

An Ultrastructural Study of the Response of Traumatized Rabbit Tibial Nerve to Epineurial Infection with *Mycobacterium leprae*¹

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Nerve damage is an essential feature of the clinical manifestations and pathology of leprosy. It results from interactions between *Mycobacterium leprae* and host cells within nerves. How these microorganisms or their antigens enter nerves has not been demonstrated yet, but this must surely be an important key toward a better understanding and management of the disease.

The possible routes for entry of *M. leprae* into nerves include a) via Schwann cells covering the terminal portions of nerve fibers in the outer dermis where they are not enclosed within a perineurial sheath, b) transperineurial transport, and c) via endoneurial blood vessels (¹³). Earlier workers have favored the first mode. However, this does not seem to be an adequate explanation for lesions that are located focally and more proximally in nerve trunks. Endoneurial blood vessels are known to function as an effective blood nerve barrier (¹²), which is not likely to be breached except following significant endoneurial inflammation or endothelial injury. A transperineurial route was considered likely from personal unpublished observations where leprosy neuritis, affecting dermal nerves or major nerve trunks, was seen to be frequently associated with dense, focal epineurial inflammation and a centripetally decreasing density of inflammatory cells passing into the endoneurium across the perineurium. It was thought that chronic inflammation in the epineu-

rium might weaken the perineurial barrier and so facilitate the passage of bacillated inflammatory cells across the perineurium. Trauma could also possibly effect a similar change in the perineurium.

It was therefore decided to test, in an animal model, whether trauma and epineurial inflammation could damage the perineurial barrier sufficiently to promote the passage of bacillated inflammatory cells from the epineurium into the endoneurium.

MATERIALS AND METHODS

Nine adult rabbits of either sex, weighing 1.25–1.5 kg each, were anesthetized by injecting pentobarbital (Nembutal) in a dosage of 30–35 mg/kg body weight into the peritoneal cavity, supplemented when necessary with ether vapor inhalation for short periods. Using sterile techniques, the right and left tibial nerves of animals 1–6 were exposed and crushed high in the leg between the jaws of an artery clamp for 10–15 sec, after which the skin was closed with interrupted cotton sutures and covered with a tincture of benzoin seal. Only the right tibial nerve was crushed as described in animals 7–9.

On the fifth post-operative day, the animals (1–9) were once more anesthetized as described above. Using sterile procedures, the tibial nerves were again exposed and the site of the nerve crush identified. This was marked by a residual patch or transverse bar of erythema or hemorrhage in the epineurium. Using a tuberculin syringe and a sterile, disposable, 24-gauge hypodermic needle, a suspension of live *M. leprae* in normal saline was inoculated into the epineurium at and just distal to the site of the crush. In animals 1–6, 1×10^7 organisms in 0.01 ml saline were inoculated bilaterally; in animals 7–9, 5×10^7 organisms in 0.01 ml saline were inoculated bilaterally. The site of inoculation into the left (uncrushed)

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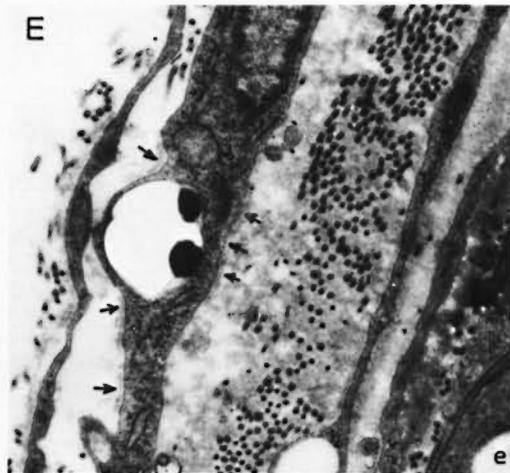


FIG. 1. *M. leprae* in the cytoplasm of a perineurial cell three days post-inoculation ($\times 30,660$). E = epineurial aspect; e = endoneurial aspect.

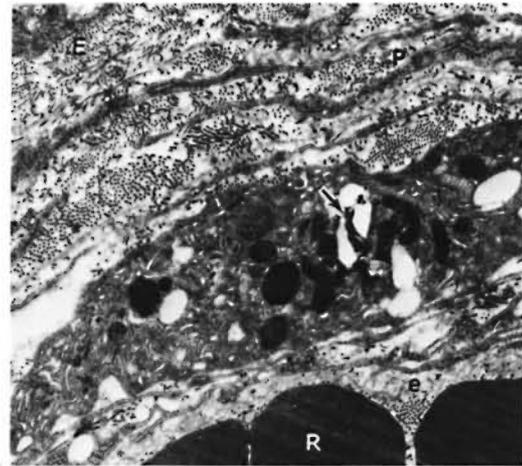


FIG. 2. Macrophage containing a leprosy bacillus (arrow) infiltrating the perineurium (P), the laminae of which are loosened and separated, three days post-inoculation ($\times 10,045$). R = erythrocyte; E = epineurial aspect; e = endoneurial aspect.

tibial nerve was identified by noting its relationship to prominent landmarks (distance from the knee joint and the upper limits of the incision). The suspension of living *M. leprae* was prepared by the method of Rees⁽¹⁴⁾ from the foot pads of T900r CBA mice inoculated with organisms originally obtained from a human lepromatous skin nodule and subsequently passaged in the foot pads of T900r CBA mice.

