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Ultrastructural Study of Schwann Cells and Endothelial Cells in the Pathogenesis of Leprous Neuropathy[1]

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ABSTRACT

Peripheral nerve biopsies from 4 borderline tuberculoid (BT) and 4 lepromatous (LL) patients who were on multidrug therapy were investigated by light and electron microscopic studies. The variation of diameters and distribution of myelinated and unmyelinated fibers between BT and LL patients were not significant. This study has shown significant changes in peripheral nerves and endoneural blood vessels. It was revealed that besides Schwann cells (SC), the endothelial cells (EC) of endoneural blood vessels frequently harbor *M. leprae*. In BT, peripheral nerves in addition to the degenerative changes of SC and presence of perineural and perivascular cuffing by mononuclear cells, the endoneurial blood vessels showed thickening of basement membrane with hypertrophy of EC leading to narrowing or complete occlusion of lumen. On the other hand, peripheral nerves of LL patients were infiltrated with large number of *M. leprae* shown to be present in the electron transparent zone (ETZ) of the SC. The EC of endoneurial blood vessels were found to be loaded with *M. leprae*, and this bacillary loaded EC was found to release *M. leprae* into the lumen through its ruptured membrane.

RESUMÉ

Les biopsies de nerfs périphériques provenant de 4 patients tuberculoïdes borderlines (BT) et de 4 patients lépromateux (LL) soumis à une polychimiothérapie (PCT) ont été étudiées par microscopies optique et électronique. Aucune différence significative fut observée en terme de diamètre et de distribution des fibres myélinisées et non myélinisées entre les patients BT et LL. L'étude a montré des différences significatives dans les nerfs périphériques en particulier au sein des vaisseaux sanguins intra-neuraux. Il a été mis en évidence que, en plus des cellules de Schwann (CS), les cellules endothéliales (CE) des vaisseaux intra-neuraux hébergaient fréquemment des *M. leprae*. Au sein des nerfs périphériques des patients BT, en plus des lésions dégénératives des CS et de la présence de manchons périvasculaires de cellules mononucléées, les vaisseaux sanguins intra-neuraux montraient un épaississement des membranes basales associées à une hyertrophie des CE, résultant en un rétrécissement voire une occlusion complète de la lumière. D'autre part, les nerfs périphériques des patients LL étaient infiltrés par un grand nombre de *M. leprae*, localisées dans les CS et les ETZ ; les CE des vaisseaux sanguins intra-neuraux étaient remplies de *M. leprae*, et certaines de ces CE surchargées montraient parfois un relarguage de *M. leprae* dans la lumière au travers de la membrane cellulaire rompue.

RESUMEN

Se analizaron, por microscopía de luz y electrónica, las biopsias de nervios periféricos de 4 pacientes con lepra tuberculoide subpolar (BT) y 4 pacientes lepromatosos (LL), que estuvieron en tratamiento con poliquimioterapia. Las variaciones en el diámetro y distribución de las fibras mielinizadas y no mielinizadas entre los pacientes BT y LL no fueron significativas. Sin embargo, el estudio mostró cambios importantes en los nervios periféricos y en los vasos sanguíneos endoneuriales. Se observó que además de las células de Schwann (CS), las células endoteliales (CE) de los vasos sanguíneos endoneuriales con frecuencia también contienen *M. leprae*. En la lepra BT, los nervios periféricos mostraron cambios degenerativos de las CS con acumulación perineural y perivascular de células mononucleares,

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además de engrosamiento de la membrana basal de los vasos sanguíneos perineurales e hipertrofia de las CE, con reducción u oclusión completa del lumen. Por otro lado, los nervios periféricos de los pacientes LL estuvieron infiltrados con gran número de *M. leprae*, sobre todo en la zona electrotransparente (ZET) de las CS. Las CE de los vasos sanguíneos endoneuriales se encontraron repletas de bacilos y los estuvieron liberando hacia el lumen, a través de sus membranas rotas.

Leprosy is a chronic disease, caused by *Mycobacterium leprae* where involvement and damage of peripheral nerves is a typical and unique feature. One of the cardinal signs of clinical diagnosis of leprosy patients depends on the recognition of thickened peripheral nerves in patients supplying an anesthetic area in the skin, hand, legs, or face. The histopathological demonstration of *M. leprae* in the nerve and the presence of inflammatory granuloma in and around a nerve are mandatory for confirmation of diagnosis. However, the bacilli have not yet been reported in the brain and spinal cord, possibly due to the unfavorable condition for survival and growth of *M. leprae* in these areas ([1]). The disease is manifested in a spectrum based on the cell mediated immunity (CMI) of the host. While a strong CMI against *M. leprae*, which limits the growth of the bacilli, is expressed in tuberculoid (TT), and borderline tuberculoid (BT) leprosy, in lepromatous leprosy (LL) there is a lack CMI against the bacilli leading to unlimited growth of *M. leprae* in the host. In the early stage of the disease, even when only limited numbers of skin lesions are present and only small number of *M. leprae* are found in the skin, the organisms preferentially localize in the peripheral nerves.

Histologically, bacilli are seen within cells, either in myelinating or nonmyelinating Schwann Cells (SC) of nerves or in macrophages ([7, 17, 18, 14]). Presence of *M. leprae* has been shown by electron microscopy in SC of myelinated (MY) and unmyelinated (UMY) nerve fibers and also in macrophages, endothelial cells (EC), and perineurial cells ([23, 12, 8, 9]). Ultrastructurally, *M. leprae* have been found in the EC of blood vessels ([5, 2, 3, 21, 19]). It has been clear therefore, that the SC are not the only target cells for *M. leprae* growth, but organisms also grow in the EC of blood vessels. Recently, in an experimental model *M. leprae* has been observed in the EC of epineurial and perineurial blood vessels and also in lymphatics ([28, 27]). However, it is known that the endoneurial blood vessels supply the nutrients to the nerves for maintaining the metabolic activity of SC and is essential for proper nerve fiber functioning. Therefore, parasitization of the EC of endoneurial blood vessels could be also an essential feature for initiation of nerve damage.

