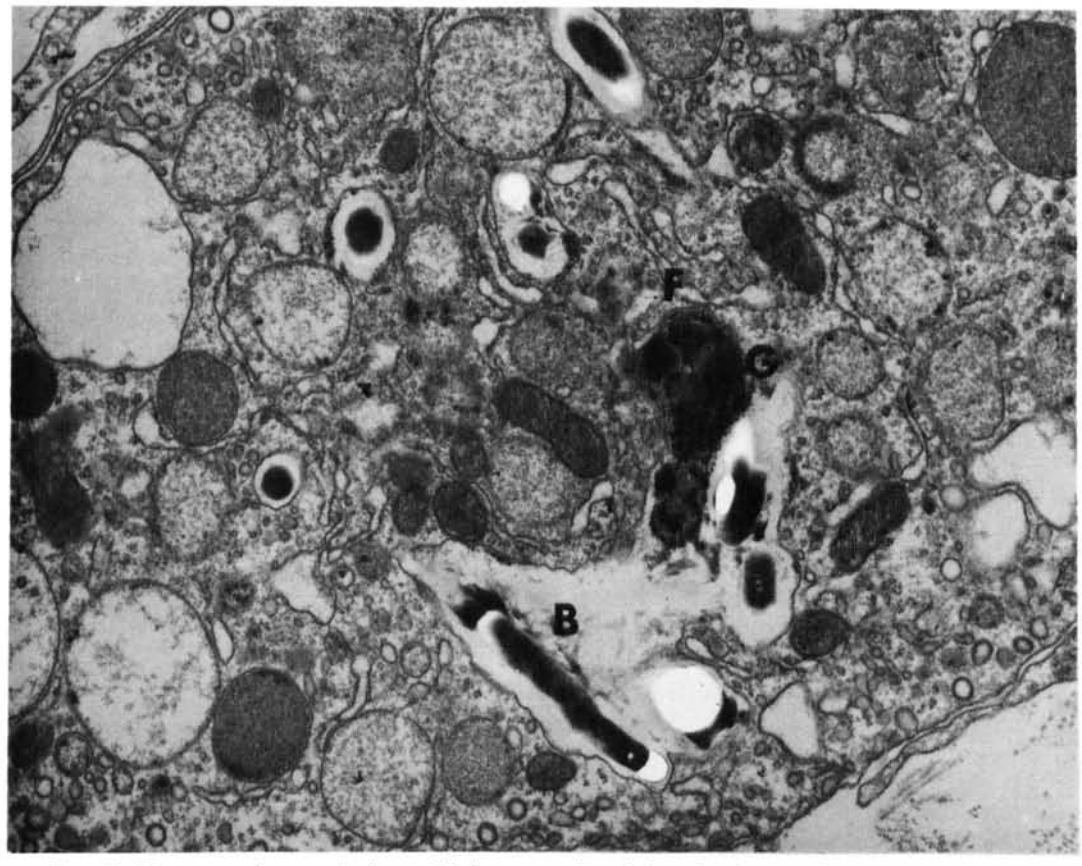

FIG. 9. Electron micrograph from *M. lepraemurium* infected culture of mouse cross section, 48 days *in vitro*. X11,940. The bacilli are found within a phagocytic vacuole, which also occasionally contains granular (G) and fibrillar (F) material similar to that shown in Figure 8.

tion of bacilli is not an entirely passive response since infected cells also contained large vacuolated bodies and myelinoform figures (Fig. 7). These two morphological changes were not observed in the cultures inoculated with *M. lepraemurium*. Extensive myelinoform figures have been observed frequently in many cell types in both naturally occurring (e.g., mammatroph cells with excess hormone) and pathologic conditions especially in cells in which the lipid metabolism has been disturbed ([13]). They have also been observed in similar cultures of the PNS after exposure to certain drugs ([1]). At the present time measurements of the periodicity of such lipid layers suggest that this material does not represent degraded PNS myelin. Our electron micrographs indicate that the myeliniform figures have a tubular structure (Fig. 7) and when the material is wound loosely the bodies show the "bridges" described by Nishiura *et al* ([12]) in skin biopsy material.