A 0.5-cm segment from both the right and left tibial nerves, including the site of inoculation at the upper end of the segment, was excised from all of the animals while they were under anesthesia. Pairs of such nerve samples were excised at 10, 20, 30, 40, 50, and 60 min post-inoculation from animals 1-6. Similar samples of crushed, and uncrushed but inoculated, tibial nerve tissue from the right and left sides, respectively, were excised from rabbits 7-9, at 60 min, 24 hr, and 72 hr post-inoculation. The nerve samples were keyed to identify the inoculated end and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. Each nerve segment was cut into 3-4 longitudinal strips and after glutaraldehyde fixation was completed, they were post-fixed in 1% osmic acid for 90 min. After dehydration and clearing, the nerve strips were oriented while embedding in araldite so as to provide transverse sections of the nerve from the inoculated area. Sections for electron microscopy were stained with uranyl

acetate and lead citrate, and examined with a Philips EM 201 electron microscope.

RESULTS

The most significant alterations were seen in the samples of crushed tibial nerves excised 40, 50 and 60 min and 72 hr post-inoculation. Changes of Wallerian degeneration resulting from crush were well developed and conformed to the descriptions of other workers^(8, 15-17).

Dense clusters of *M. leprae* were present focally in the epineurium associated with edema and the presence of a few inflammatory cells, including macrophages and neutrophils. The bacilli were located within phagosomes of macrophages, neutrophils and fibroblasts. A very mild, inflammatory cell infiltrate was seen in a few other sectors of the epineurium of some nerves.

The perineurium was composed of 4-7 lamellae of perineurial cells. In most areas, these cells and layers were unaltered. Solitary bacilli or clusters of 2-5 bacilli were seen in the perikaryon of some perineurial cells (Fig. 1) in the area where bacilli were present in the epineurium. These bacillated perineurial cells were located in the mid- and outer lamellae, as well as occasionally in the inner-most layer. In the 50 min, 60 min, and 72 hr post-inoculation nerve samples, there were a few macrophages lodged

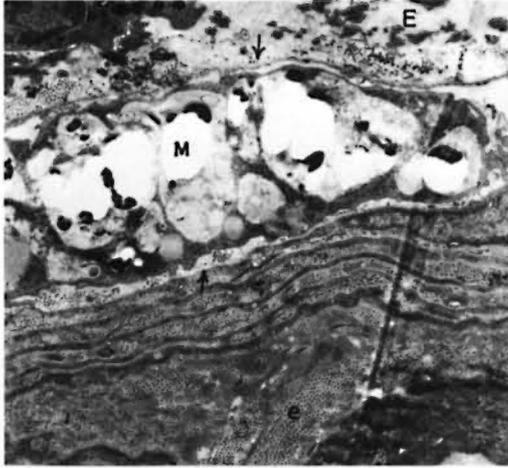


FIG. 3. A heavily bacillated macrophage (M) which has infiltrated the perineurium (arrows) 60 min post-inoculation ($\times 10,430$). E = epineurium; e = endoneurium.

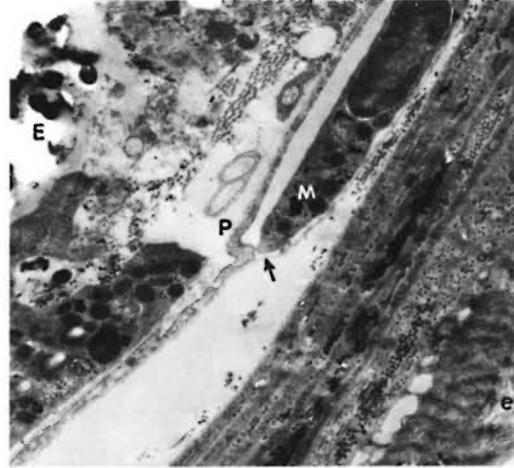


FIG. 4. A macrophage (M) has lifted off the basement membrane (arrow) of an adjacent perineurial cell (P); clusters of *M. leprae* are present in the epineurium (E); a large myelinated nerve fiber is seen in the endoneurial compartment (e); 60 min post-inoculation ($\times 13,410$).

between the perineurial lamellae 3–6, which were consequently separated from each other with what appeared to be edema fluid in between. Some of these macrophages contained phagocytosed bacilli (Figs. 2 and 3). In one instance, an infiltrating macrophage had insinuated itself between a perineurial cell and its covering basement membrane (Fig. 4). Individual perineurial cells in the vicinity were otherwise ultrastructurally unremarkable with no evidence of proliferative or reactive changes. There was no inflammatory cell infiltrate in other sectors of the perineurium. Very occasionally a few bacilli were seen in the cytoplasm of endoneurial phagocytic cells.