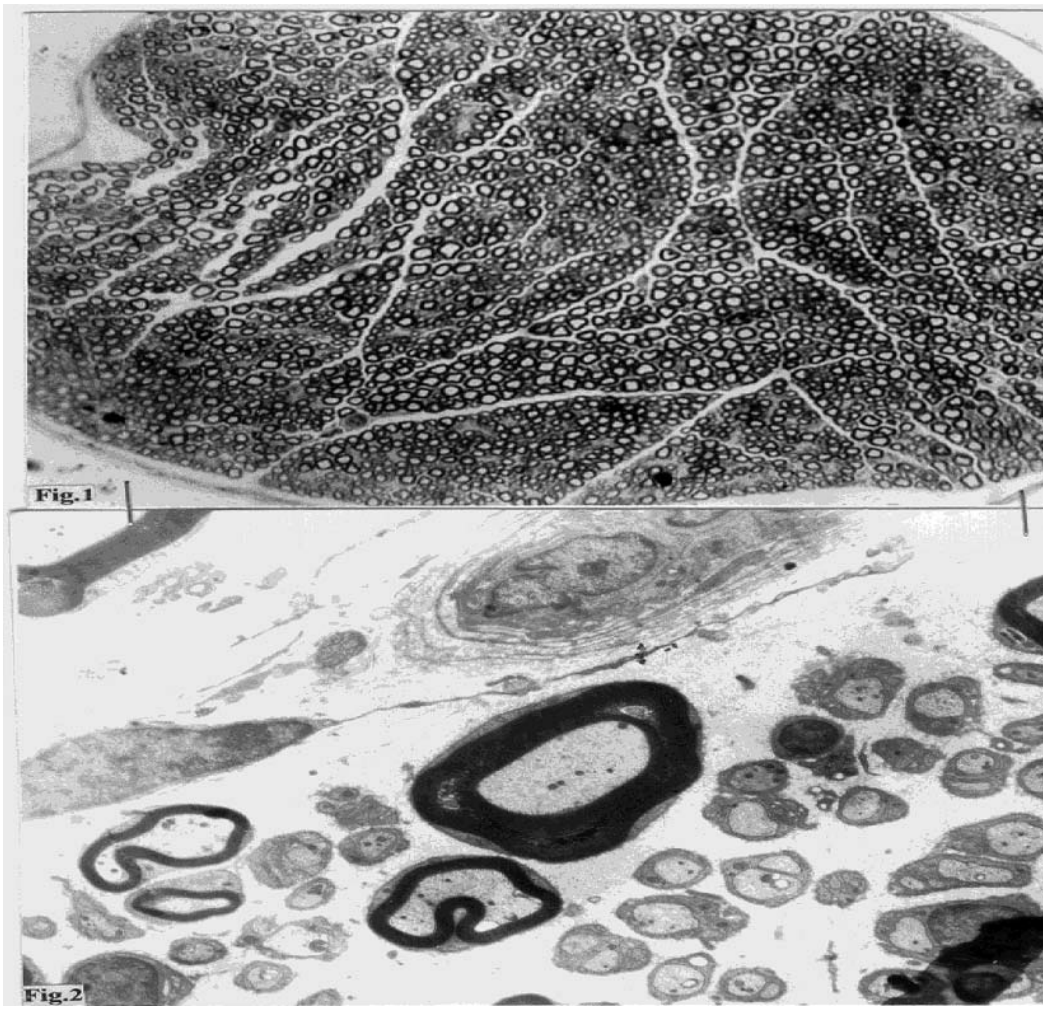
The present study has been carried out to record the detailed ultrastructural changes in the SC and EC of endoneurial blood vessels which might help in understanding the morphological significance of pathogenesis and dissemination of *M. leprae*.

MATERIALS AND METHODS

Nerve biopsies. Eight patients of leprosy (4 BT and 4 LL) who volunteered for biopsy of the peripheral nerves were included in this study. The patients were selected from the out patient clinic of the

THE TABLE. *Details of selected patients.*

No. of patients	Type of disease	Age in years	Duration of the disease	Status of nerves	Nerve biopsied
1	BT	23	3 months	Thickened	Medial cutaneous
2	BT	27	6 months	Thickened	Superficial peroneal
3	BT	38	8 months	Thickened	Infra patellar
4	BT	25	8 months	Thickened	Medial cutaneous
5	LL	47	1 year	Thickened	Superficial peroneal
6	LL	36	1 year	Thickened	Infra patellar
7	LL	48	1year 6 months	Thickened	Medial cutaneous
8	LL	52	2 years	Thickened	Superficial peroneal