Although this difference in response to *M. leprae* from *M. lepraemurium* is interesting, it is also disappointing that no obvious affinity between *M. leprae* and the Schwann cell or the muscle satellite cell has thus far been detected. Although both of these cell types were indeed found to contain bacilli, neither demyelination nor breakdown of muscle tissue was observed, and the majority of organisms were found in macrophages and fibroblasts. However, the results of *in vivo* studies on the relationship between *M. leprae* and the PNS suggest that the 50-day culture period in these preliminary experiments is not sufficiently protracted to reveal extensive nerve or muscle damage. In the electron microscope the presence of vacuolated bodies, myeliniform figures and an occasional giant mitochondrion are indicative of interference with the normal physiological activities of the host cell. Similar changes were not observed in the cells which had phagocytosed *M. lepraemurium*, al-

though fibrillar material (Fig. 9) similar to that described by Yang and Skinsnes ([22]) was observed within the phagocytic vacuoles; there was no myeliniform material in the remainder of the cytoplasm.

SUMMARY

Organotypic cultures of dorsal root ganglia and of whole cross sections (muscle somite, cord and ganglia) were prepared from rat and mouse fetal tissue. Duplicate cultures were inoculated with *M. leprae* and *M. lepraemurium* respectively and compared with controls (uninfected cultures) over an incubation period of 50 days. There was no evidence of a cytotoxic reaction to the bacilli. Following fixation and staining, the cultures inoculated with *M. leprae* were found to contain large numbers of bacilli at the end of the 50-day incubation period, whereas those inoculated with *M. lepraemurium* were comparatively free of bacilli. With the electron microscope, cultures inoculated with *M. leprae* can be further distinguished from those inoculated with *M. lepraemurium* by the formation of large vacuolated inclusion bodies and by the presence of extremely large myeliniform figures, contained mainly within macrophages and fibroblasts.

Rat and mouse dorsal root ganglia were equally susceptible to infection with *M. leprae*. No infection of cord tissues was observed. It is suggested that this exposure period (of less than two months) may not be long enough to encompass major involvement of Schwann and satellite cells.

RESUMEN

Se preparan cultivos organocíticos de ganglios de raíz dorsal y cortes seccionales completos (somitos musculares, médula y ganglios) a partir de tejido fetal de rata y ratón. Se inocularon cultivos duplicados con *M. leprae* y *M. lepraemurium* respectivamente y se compararon con controles (cultivos no infectados) durante un período de incubación de 50 días. No hubo evidencia de una reacción citotóxica hacia el bacilo. Después de fijación y tinción, se encontró que los cultivos con *M. leprae* tenían grandes cantidades de bacilos al final del período de incubación de 50 dias, mientras que los inoculados con *M. lepraemurium* estaban comparativamente libres de bacilos. Con el microscopio electrónico se pudieron establecer mayores diferencias entre los cultivos inoculados con *M. leprae* y los inoculados con *M. lepraemurium*, ya que en los primeros se observaron grandes cuerpos de inclusión vacuolados y, además, figuras mieliniformes extremadamente grandes, contenidas principalmente dentro de macrófagos y fibroblastos.

Los ganglios de la raíz dorsal de la rata y del ratón fueron igualmente susceptibles a la infección con *M. leprae*. No se observó infección de los tejidos medulares. Se sugiere que este tiempo de exposición (de menos de 2 meses) puede no ser lo bastante largo como para permitir un compromiso mayor de las células de Schwann y las células satélites.

RÉSUMÉ

On a préparé des cultures organotypiques de ganglions radiculaires dorsaux, et de coupes transversales entières (de segment musculaire, de corde dorsale et de ganglion), à partir de tissu foetal recueilli chez le rat et chez la souris. Des cultures appariées ont été inoculées respectivement à *M. leprae* et à *M. lepraemurium*. Elles ont alors été comparées avec des cultures témoins non infectées, au cours d'une période d'incubation de 50 jours. On n'a pas observé de signes d'une réaction cytotoxique envers les bacilles. Par fixation et coloration, on a mis en évidence dans les cultures inoculées avec *M. leprae*, un grand nombre de bacilles, à la fin de la période d'incubation s'écoulant sur 50 jours. Par contre, les cultures inoculées avec *M. lepraemurium* se sont révélées, par comparaison, libres de bacilles. Par microscopie électronique, on a constaté que les cultures inoculées avec *M. leprae* pouvaient en outre être distinguées de celles inoculées par *M. lepraemurium*, grâce à la formation de corpuscules d'inclusion vacuolés, de grande dimension, et par la présence d'images myéliniformes extrêmement grandes, contenues principalement à l'intérieur des macrophages et des fibroblastes.

Le rat et la souris DRG étaient également susceptibles à l'infection par *M. leprae*. Aucune infection des tissus de la corde dorsale n'a été observée. On suggère que la durée d'exposition, inférieure à deux mois, pourrait ne pas être assez longue pour permettre une atteinte majeure des cellules de Schwann et des cellules satellites.

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