DISCUSSION

The inflammatory cellular infiltrate in the epineurium could have been evoked as a response to the inoculation of *M. leprae* or the nerve crush injury which preceded it. Since the infiltrate was predominantly in the area of the inoculation, it may be attributed to the former cause. Similarly, perineurial infiltration by inflammatory cells was not seen in sectors of the nerve other than that adjacent to the location of bacilli in the epineurium. It is therefore unlikely that this resulted from mechanical nerve injury. However, it must still be conceded that the inflammatory infiltrate could have been at least partly elicited by physical tissue injury

and not by the relatively inert *M. leprae*, implanted later, only a short while before the tissues were fixed.

Bacilli were seen engulfed by inflammatory cells in the 40 min post-inoculation sample. An earlier study⁽⁵⁾ has shown that macrophages may accomplish this task within 20 min from exposure to the organism.

The most significant observation was that macrophages were capable of finding a way into the perineurial sheath. Whether this occurred as a result of physical tissue injury or epineurial instillation of bacilli is only of secondary importance. That such macrophages may have a role in transporting *M. leprae* across the perineurium is suggested by the finding of bacillated macrophages in the mid-zone of the perineurium. Deeper passage of macrophages into the perineurium was probably limited by the relatively short period of observation following bacillary instillation which was adopted in this study.

The presence of small numbers of *M. leprae* in the perineurium and endoneurium could have occurred passively and accidentally during inoculation of the epineurium or, alternatively, may have resulted from active transport across the perineurium. That perineurial cells may phagocytose *M.*

leprae has been observed in this study and others (2, 7, 9, 10, 18). In an ultrastructural study of chronic Wallerian degeneration in mice, Williams and Hall (17) observed that fat globules from myelin degradation were taken up by macrophages and transported to a subperineurial location. These globules were subsequently seen in the perineurium and, much later, in epineurial macrophages. In light of the above and in light of the present findings, the centripetal transperineurial transport of *M. leprae* from the epineurium to the endoneurial compartment seems a real possibility, although not as yet demonstrated conclusively.

In leprosy neuritis of humans, structural lesions and bacillary concentrations are generally found in those portions of peripheral nerves which have a superficial anatomical location (3, 4, 6). Such locations predispose to frequent trauma to the nerve. It has been shown that the blood nerve barrier presented by endoneurial blood vessels can be damaged by trauma (1). The perineurial barrier may also be rendered defective by crush injury according to Olsson and Kristensson (11). If this is so, transperineurial transport of *M. leprae* may be facilitated from epineurial foci of inflammation containing bacilli.

SUMMARY

Crushed rabbit tibial nerves were inoculated with a suspension of living *Mycobacterium leprae* at and just distal to the site of nerve trauma. The resulting changes occurring over a period of time from 40 min to 72 hr post-inoculation were studied electron microscopically. Bacilli were seen in perineurial cells and in macrophages that had infiltrated the perineurium adjacent to epineurial deposits of *M. leprae*.

It is suggested that trauma may weaken the perineurial barrier and facilitate the transperineurial passage of phagocytes, some of which may be laden with *M. leprae*, and may thus be a means whereby *M. leprae* enter the endoneurium of peripheral nerves.

RESUMEN

Se traumatizaron los nervios tibiales en algunos conejos y éstos se inocularon con *Mycobacterium leprae* vivos tanto en el sitio del trauma como en un sitio distante al mismo. Los cambios ocurridos entre 40 min

y 72 horas post-inoculación se estudiaron por microscopía electrónica. Se observaron bacilos tanto en las células perineuriales como en los macrófagos infiltrantes del perineurio adyacentes a los depósitos epineuriales del bacilo.

Se sugiere que los traumatismos, en general, pueden debilitar la barrera perineurial y facilitar el paso transperineurial de los fagocitos, algunos de los cuales pueden contener y ser portadores del *M. leprae*. El bacilo puede entrar al endoneurio de los nervios periféricos a través de este mecanismo.

RÉSUMÉ

On a inoculé des nerfs tibiaux broyés provenant du lapin, avec une suspension de *Mycobacterium leprae* vivant, en un endroit immédiatement distal par rapport au site du traumatisme nerveux. On a alors étudié au microscope électronique les changements qui se produisent au cours d'une période de 40 minutes à 72 heures après l'inoculation. On a pu observer des bacilles dans les cellules périneurales et dans les macrophages qui infiltraient le périnèvre adjacent aux dépôts épineuraux de *M. leprae*.

On suggère dès lors que le traumatisme peut affaiblir la barrière périneurale, et faciliter ainsi le passage transperineurial des phagocytes, dont certains peuvent contenir *M. leprae*. Ceci pourrait constituer un moyen pour *M. leprae* de pénétrer dans l'endonevre des nerfs périphériques.

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