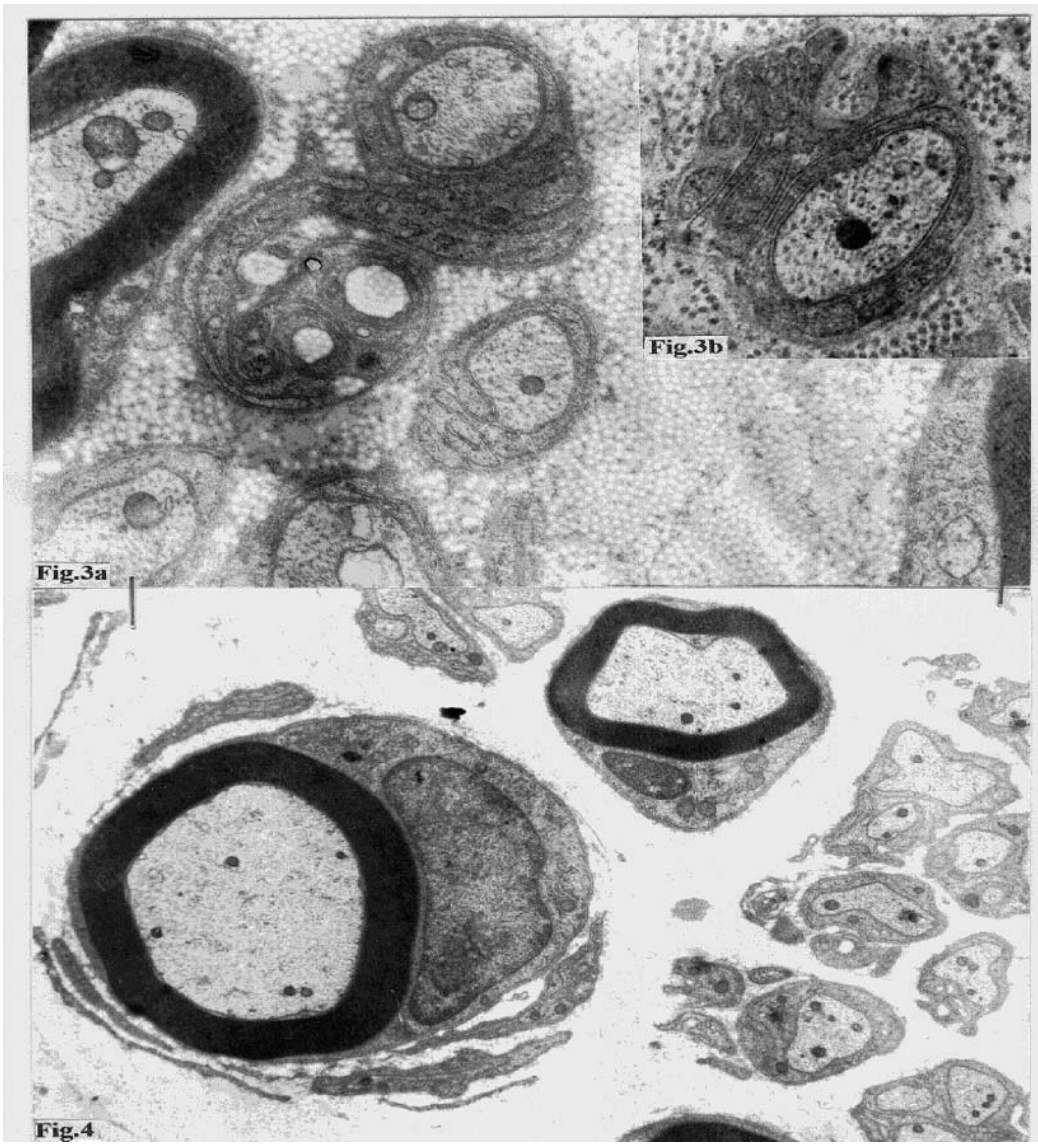


FIGS. 1–2. **1,** Semithin section of peripheral nerve fascicle from BT patient showing distribution of myelinated fibers with small endoneurial blood vessels (BV). ×600. **2,** Electron micrograph of peripheral nerves from BT patient showing unmyelinated Schwann cells (USC) with axons of smallest diameters, few myelinated sheath (MS) and endoneurial blood vessels (BV). The endoneurial blood vessel showed closed lumen with enlarged nucleus and multilayering basement membrane (BM). ×5000.

Central JALMA Institute for Leprosy, Agra. Peripheral nerves from all of these patients were biopsied by employing standard surgical procedures (Table 1). Before conducting the study an ethical clearance was obtained from the Institutional Ethical Committee.

One part of the biopsy was fixed in formol-Zenker's fluid and processed for histopathology. The other part was fixed in 2.5% glutaraldehyde for electron microscopic studies.

Tissue preparation for electron microscopy. Small pieces of the nerve biopsies were fixed overnight in 2.5% glutaraldehyde (TAAB Laboratory equipment, U.K). Subsequently, the tissues were washed in phosphate buffered saline (PBS), (Itron Laboratory Inc., Japan) post fixed in 1% osmium tetra oxide (OsO_4) (John Matthey Chemical, U.K.) and again washed in PBS. The fixed tissues were then dehydrated in ascending grades of alcohol. Later, these were immersed in propylene oxide (Fluka AG, Chemische Fabrik, Switzerland) and embedded in Spurr's resin (TAAB Laboratories Equipment, England). Finally, the tissues were polymerized overnight at 70°C and blocks were made. Cross sections, 1 μ



FIGS 3–4. **3a,** Ultrathin sections of peripheral nerve from BT showing Schwann cells with well preserved collagen fibers (CF) surrounded by unmyelinated fibers. The connections of mesaxon (Mx) are the extensions of the Schwann cell membrane are clearly seen. ×10,000. **3b,** The Schwann cell observed on wrapping (WR) to the surrounding collagen fibers. ×15,000. **4,** Ultrathin section of peripheral nerves in BT showing myelinated and unmyelinated Schwann Cells. The myelinated Schwann cells, showed attenuated Schwann cell processes arranged circumferentially forming an "Onion bulb" (OB). The cluster of unmyelinated Schwann cell can be seen with atrophied axon. The axonal cytoplasm is less electron dense than Schwann cell cytoplasm. ×7000.

thick, of the entire fascicles embedded in Spurr's resin were examined after staining with toluidine blue (Himedia Laboratories Pvt. Ltd, Bombay). These preparations were used for a general survey and for counts of MY and UMY fibers. Photographs of the entire cross section of each fascicle along with a micrometer scale were obtained by a PM-10 ADS camera, Olympus microscope and enlarged to 1000 times. From these photographs myelinated (MY) and unmyelinated (UMY) fibers were counted and the measurement of their diameters were made by the method of Espir and Harding ([10]).

The frequency distribution of the UMY and external diameters of all the MY fibers in each fascicle was ascertained by separating them into groups increasing by 1 μ. The photographs of the fascicle within the perineurium area were enlarged after finding their mean radius, and the densities of the UMY and MY fibers were calculated per sq. mm of the intraperineurial area.

Ultrathin sections were cut in an ultramicrotome (MT2, Porter blum, U.S.A.) and stained with uranyl acetate and lead citrate solutions (E. Merk, India). The sections were observed under an electron microscope H-300. The accuracy of the observations by light microscopy was checked. Montages were made of electron micrographs taken at about 2000 times magnification and enlarged to about 8000 times. Measuring the respective grids-spaces by light microscopy controlled the exact final enlargement and was compared with enlarged electron micrographs. One to 4 areas, equivelent to about 5000–10,000 sq. μ per fascicle, of 2 or 3 fascicles were studied in each case. Quantitative studies of UMY and MY fibers were made. Using the same apparatus, both UMY and MY fibers were counted. Their diameters were measured on electron micrographs after being enlarged to near 8000 times, at which magnification they could be separated reliably into groups differing by 1 millimeter. Discrimination of the frequency distribution of the diameter of UMY axon (AX) was thus possible into about 8 groups increasing by 0.2 μ. Usually, over 300 UMY AX were counted in each case. Densities of the UMY and MY were calculated as numbers per sq. mm. No correction was made for shrinkage of the tissue during preparation.

RESULTS

In Borderline Tuberculoid (BT). The semi-thin sections of the nerves revealed uniformly distributed MY fibers with few dispersed blood vessels (Fig. 1). The significant change in the diameter of the AX and MY sheath varied between BT and LL patients. Using light and electron microscopy of the entire cross sections the quantitative measurements of UMY and MY fibers in BT patients were made. The diameter of UMY fibers ranged from 0.5 μm to 3.8 μm. Their distribution ranged from 6% to 32% (Fig. 5a). The diameters of UMY fibers that were in close relation to endoneurial blood vessels were generally very small. The external diameter of MY fibers was measured by extrapolating the population of UMY fibers in the partial areas to the whole area of the fascicles. It ranged from 2 μm to 22 μm and their distribution varied from 9% to 22% (Fig. 5b). At the ultrastructural level, it was observed that the UMY fibers were extensively involved with degenerative and regenerative changes. UMY fibers manifested regeneration in the form of dense groups of very small and intact UMY AX (Fig. 2). In the regenerative AX, mesaxon connecting the AX and the basement membrane of SC was clearly seen (Fig. 3a). Proliferation of SC and prominence of endoneurial collagen fibers were also noticed. In some cases, collagen fibers increased and few SC, phagocytic in nature, were in the process of engulfing the adjacent collagen fibers (Fig. 3b). Onion-like bodies were observed, which are probably bionecrotic parts of nerves (Fig. 4). The MY group showed hypertrophy of Schwann cells with prominent nucleus and well-preserved collagen fibers. The regenerating AX were oval-shaped and some of them revealed the beginnings of MY sheath formation. However, *M. leprae* organism and their debris were not observed in any of these sections (Figs. 7 and 8).

Multiple layers of basal lamellae separated by ground substance accompanied the proliferation of EC of endoneurial blood vessels. The EC and basement membrane contained many granules and were surrounded with prominent inflammatory cells. The mononuclear leukocytes were often observed forming a perivascular cuff around blood vessels in the epineurium and perineurium. Such cuffing consisted primarily of macrophages. Occasionally, circulating infected monocytes were also observed occasionally within vascular lumen. Endothelial membranes were seen with multiple infolding and often with finger like protrusion around the blood vessels. The EC were hypertrophied with enlarged nuclei, often causing narrowing of the vascular lumina. (Fig. 9). In some cases, this hypertrophy of EC was up to such an extent that the lumen of the blood vessels got completely obliterated (Fig. 10). However, the degree of obstruc-

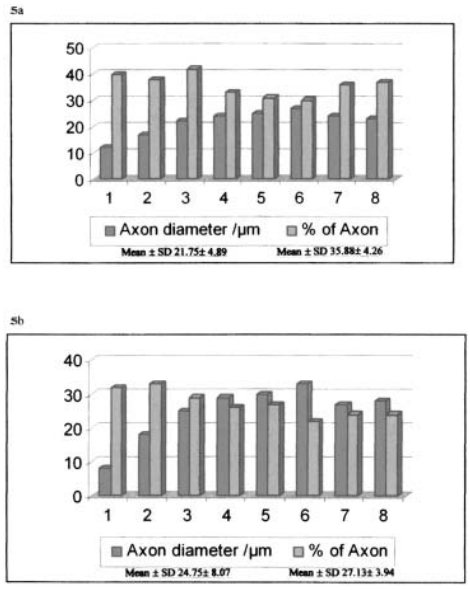


FIG. 5.

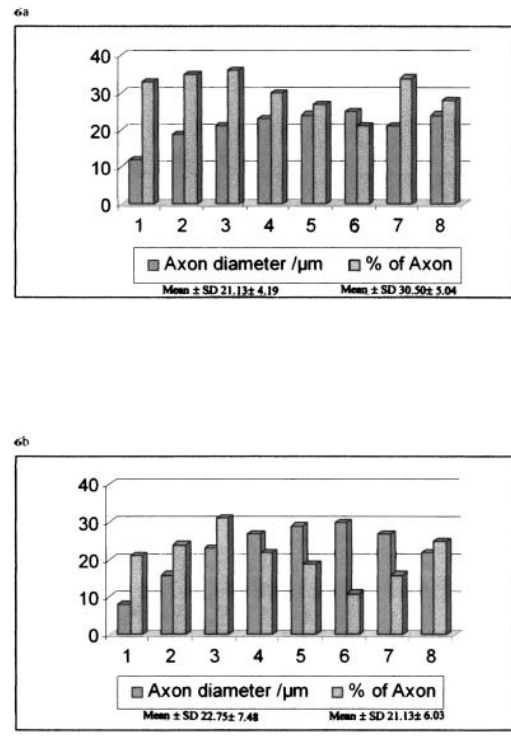


FIG. 6.

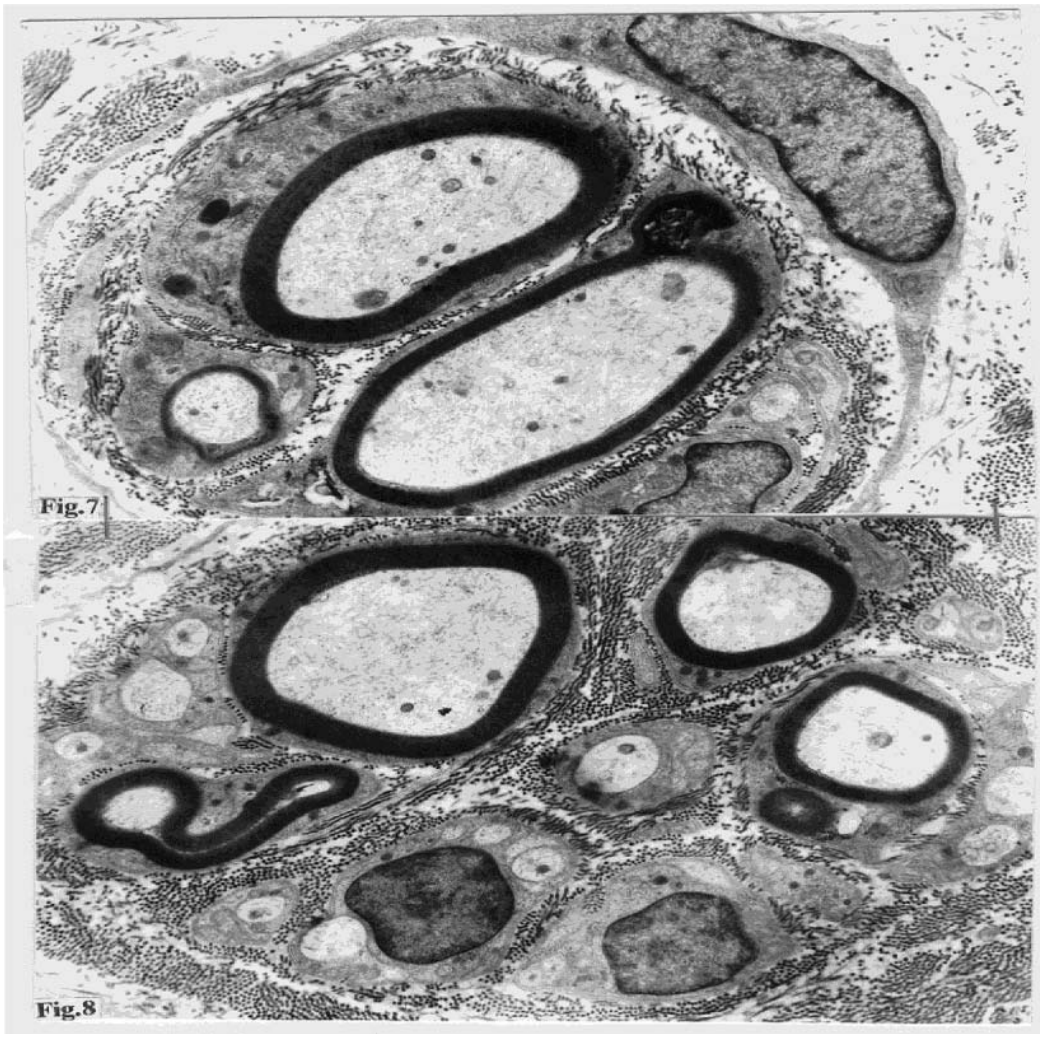
tion of the lumen of the blood vessels due to the hypertrophy of EC varied extensively. In some cases, the lumen was completely closed and in others the lumen had narrowed, but not closed completely.

In Lepromatous Leprosy (LL). The quantitative variation of the diameter of UMY fibers in LL patients varied between 0.5 µm to 3.8 µm and their distribution in entire cross section ranged from 2% to 20% (Fig. 6a). On the other hand, the external diameter of MY fibers ranged from 2 µm to 22 µm, and their distribution varied between 3% and 25% (Fig. 6b). The ultrastructural changes were characterized by degeneration of the SC, AX, and MY sheath. *M. leprae* were present singly or in clusters. Clumping of cytoplasm, neural filaments, and neural tubules indicated degenerative changes of SC. The organisms were usually seen within the electron transparent zone (ETZ) around the bacilli in the SC, cytoplasm (Figs. 11 and 12). The endoneurial blood vessels in LL showed patent lumen lined by degenerative EC. The nucleus was small and no hypertrophy of EC was observed. The basement membrane was thin, with visible endothelial cell junction (Fig. 13). Large numbers of intact bacilli were also noticed in the EC with ETZ. In some EC, the bacilli were seen being released into the lumen through endothelial cell membrane rupture (Fig. 14).

DISCUSSION

The quantitative study showed the correlation between axon diameter and myelin sheath diameter, and indicated a linear correlation with the thicker sheath surrounding the larger AX. This feature is probably useful for deciding whether an AX that has no myelin sheath is truly UMY or it has become degenerated. This might be a reflection of the changes in nerve conduction velocities and axonal degeneration and regeneration or segmental demyelination in neuropathies. These observations are in agreement with earlier findings ([9]).

The ultrastructural study has shown significant changes in the peripheral nerves and their endoneurial blood vessels in BT and LL patients. Various workers ([23, 12, 30, 20]) have reported that SC are the main target cell in leprosy. Our study using electron microscopic techniques has convincingly confirmed that beside the SC, the EC of blood vessels also harbor *M. leprae* frequently.

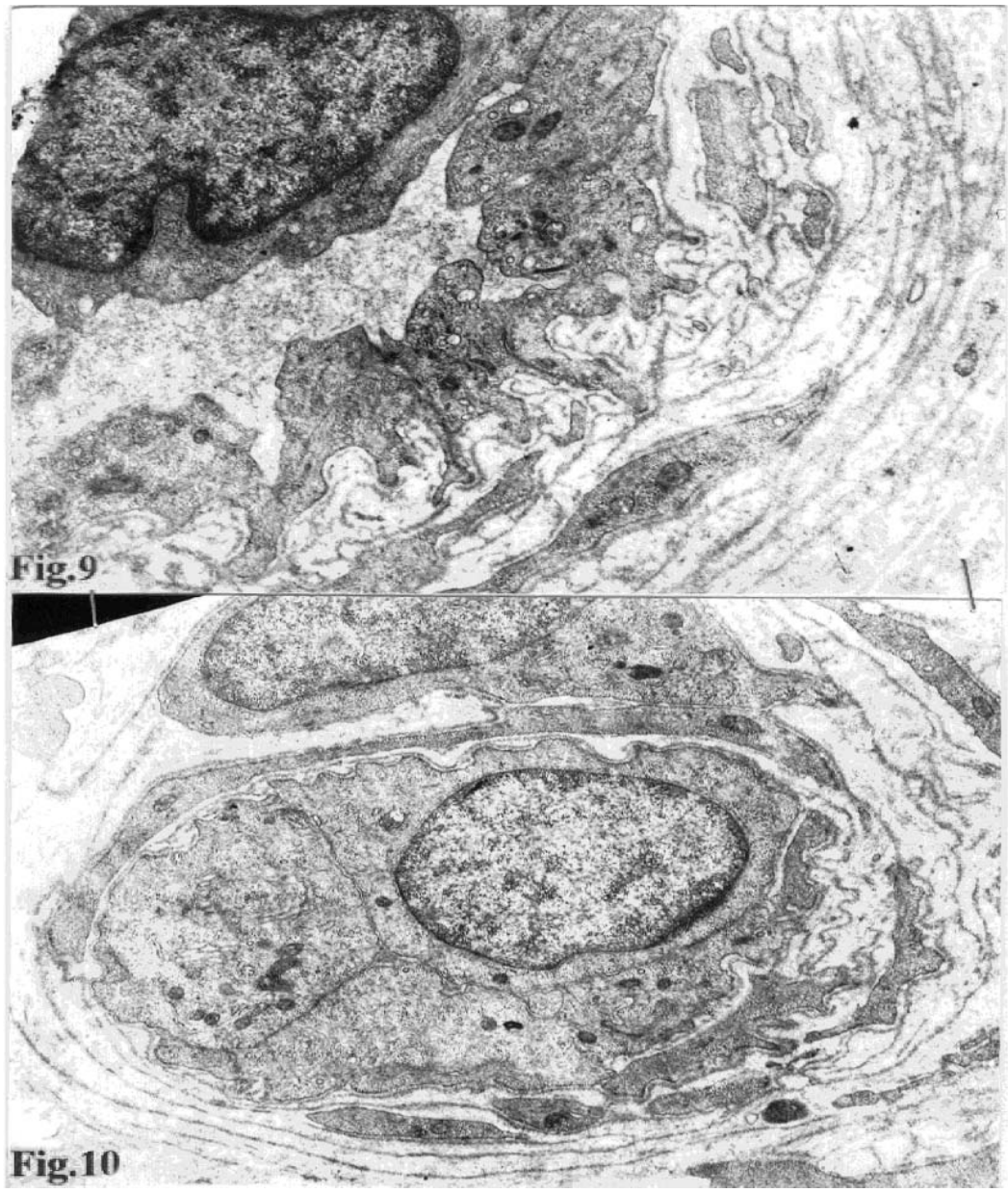


FIGS. 7–8. **7,** Ultrathin section of BT nerves showing two myelinated nerves surrounded by many collagen fibers. Small newly formed myelin sheath and elongated nucleus of Schwann cell is also noticed. ×8000. **8,** Ultrathin sections of BT nerve showing unmyelinated Schwann cells with prominent nucleus (N) containing many small oval shaped axons (AX). In the myelinated Schwann cell the neural filaments and neural tubules (NT) are well preserved. Some of the axons seem to be in the process of small myelin sheath formation. ×7000.

Electron microscopic changes of peripheral nerves in BT and LL patients have indicated interesting findings in understanding the interrelationship between bacilli and the host cells. In BT nerves, the UMY SC showed degenerative and regenerative changes with severe destruction of the nerve elements.

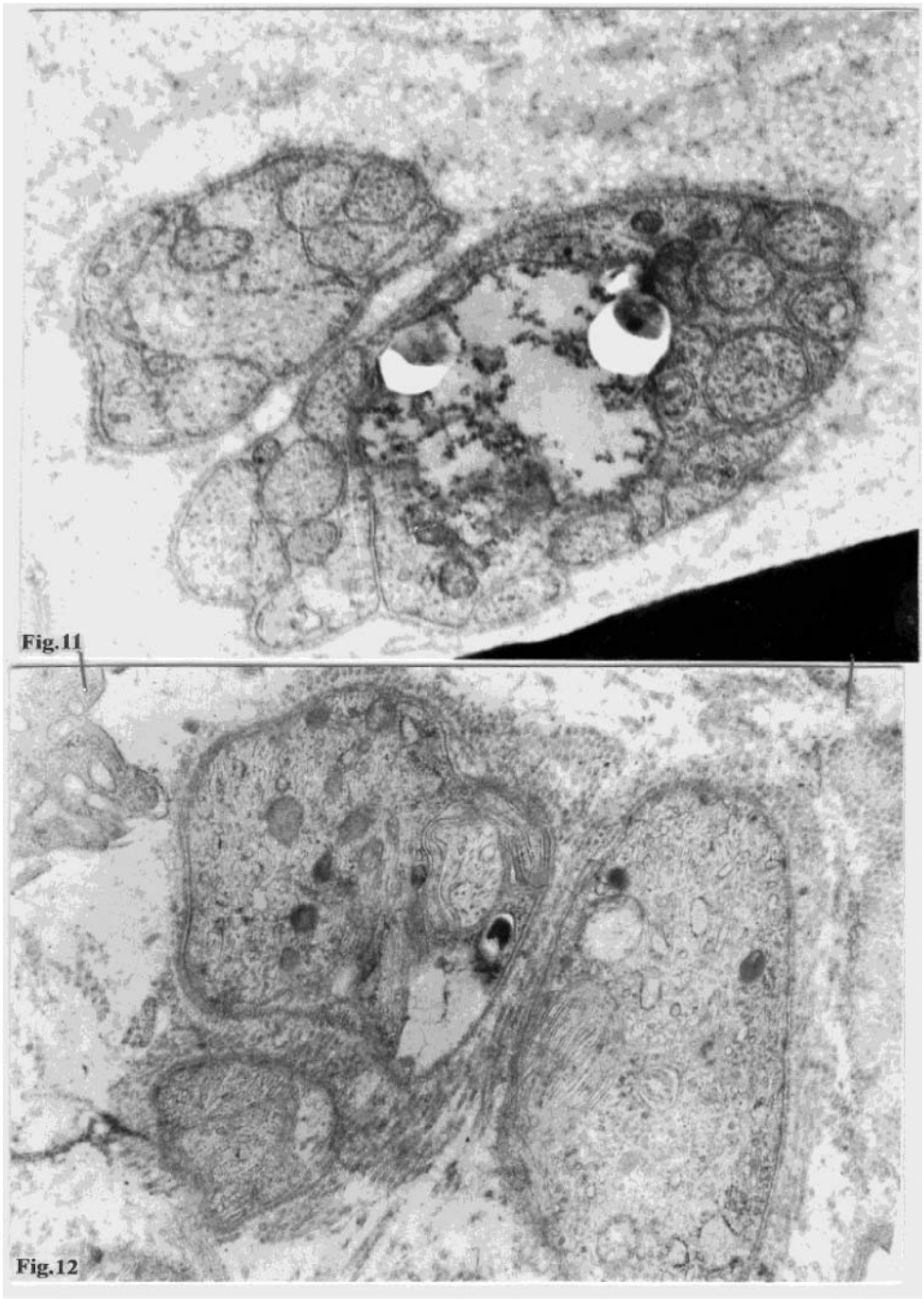
The increases in collagen fibers suggest that the SC may play a very important role in the production of collagen in neuropathy, possibly due to lack of blood supply. The continued destruction of SC in absence of AX multiplication and excessive production of collagen fibers may be responsible for the destruction of normal nerve architecture and for the prevention of axonal regeneration. Other workers ([15, 29, 30]) have proposed a similar concept. Further, they have suggested that SC in these nerves give off multiple cytoplasmic processes, which form close relationship with AX and also with the collagen formation.

The reason for greater phagocytic activity of surrounding matter by SC in UMY fibers is not clearly understood. Similar phagocytic activity of SC was also noticed by earlier

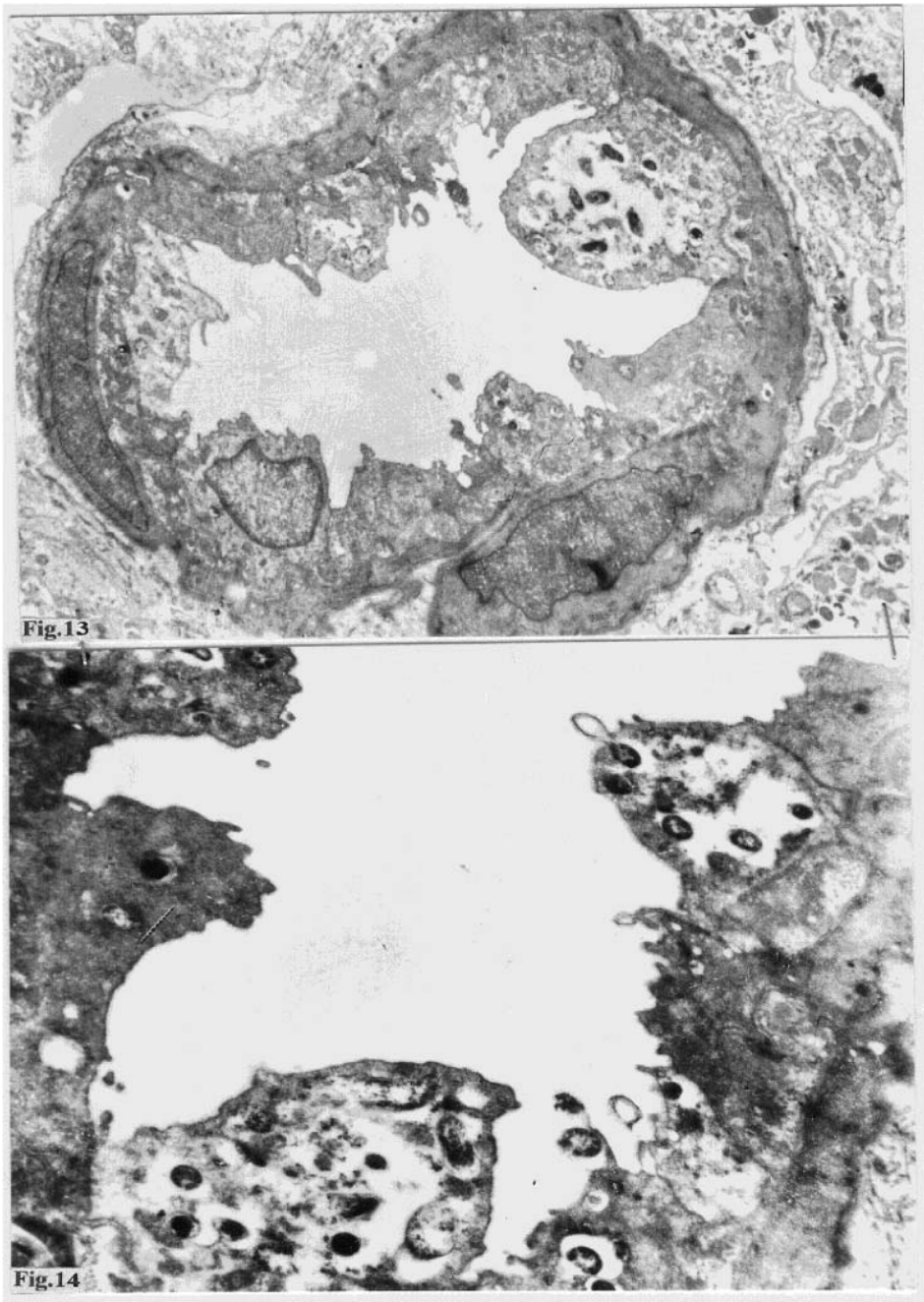


FIGS. 9–10. **9,** Higher magnified ultrastructural view of endoneurial blood vessel in BT patients showing narrowing of lumen (L) with enlarged nucleus. The basement membranes are multilayering with finger like protrusion. ×17,000. **10,** Cross section of endoneurial blood vessel in BT patients showing hypertrophy of endothelial cells containing many small mitochondria (MT), golgi complex with (GC) enlarged nucleus. The total obliteration of lumen and reduplication of basal lamina is clearly seen. ×10,000.

workers ([24 32, 33]). However in addition, the phagocytic activity by the perineurial cells was also noticed by other workers ([31]) who suggested that the bacilli are ingested into axoplasm by the phagocytic activity in the growth cones of the regenerating AX. Further, it was noted that regenerating AX were small in size and the mesaxons were connected with the regenerating AX, and the surface membrane of the SC. The growing tip of the regenerating AX migrate freely in the body fluid until they reach the final posi-



FIGS. 11, 12. **11,** Ultrathin sections of peripheral nerve from LL patient showing unmyelinated Schwann cell containing numerous axons and two intact bacilli (B) in Electron transparent zone. The clumping of cytoplasm and neural fibers were also seen. ×10,000. **12,** Ultrathin sections of LL nerve showing unmyelinated Schwann cell with well preserved basement membrane (BM), and single intact *M. leprae* (ML). ×12,000.



FIGS. 13, 14. **13,** Ultrathin sections of endoneurial blood vessel in LL patients showing open lumen with small nucleus. Many *M. leprae* organisms are seen inside endothelial cell (EC) in Electron transparent zone (ETZ). The endothelial cell junctions (EJ) are also clearly noticed. ×10,000. **14,** Higher magnification of endothelial cell of endoneurial blood vessels from LL patients containing many *M. leprae* organisms. The bacilli are being release from the endothelial cell into lumen of blood vessel. ×30,000.

tion in the nerve. Subsequently, SC infold the entire length of the regenerating AX behind the growing tip. It seems that in this free and naked stage of regeneration, AX engulf leprosy bacilli in the peripheral nerves. The onion-like bodies found in the BT nerve lesions resembled the plasmosome of alveolar cells. These onion-like bodies may be the remnants of the myelin (Figs. 11 and 12). These observations are in agreement with previously published findings ([12, 20, 23]). In BT leprosy, it was observed that almost every dermal nerve present in the localized lesion showed a presence of inflammatory cells, which destroyed large portion of nerves. The perivascular and perineurial cuffing by mononuclear cells is an indication of immune mediated inflammatory process.

Ultrastructural examination of the endoneurial blood vessels revealed that the basement membranes are multilayered, separated by ground substance indicating repeated episodes of injury to the vascular endothelium. The most interesting findings were the hypertrophy of EC noticed at various levels as observed by others ([2, 4, 6, 22]). Recently, ([28]) in an experimental study it was observed that the activation of infected EC led to thickening of the cells and narrowing of the lumen of blood vessels. In addition, these workers suggested that, since the endoneurial blood circulation is responsible for suppling the nutrients for maintaining metabolic activity of the nerves, their occlusion could be a major cause of nerve damage. However, the extent of swelling of EC in the experimental study did not lead to closure of the vessel. We hereby report a complete obstruction of the endoneurial blood vessels by hypertrophied EC which is frequently noticed in the nerves of BT patients. The ischemia caused by this, could play a major role in nerve degeneration leading to neuropathy and neural pain in these patients even after chemotherapy.

In contrast, peripheral nerves in LL cases were infected with large numbers of *M. leprae*. The number of inflammatory cells was very few when compared with that of BT cases. Further, an abundance of the organisms in SC showed foamy degeneration, followed by disintegration of AX leading to endoneurial fibrosis. The proliferation of perineurial cells, increase in endoneurial collagen, and growth of macrophage granuloma caused a pronounced thickening of the nerve. Ultrastructurally, leaving aside the SC, parasitization of other cells like EC of blood vessels and the perineurial cells was also noticed. The presence of *M. leprae* in SC in the ETZ confirmed the finding of other workers ([11, 20, 15]). The bacilli in various states of degenerations were seen inside phagosomal vacuoles of SC. However, some SC were found loaded with intact bacilli indicating their incapability in killing *M. leprae*. Some workers ([25, 26]) have described a molecular mechanism of *M. leprae* gaining entry into the SC of peripheral nerves. Further, they have demonstrated that the binding of *M. leprae* to the SC is mediated by surface proteins of the bacillus binding via α-dystroglycan to the α-2 isoform of laminin found in SC. The present study has also indicated that there was no significant hypertrophy of EC and therefore the lumen of the blood vessels remain patent. However, EC contained many bacilli in the ETZ. These organisms appeared to be solid and are therefore probably viable. Further, the bacilli were found in large numbers inside the cells, indicating their intracellular multiplication. In certain situations, the rupturing of EC due to excessive bacterial growth and release of *M. leprae* in the lumen of blood vessels was clearly noticed. It is therefore obvious that the released bacilli in the blood vessels are the direct evidence for hematogenous spread of the disease. Therefore, the present study provides a proof for transmission of *M. leprae* infection in the nerve through blood stream leading to active phagocytosis of *M. leprae* by SC from the circulation due to blood nerve barrier damage ([3]). The present study also suggest that the small endoneurial blood vessels may play an important role for propagation of *M. leprae* infection and progression of the disease in the nerve in LL.

In conclusion, we have now shown that the mechanism of nerve damage in BT and LL forms of leprosy are different. In BT, it is probably the result of immune-mediated inflammation of the nerves damaging SC and further compounded by vascular occlusion, causing ischemia. In contrast, in LL there is infection of SC by *M. leprae* leading to their damage. There is no vascular occlusion but the EC appear to be a source of propagation of infection as the organisms actively multiply in them and are then re-

leased into the lumen of endoneurial blood vessels.

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